HARALD VON BOEHMER
MIDWINTER
CONFERENCE
Advances in Immunobiology

CONFERENCE PROGRAM

HARALD VON BOEHMER
MIDWINTER CONFERENCE

Seefeld in Tirol, Austria
January 20 - 24, 2024

HDACs
IMMUNO-
MODULATION
TCELLS
SFB-F70

midwinterconference.org

Contents

Contents

About the MWC

Dear friends and colleagues,

We are pleased to welcome you to the 4th Harald von Boehmer Midwinter Conference: Advances in Immunobiology (MWC). This conference was initiated in 2014, when Harald von Boehmer, Ludger Klein and Lisa von Boehmer founded the Association for the Advancement of Immunobiology, a non-profit organization based in Munich, Germany. The aim of the Association is to provide a platform for the exchange of ideas in immunobiology by enabling established senior scientists and fledgling researchers to interact in a science-focused environment. The Midwinter Conference is designed to encourage open discussion, networking with peers from around the world and leveraging knowledge from leading experts in this field.

In memoriam of Harald von Boehmer and in honor of his fundamental contributions to the field of immunology, we have renamed the congress the "**Harald von Boehmer Midwinter Conference**".

The Harald von Boehmer MWC 2024 will feature 24 invited presentations and a similar number of talks selected from abstracts as well as two poster sessions. This year the conference is co-organized with the DFG-funded Collaborative Research Centre 1054 "Control and Plasticity of Cell Fate Decisions in the Immune System" and the the FWF-funded Special Research Programme F70. We are very pleased that – despite the pandemic-induced 5 year hiatus – the interest in the MWC continues to grow and the number of participants has reached a new record in 2024.

We sincerely hope you will enjoy your time in Seefeld,
the MWC committee


Ludger Klein


Thomas Brocker


Wilfried Ellmeier


Lisa von Boehmer

Conference Speakers

Burkhard Becher
University of Zurich

Thomas Boehm
Max Planck Institute for Immunobiology and Epigenetics

Chunaram Choudhary
University of Copenhagen

Adrian Erlebacher
UC San Francisco

Donna Farber
Columbia University

Ananda Goldrath
UC San Diego

Muzlifah Haniffa
Wellcome Sanger Institute/
Newcastle University

Matteo Iannacone
San Raffaele Scientific Institute

Nicole Joller
University of Zurich

Susan Kaech
Salk Institute for Biological Studies

Axel Kallies
University of Melbourne

Taras Kreslavskyi
Karolinska Institute

Maria Mittebrunn
Severo Ochoa Molecular Biology Center

Marion Pepper
University of Washington

Conference Speakers

Ellen Robey
UC Berkeley

Chiara Romagnani
DRFZ Berlin

Romain Roncagalli
Centre d'Immunologie de Marseille-Luminy

Alexander Scheffold
Christian-Albrechts-Universität zu Kiel

Michael Sixt
Institute of Science and Technology Austria

Caetano Reis e Sousa
The Francis Crick Institute

Georg Stary
Medical University of Vienna

Martin Turner
The Babraham Institute

Gabriel Victora
The Rockefeller University

Carola Vinuesa
The Francis Crick Institute

Conference Program

Saturday, January 20, 2024

16.00 Registration desk open

KEYNOTE LECTURE AND WELCOME RECEPTION

18.00	MWC committee	Introductory remarks
18.15	**Thomas Boehm**	**Evolutionary novelties in vertebrate adaptive immune systems**
19.15	*Mix and Meet*	*Fingerfood will be served*

Conference Program

Sunday, January 21, 2024

LYMPHOCYTE DEVELOPMENT AND MALIGNANCIES

09.00	**Ellen Robey**	**A multi-omics approach to dissecting T cell fate in the thymus**
09.30	Andreas Krueger	High-resolution mapping of cell-cycle dynamics during steady-state T-cell development and regeneration in vivo
09.45	Robin Thiele	Constrained B-cell development promotes malignant transformation
10.00	Carlota Farre Dias	Using sequential mutagenesis to model human multiple myeloma subgroups in mice
10.15	*Coffee Break*	

HUMORAL IMMUNITY

10.45	**Carola Vinuesa**	**Signals driving pathogenic B cells in autoimmunity**
11.15	**Gabriel Victora**	**B cell selection in germinal centres**
11.45	Louise Webb	Rejuvenation of the Germinal Center Reaction and Affinity Maturation Following Depletion of T Follicular Helper Cells
12.00	Mirela Kuka	IFNγ Suppresses T Follicular Helper Cell Differentiation and Antibody Responses
12:15	*Lunch Break*	

CONTROL OF GENE EXPRESSION AND PROTEIN FUNCTION

14.00	**Chunaram Choudhary**	**Lysine acetylation in gene regulation**
14.30	**Martin Turner**	**RNA binding proteins limiting T cell differentiation and function**
15.00	Borja Jimenez Lasheras	Neddylation as a relevant agent in metabolism and antitumor capacity of CD8+ T cells

Conference Program

Sunday, January 21, 2024

15.15	Daniel Greenwood	Mapping the heterogeneity of NF-kB response in primary human immune cells with deep-learning assisted high content microscopy
15.30	*Coffee Break*	

T CELL ACTIVATION

16.00	**Romain Roncagalli**	**Insights into the molecular mechanisms encoding TCR ligand discrimination**
16.30	Alexander Leithner	A second-generation supported lipid bilayer system to study the effects of ligand lateral mobility on T cell activation
16.45	Katarzyna Jobin	Post priming long-term T cell interaction niches scale CD8 and CAR Teff formation

Conference Program

Monday, January 22, 2024

AT THE CROSSROADS OF INNATE AND ADAPTIVE IMMUNITY

09.00	**Taras Kreslavskiy**	**Unusual specificities of γδ T cells**
09.30	**Chiara Romagnani**	**Adaptive features of NK cells**
10.00	Immo Prinz	Resident γδ and αβ T cells in human lung and associated lymph nodes adapt into complementary regulatory and cytotoxic phenotypes
10.15	Georg Gasteiger	Differentiation of innate and adaptive tissue-resident lymphocytes during ontogeny, infection and allergy
10.30	*Coffee Break*	
11.00	Gleb Turchinovich	Power of two: new binary Cre mouse model s pecifically targeting type 3 Innate Lymphoid Cells
11.15	Julie Ribot	IL-17 regulates peripheral nerve regeneration
11:30	**Burkhard Becher**	**Cytokines in inflammation and cancer**
12:00	*Lunch Break*	

TRANSCRIPTIONAL CONTROL OF IMMUNE CELL FUNCTION

17.00	**Ananda Goldrath**	**Transcriptional programming of T cell tissue immunity in space and time**
17.30	Ari Glasner	Conserved transcriptional connectivity of regulatory T cells in the tumor microenvironment informs new combination cancer therapy strategies
17.45	Carmen Gerlach	CD8+ T cell differentiation and activation represent distinct axes of diversification
18.00	*Poster Session I*	*Fingerfood will be served*

Conference Program

Tuesday, January 23, 2024

MYELOID CELL BIOLOGY

09.00	**Caetano Reis e Sousa**	**DaNgeRous necrophagy, coprophagy and immunity to cancer**
09.30	**Michael Sixt**	**Coordination of mechanical forces in migrating leukocytes**
10.00	Barbara Schraml	Identification of unique RORγt expressing antigen presenting cells conserved across tissues and species
10.15	Michael Kern	Host intestinal perivascular macrophages protect against murine acute graft-versus-host disease via PD-L1
10.30	*Coffee Break*	

TOLERANCE AND AUTOIMMUNITY

11.00	**Adrian Erlebacher**	**Mechanisms of fetomaternal tolerance**
11.30	Andrew Koh	Thymic epithelia destabilize chromatin via p53 repression to amplify transcriptional noise for AIRE-mediated immune tolerance
11.45	Stefano Angiari	Coenzyme A fueling with pantethine limits auto-reactive T cell pathogenicity in experimental neuro-inflammation
12.00	*Lunch Break*	

IMMUNITY AND AGING

17.00	**Donna Farber**	**Tissue and age as determinants for human immunity**
17.30	**Maria Mittelbrunn**	**Decoding the contribution of the immune system to aging**
18.00	*Poster Session II*	*Fingerfood will be served*

Conference Program

Wednesday, January 24, 2024

HUMAN IMMUNOLOGY

09.00	**Muzlifah Haniffa**	**Decoding the developing human immune system**
09.30	**Alexander Scheffold**	**Exhausted autoreactive T cells in human auto-immunity**
10.00	**Georg Stary**	**Tissue-resident T cells as guardians of cutaneous immunity**
10.30	*Coffee Break*	

CANCER IMMUNOLOGY

11.00	**Susan Kaech**	**Stressing out our immune system in cancer**
11.30	**Axel Kallies**	**Differentiation and function of cytotoxic T cells in chronic infection and cancer**
12.00	Patrizia Stoitzner	Tumor-targeted therapy with BRAF-inhibitor remodels the myeloid landscape to promote tumor immunity in melanoma
12.15	Lydia Dyck	Analysis of mechanisms promoting T cell tolerance against autochthonous cancer in aged mice
12:30	*Lunch Break*	

IMMUNITY IN TISSUES

14.00	**Matteo Iannacone**	**Immune surveillance of the liver**
14.30	**Nicole Joller**	**The co-inhibitory receptor TIGIT promotes tissue protection and repair**
15.00	*Coffee Break*	
15.30	Jan Dobeš	Segmented filamentous bacteria-induced epithelial MHCII regulates cognate CD4+ IELs and epithelial turnover

Conference Program

Wednesday, January 23, 2019

15.15	Anneli Peters	T:B cell cooperation in ectopic lymphoid follicles in CNS autoimmunity
16.00	**Marion Pepper**	**Memory T cells in tissues**
19.00	*Farewell Dinner*	

Poster Abstracts

Ali Afzali
TU Munich
ali.afzali@tum.de

B cells control autoimmunity against AQP4 by negative selection of antigen-specific thymocytes

Ali Maisam Afzali[1,2,3], Lucy Nirschl[1], Christopher Sie[1], Christine Federle[4], Elisabetta Petrozziello[4], Hsin-Hsiang Chen[1], Sofia Tyystjärvi[1], Andreas Muschaweckh[1], Katja Lammens[5], Katja Steiger[6], Gönül Seyhan[7], Rupert Öllinger[8], Roland Rad[8], Adrian Straub[9], Anton Mühlbauer[9], Simon Grassmann[10], Bernhard Hemmer[2,3], Jan P. Böttcher[11], Doron Merkler[12], Marc Schmidt-Supprian[7], Veit R. Buchholz[9], Sylvia Heink[1], Dirk H. Busch[9,13], Ludger Klein[4], Thomas Korn[1,2,3,*]

1 Institute for Experimental Neuroimmunology, Technical University of Munich School of Medicine, Munich, Germany
2 Department of Neurology, Technical University of Munich School of Medicine, Munich, Germany
3 Munich Cluster for Systems Neurology, Munich, Germany
4 Institute of Immunology, University Hospital and Biomedical Center, Ludwig-Maximilians-University Munich, Planegg-Martinsried, Germany
5 Department of Biochemistry at the Gene Center, Ludwig-Maximilians-University, Munich, Germany
6 Institute of Pathology, Technical University of Munich, Munich, Germany
7 Institute for Experimental Hematology, Technical University of Munich School of Medicine, Munich, Germany
8 Institute of Molecular Oncol. and Func. Genomics, TranslaTUM Cancer Center, Technical University of Munich School of Medicine, Munich, Germany
9 Institute for Medical Microbiology, Immunology, and Hygiene, Technical University of Munich, Munich, Germany
10 Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA
11 Institute of Molecular Immunology and Experimental Oncology, Technical University of Munich School of Medicine, Munich, Germany
12 Department of Pathology and Immunology, Div. of Clin. Pathology, Geneva Faculty of Medicine, Centre Médical Universitaire, Geneva, Switzerland
13 National Center for Infection Research (DZIF), Munich, Germany

Neuromyelitis optica (NMO) is a debilitating autoimmune disease of the CNS that is mediated by autoantibodies against the water channel protein AQP4 (NMO-IgG). Tolerance mechanisms against AQP4 are poorly understood. While AQP4-specific T cells are exceedingly rare in the natural T cell repertoire, there is evidence suggesting that an antigen-specific T cell response is required for the generation of NMO-IgG. The major focus of this project is to understand how T cells are tolerized against AQP4 and how fate decisions of AQP4-specific thymocytes can be misguided so that AQP4-specific T cells can seed the peripheral immune compartment and eventually - through cooperation with AQP4-specific B cells - induce the production of anti-AQP4 antibodies. Here, we show that a key mechanism for the deletion of AQP4-specific thymocytes is the presentation of endogenous AQP4 by thymic B cells. AQP4 belongs to a class of autoantigens that are upregulated and presented through MHC class II in B cells in response to CD40 and IL-21 stimulation. Since thymic B cells receive an anti-CD40 signal during licensing, their transcriptome overlaps with the transcriptome of peripheral CD40-stimulated B cells. The presentation of AQP4 by thymic B cells is sufficient to purge the T cell receptor (TCR) repertoire of AQP4-reactive TCRs. Lack of AQP4 expression in B cells rescues the absolute number of AQP4-specific T cells in secondary lymphoid tissues to the level of Aqp4–/– mice that totally lack thymic negative selection of AQP4-specific thymocytes. We propose that thymic tolerance against antigens expressed in CD40-activated B cell is an efficient means to withhold T cell help from forbidden T cell – B cell interactions and prevents the engagement of antigen-specific T cells in germinal center (GC) reactions and their development into T follicular helper cells. Failure of this tolerance mechanism might – as in the case of NMO – result in productive T cell-dependent autoantibody production and clinical disease. Therefore, our data lay the ground for a novel patho-mechanistic interpretation of a class of autoimmune disorders of the CNS and perhaps other organs.

Batuhan Akçabozan
Institute for Immunology, LMU
bakcabozan@gmail.com

Reciprocal regulation of mTORC1 and ribosomal biosynthesis determines cell cycle progression in activated T cells

Teresa Rosenlehner[1], Stefanie Pennavaria[1], Batuhan Akçabozan[1], Shiva Jahani[1], Thomas J O'Neill[2], Daniel Krappmann[2], Mario Pende[3], Tobias Straub[4], Jan Kranich[1], Reinhard Obst[1]

1 Institute for Immunology, Medical Faculty, Biomedical Center, Ludwig-Maximilians-Universität München, Germany.
2 Research Unit Signaling and Translation, Helmholtz Zentrum München, Germany.
3 Institut Necker Enfants Malades, INSERM U1151, Université de Paris, Paris, France.
4 Bioinformatics Core Facility, Biomedical Center, Medical Faculty, Ludwig- Maximilians-Universität München, Planegg-Martinsried, Germany

Ribosomal biosynthesis is a highly energy demanding process driven by the activities of all RNA polymerases and around 300 RNAs and proteins. How ribosomal biosynthesis is integrated into the process of T cell activation is incompletely understood. We have investigated how the mTOR complexes affect T cell proliferation, TCR signaling and ribosomal biosynthesis. We found that the proliferation of mTORC1- but not mTORC2-ablated T cells is slowed, with each consecutive division being delayed. mTORC1 signaling is affected to different extents by conventional TCR signaling via the PI3K, MAP kinase and CBM complex pathways, while reciprocally TCR sensitivity is hampered by mTORC1 inhibition. Upon activation the amount of RNA per cell increases at least 9-fold, which is reduced by half in mTORC1-ablated cells. RNA-seq data showed that this reduction affects all RNA biotypes and that rRNA processing is no bottleneck in activated T cells, while the reduced number of ribosomes correlates with lower levels of protein synthesis. Using FISH probes of nascent pre-rRNA in imaging cytometry revealed that mTORC1 but not mTORC2 ablation reduces the number and the expansion of nucleolar sites of active transcription. The ablation of the CBM complex component MALT1 in T cells similarly decreases rRNA transcription. We also found that inhibiting RNA Polymerase I blocked not only proliferation but also mTORC1 activity, suggesting an integration of nucleolar activity with TCR signaling. Our data thus show that TCR signaling, mTORC1 activity and ribosomal biosynthesis are tightly interlinked and regulate each other.

Stefano Angiari
Medical University of Graz
stefano.angiari@medunigraz.at

Coenzyme A fueling with pantethine limits autoreactive T cell pathogenicity in experimental neuroinflammation

Stefano Angiari[1,2], Tommaso Carlucci[1], Simona L. Budui[1], Simone D. Bach[1], Silvia Dusi[1], Julia Walter[2], Elena Ellmeier[2], Alyssa Schnabl[2], Cansu Tafrali[3], Rina Demjaha[3], Michael Khalil[3], Natalie Bordag[4], Carlo Laudanna[1,5], Barbara Rossi[1], Gabriela Constantin[1]

1 Department of Medicine, Division of General Pathology, University of Verona, Italy
2 Otto Loewi Research Center, Division of Immunology, Medical University of Graz, Austria
3 Department of Neurology, Division of General Neurology, Medical University of Graz, Austria
4 Department of Dermatology and Venereology, Medical University of Graz, Austria
5 The Center for Biomedical Computing (CBMC), University of Verona, Italy

Immune cell metabolism governs the outcome of immune responses and contributes to the development of autoimmunity by controlling lymphocyte pathogenic potential. In this study, we evaluated the metabolic profile of myelin-specific murine encephalitogenic T cells, to identify novel therapeutic targets for autoimmune neuroinflammation. By performing metabolomics analysis, we detected a potential break in the coenzyme A (CoA) synthesis pathway in actively-proliferating encephalitogenic T cells, compared to resting T cells. CoA fueling with the CoA precursor pantethine affected essential immune-related processes of autoreactive T cells, such as antigen-specific proliferation, cytokine production, and cell adhesion, both in vitro and in vivo. Mechanistically, pantethine exerted its immunomodulatory effects in encephalitogenic T cells by linking metabolic reprogramming to alteration of intracellular signaling pathways. Preventive treatment with pantethine inhibited the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of human multiple sclerosis (MS). Importantly, pantethine also significantly ameliorated the disease course when administered after disease onset in a therapeutic setting. Finally, pantethine limited pro-inflammatory cytokine production by human T helper 1 (Th1) and Th17 cells in vitro, as well as by T cells from MS patients, confirming its translational potential. In conclusion, we demonstrated that CoA fueling with pantethine in pro-inflammatory and autoreactive T cells may represent a novel therapeutic approach for the treatment of autoimmune neuroinflammation.

Pinar Ata
Marmara University School of Medicine
pinaren@gmail.com

Genetic Findings of Primary Immune Disturbances in a Third Grade Genetic Diagnosis Center

Esra Hilal Ceylan[1], Pinar Ata[1], Senol Demir[1], Mehmet Ali Soylemez[1], Bilge Geckinli[1], Ilter Guney[1], Ahmet Arman[1]

1 Genetic Diseases Diagnosis Center, Marmara University School of Medicine, Istanbul Turkey

Immunodeficiency syndromes can be diagnosed using cell population counts by flow-cytometry, antibody quantification and serological techniques in routine diagnostic workup. Genetic Diagnosis is important tool for the clinicians to consider the patient and the family as an integrative aproach. Herein we have searched our patients with immune system disturbances and analyzed a spectrum of mutations that are effective for their diagnosis.

Materials and Methods: In this study we evaluated patients with immune dysregulation who are admitted to our Genetic Disorders Diagnosis Center from June 2018 to December 2023, retrospectively. The QIAamp DNA Mini Kit (Qiagen, MD, USA) was used for DNA isolation from patients' peripheral blood. Quantification of extracted DNA was performed using Qubit 3.0 Florometre (Thermo Fisher Scientific, Waltham, MA). We analyzed using their clinical exome sequencing data according to Severe combined immunodeficiency, Primary immune Dysregulation and Primary Antibody deficiency gene panels. SOPHIA Clinical Exome Solution V2 (Boston, USA) kit was used for single nucleotide variants (SNVs) and copy number variants (CNVs) of 51 genes as Severe Combined immunodeficiency panel, 178 genes as Primary immune dysregulation panel, 32 genes as primary antibody deficiency panel. The sequencing data was classified according to ACMG criteria.

FINDINGS: Patients were aged at an average of 18 years and there were 23 females and 52 males. Among 75 patient analysis we detected 30 clinically pathogenic mutations and 21 variants of unknown significance. Eighteen of the pathogenic mutations were homozygote and remaining 12 were heterozygous and 4 had homozygote/hemizygote variants and 17 had heterozygous variants.

CONCLUSIONS: Herein most of the pathogenic mutations in our cohort was detected in TNFRSF13B, BTK, LRBA and ADA genes. According to timely databases in our cohort genetic diagnostic workup revealed our patients' final diagnosis. It is important to reanalyze patient sequencing data periodically when exome sequencing performed instead of specific gene panels.

Anne-Marie Bührer
Helmholtz Munich
annemarie.buehrer@helmholtz-munich.de

During islet autoimmunity *all-trans* retinoic acid restores impaired Treg induction potential *in vitro*

Anne-Marie Bührer[1,2], Maria Kral[3], Isabelle Serr[1,2] and Carolin Daniel[1,2,4]

1 Research Unit Type 1 Diabetes Immunology, Helmholtz Diabetes Center, Helmholtz Munich, Munich, Germany
2 Deutsches Zentrum für Diabetesforschung, Neuherberg, Germany
3 Institute for Cardiovascular Prevention, Ludwig-Maximilians-Universität München, Munich, Germany
4 Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-Universität München, Munich, Germany

Type 1 diabetes (T1D) is characterized by a loss of immune tolerance and aberrant immune activation promoting the destruction of insulin-producing beta cells in the pancreas. Main mediators of peripheral immune tolerance are Foxp3+ regulatory T cells (Tregs), but critical impairments in Treg induction were identified during islet autoimmunity. Importantly, Treg induction defects were linked to changes in the activation threshold of naïve T cells, indicating alterations prior to autoimmune activation. However, the underlying cellular mechanisms remain poorly understood. Thus, identifying signaling pathways that can be targeted to foster Tregs is essential to advance the development of innovative Treg-based immunomodulation. The crucial role of vitamin A in regulating immune function is well-established. In this regard, it has been shown that T1D patients are at risk for vitamin A deficiency and that *all-trans* retinoic acid (atRA), as active metabolite of vitamin A, favors Treg differentiation in presence of TGFβ. Accordingly, we observed reduced vitamin A levels in the serum of T1D patients compared to healthy controls ($p=0.05$) as well as in autoimmune-prone non-obese diabetic (NOD) mice compared to Balb/c controls ($p=0.03$). Using Treg induction *in vitro*, we showed that atRA induces Tregs in T cells from T1D individuals despite the absence of TGFβ ($p=0.02$). This aligns with our murine data where atRA fosters Treg induction in NOD mice even without the addition of TGFβ, and this effect is more pronounced in NOD mice compared to Balb/c controls ($p<0.0001$). *In vivo*, a short-term vitamin A excess diet led to a trend towards increased anti-inflammatory signatures of pancreatic Tregs. Moreover, atRA-treatment in NOD mice resulted in reduced lymphocyte infiltration into the pancreas ($p=0.0002$) and upregulation of Tregs with an anti-inflammatory and suppressive phenotype, e.g. HELIOS+ Tregs ($p=0.001$). Overall, these initial findings illustrate the importance of vitamin A and the *in vitro* data suggest a TGFβ-independent tolerogenic effect of atRA during islet autoimmunity.

Luisa Bach
University Hospital Bonn, Medical Clinic III
luisa.bach@ukbonn.de

TGFb specifies Tfh versus Th17 cell fates of murine CD4+ T cells through c-Maf

Yinshui Chang, Luisa Bach, Marko Hasiuk, Lifen Wen, Tarek Elmzzahi, Carlson Tsui, Nicolás Gutiérrez-Melo, Teresa Steffen, Daniel T. Utzschneider, Timsse Raj, Paul Jonas Jost, Sylvia Heink, Jingyuan Cheng, Oliver T. Burton, Julia Zeiträg, Dominik Alterauge, Frank Dahlström, Jennifer-Christin Becker, Melanie Kastl, Konstantinos Symeonidis, Martina van Uelft, Matthias Becker, Sarah Reschke, Stefan Krebs, Helmut Blum, Zeinab Abudullah, Katrin Paeschke, Caspar Ohnmach, Christian Neumann, Adrian Liston, Felix Meissner, Thomas Korn, Jan Hasenauer, Vigo Heissmeyer, Marc Beyer, Axel Kallies, Lukas T. Jeker, Dirk Baumjohann

T follicular helper (Tfh) cells are essential for effective antibody responses but deciphering the intrinsic wiring of mouse Tfh cells has long been hampered by the lack of a reliable protocol for their generation in vitro. We report that TGFb induces robust expression of Tfh hallmark molecules CXCR5 and Bcl6 in activated mouse CD4+ T cells in vitro. TGFb-induced mouse CXCR5+ Tfh cells are phenotypically, transcriptionally, and functionally similar to in vivo generated Tfh cells and provide critical help to B cells. The study further reveals that CXCR5 expression is independent of Bcl6 but requires c-Maf. Notably, classical TGFb-containing Th17 induction conditions also yield separate CXCR5+ and IL-17A-producing cells, highlighting shared and distinct cell fate trajectories of Tfh and Th17 cells. We demonstrate that excess IL-2 in high-density T cell cultures interferes with the TGFb-induced Tfh cell program, that Tfh and Th17 cells derive from a common precursor, and that c-Maf acts as a switch factor for Tfh versus Th17 cell fates in vitro and in vivo.

Jana Balounova
Czech Centre for Phenogenomics, Institute of Molecular Genetics of the Czech Academy of Sciences
jana.balounova@img.cas.cz

Fam83h contributes to normal lymphopoiesis in postnatal hematopoietic niches

Ogan B[1], Šímová M[1], Dowling L[1], Forstlová V[1], Vičíková K[1], Špoutil F[1], Turečková J[1], Labaj J[1], Procházka J[1], Sedláček R[1] and Balounová J[1]

1 Czech Centre for Phenogenomics, Institute of Molecular Genetics of the Czech Academy of Sciences

The scaffold protein FAM83H is implicated in various biological processes, spanning intracellular transport, cytoskeletal network regulation, cell proliferation, and enamel formation. While widely expressed in epithelial cells, FAM83H plays a crucial role in recruiting casein kinase 1 (CK1) to the keratin cytoskeleton. Truncation mutations within the *FAM83H* gene are identified as the primary cause of autosomal dominant hypocalcified amelogenesis imperfecta (AI). Remarkably, two AI patients within a Czech family, confirmed to have a *FAM83H* mutation, developed juvenile rheumatoid arthritis. To elucidate the role of FAM83H in immune system homeostasis, we generated *Fam83h* knockout animals. These exhibit a spectrum of phenotypic abnormalities, including reduced body size, sparse coat, scaly skin, hypoactivity and early lethality, but no evident dental phenotype. Notably, the majority of *Fam83h-/-* animals develop transient soft tissue lesions in their forelimbs by two weeks of age. Simultaneously, all *Fam83h-/-* animals display elevated neutrophils and monocytes, coupled with decreased lymphocytes in the peripheral blood. Of note, lymphoid cell development is arrested in the bone marrow (BM) and thymus. While fetal hematopoiesis appears unaffected, the development of B cells and NK cells in BM is partially blocked at pro- and pre-B cell stages and the immature NK cell stage. Although the thymus size is normal in *Fam83h-/-* animals at birth, its growth is limited within the first three weeks of life. Furthermore, the development of T cells is severely impaired in *Fam83h-/-* thymi, featuring a profound, yet not complete, arrest at the DN stage. Thymic epithelial cells deficient in *Fam83h* exhibit increased beta-catenin mRNA levels, along with elevated expression of Wnt target genes *Axin2* and *Sox9*, and reduced levels of *Foxn1*, linked to thymus involution. In summary, while FAM83H proves dispensable in fetal thymic development, our findings suggest its potential role in attenuating Wnt signaling through CK1-dependent degradation of beta-catenin in the postnatal thymus.

Aidan Barrett
University of Gothenburg, Sahlgrenska Academy, Institute of Medicine, Department of Rheumatology and Inflammation Research
aidan.barrett@gu.se

Importance of estrogen signaling in fibroblastic reticular cells for innate and adaptive immune responses in experimental arthritis

Aidan Barrett[1], Karin Horkeby[2], Carmen Corciulo[3], Hans Carlsten[1], Marie K Lagerquist[2], Julia M. Scheffler[1], Ulrika Islander[1,4*]

1 Institute of Medicine, Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
2 Institute of Medicine, Department of Internal Medicine and Clinical Nutrition, Sahlgrenska Osteoporosis Center, Centre for Bone and Arthritis Research, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
3 Institute of Neuroscience and Physiology, Department of Pharmacology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
4 SciLifeLab, University of Gothenburg, Gothenburg, Sweden

Women are more prone to develop rheumatoid arthritis, with peak incidence occurring around menopause. Estrogen has major effects on the immune system and is protective in arthritis. Fibroblastic reticular cells (FRCs) are stromal cells that generate the three-dimensional structure and different microenvironmental niches in lymph nodes. They are vital for coordinating immune responses from within lymph nodes and are characterized by expression of the chemokines CCL19 and CCL21, which drive immune cell migration into the lymph node. Previous studies in the lab have shown that treatment with estrogen ameliorates joint destruction and frequencies of inflammatory cells present in synovium and spleen in the murine antigen-induced arthritis (AIA) model, although the mechanisms contributing to this effect remain unknown. The aim of this study was to determine the influence of estrogen signaling via FRCs in regulating innate and adaptive immune response in the AIA model. Conditional knockout mice lacking estrogen receptor alpha (ERalpha) in CCL19-expressing FRCs, and littermate controls, were ovariectomized, treated with vehicle or estradiol, and subjected to the AIA model. Various innate and adaptive immune cell populations in the lymph node were profiled by flow cytometry. The results reveal that ERalpha signaling in FRCs is important for the estrogen-induced decreased frequency of CD8+ conventional dendritic cells, and for the increased levels of B cells, plasma cells, and IgM-producing cells in lymph nodes. Overall, this study contributes to improved knowledge about the mechanisms involved in the effects of estrogen on immune responses during inflammatory diseases.

Maike Becker
Helmholtz Zentrum München GmbH, Deutsches Forschungszentrum für Gesundheit und Umwelt
maike.becker@helmholtz-munich.de

Regulatory T cells require IL-6 receptor alpha signaling to control skeletal muscle function and regeneration

Maike Becker[1,2], Francisco Garcia-Carrizo[2,3], Anne H. Neubig[1,2], Sini S. Joseph[2,4,5], Daria Opaleva[1,2], Isabelle Serr[1,2], Susanna M. Hofmann[2,6,7], Tim J. Schulz[2,3,8], and Carolin Daniel[1,2,9]

1 Research Unit Type 1 Diabetes Immunology, Helmholtz Diabetes Center at Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Munich-Neuherberg, Germany
2 German Center for Diabetes Research (DZD), 85764 Munich-Neuherberg, Germany
3 Department of Adipocyte Development and Nutrition, German Institute of Human Nutrition Potsdam-Rehbrücke, 14558 Nuthetal, Germany
4 Institute for Diabetes and Obesity, Helmholtz Diabetes Center at Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Munich-Neuherberg, Germany.
5 Division of Metabolic Diseases, Department of Medicine, Technische Universität München, 80333 Munich, Germany
6 Institute for Diabetes and Regeneration, Helmholtz Diabetes Center at Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Munich-Neuherberg, Germany
7 Department of Medicine IV, University Hospital, LMU Munich, 80336 Munich, Germany
8 University of Potsdam, Institute of Nutritional Science, Potsdam-Rehbrücke, 14558 Nuthetal, Germany
9 Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-Universität München, 80337 Munich, Germany

Muscle-residing regulatory T cells (Tregs) control local tissue integrity and function. However, the molecular interface connecting Treg-based regulation with muscle function and regeneration remains largely unexplored. Therefore, we subjected mice to voluntary wheel running. This exercise was performed for 4 weeks (ex) or followed by 4 weeks of rest (pre-ex) to test for beneficial long-term effects. Control mice were kept sedentary (sed). Here, we show that exercise fosters a stable induction of highly functional muscle-residing Tregs with increased expression of amphiregulin (Areg, sed vs pre-ex p= 0.0064; ex vs pre-ex p= 0.0009), EGFR (sed vs ex p=0.0192, sed vs pre-ex p<0.0001, ex vs pre-ex p<0.0001) and ST2 (sed vs. pre-ex p=0.0031, ex vs. pre-ex p=0.0002). During exercise, the myokine IL-6 is secreted locally from myofibers and leads to upregulation of IL6Rα on T cells. Mechanistically, we find that mice lacking IL6Ra on T cells (TKO) show significant reductions in muscle Treg maturation, satellite cells (p=0.046) and fibro-adipogenic progenitor cells (p=0.0021), which are all required for efficient muscle regeneration. Using exercise and sarcopenia models, IL6Rα TKO mice demonstrate deficits in phenotypic adaptation of Tregs, especially their functional maturation which leads to a more pronounced decline in muscle function as assessed by grip strength tests (p=0.0146). Furthermore, a muscle injury model shows that IL6Rα TKO mice have significant impairments in muscle regeneration 14 dpi as indicated by reduced fiber cross sectional areas (p=0.0017). Importantly, Treg gain-of-function restores impaired muscle repair in IL6Rα TKO mice. Of note, pharmacological IL6R blockade in WT mice phenocopies impairments in muscle function (p=0.0014) identified in IL6Rα TKO mice highlighting the clinical implications of these findings. Thus, the results of this study underscore the relevance of dissecting muscle-specific immune regulation and will be of importance for the design of tailored precision medicines targeting niche-specific Tregs in the future.

Bettina Elisabeth Bernard
Insitute for Medical Microbiology, Immunology and Hygiene, MRI, TUM
bettina.bernard@tum.de

In vitro exhaustion model based on induced mitochondrial dysfunctionality and TOX signatures deciphers human CD8+ T cell exhaustion

Bettina E. Bernard[1], Lea M. Seidel-Raub[3,4,5,6], Corinna Seitz[1,2], Frances Winkler[3,4,6], Bertram Bengsch[3,6*], Kathrin Schumann[1, 2*]

1 Technical University of Munich (TUM), School of Medicine and Health, Department of Preclinical Medicine, Institute for Medical Micro biology, Immunology and Hygiene, Munich 81675, Germany
2 TUM, Institute for Advanced Study, Garching 85748, Germany
3 Department of Medicine II (Gastroenterology, Hepatology, Endocrinology and Infectious Diseases), Freiburg University Medical Center, Faculty of Medicine, University of Freiburg, Freiburg 79106, Germany
4 Faculty of Biology, University of Freiburg, Freiburg 79106, Germany
5 SGBM – Spemann Graduate School of Biology and Medicine, University of Freiburg, Freiburg 79106, Germany
6 Signalling Research Centres BIOSS and CIBSS, University of Freiburg, Freiburg 79106, Germany
* equal contribution

In chronic infections and solid tumors, CD8 T cells can acquire an exhausted cell state with negative implications for disease clearance. The interrogation of genes important for human exhausted T cells (Tex) is challenging due to the low numbers of Tex that can be isolated in human disease. An in vitro model for human T cell exhaustion would allow high-throughput screening for genes involved in the exhaustion program. Here, we used an approach that integrates major pathways driving exhaustion, in particular TCR signaling, mitochondrial dysfunction and TOX overexpression to recapitulate human Tex development in vitro. Human Tex cells are generated from healthy donor T cells ex vivo by inducing mitochondrial dysfunction using the mitochondrial translation inhibitor Azithromycin (AZM). Additionally, we included an ectopic expression of TOX, a transcription factor described as a key player of exhaustion. Using immune-metabolic flow analysis, CyTOF profiling and RNAseq analysis we identified an induction of exhaustion features, including terminally exhausted CD127-PD-1hi Tex cells and reduced cytokine production after AZM treatment. Ectopic TOX expression further increased inhibitory receptor expression and reduced cytokine release. Both AZM- and TOX-specific effects contributed to the exhaustion phenotype, but also synergistic effects were detected. Interestingly, our approach also induced substantial numbers of Tpex-like CD127+PD-1+ cells, highlighting our model as suitable to dissect distinct stages of Tex development. Using arrayed CRISPR Cas9 RNP-mediated KOs of exhaustion related transcription factors we now systematically dissect transcriptional signatures inducing or blocking T cell exhaustion. These highly controlled assays allow us to genetically dissect human T cell exhaustion ex vivo, to find novel exhaustion-related target genes and to improve therapies to fight chronic infections and cancer.

Sarah Braun
Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich (TUM), Munich, Germany
sa.braun@tum.de

Isolation and characterization of neoepitope-specific TCRs for T cell therapy in gastrointestinal cancer

Sarah Braun[1], Karolin I. Wagner[1], Sebastian Scheu[1], Anna Brutau-Abia[1], Raquel Mejias-Luque[1], Markus Gerhard[1,2], Elvira D'Ippolito[1,2] and Dirk H. Busch[1,2]

1 Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich (TUM), Munich, Germany
2 German Center for Infection Research (DZIF) partner site Munich

TCR-engineered T cells for adoptive cell therapy represent a major promise in cancer immunotherapy, in particular for aggressive tumor types with limited therapeutic options like gastrointestinal cancers. Neoantigens arising from frequently occurring frameshift-mutations in tumor suppressor genes are interesting immunological targets as they should provide high tumor specificity and immunogenicity. In addition, the derived neoepitopes could be potentially shared among different mutations and individuals. We developed a platform for the isolation of extremely rare CD8 naïve neoepitope-specific T cells from peripheral blood of healthy donors via single-cell sorting on combinatorial pMHC multimer staining. As proof-of-principle, we targeted a HLA-A*02:01-restricted neoepitope deriving from frameshift mutations is the tumor suppressor gene Ring Finger Protein 43 (RNF43). After re-expression of the identified TCRs via CRISPR-Cas9-mediated orthotopic TCR replacement, we identified functional TCRs with sufficient epitope sensitivity and cytotoxicity. Expanding on this, we extracted from the TCGA database the most frequent mutations in tumor suppressor genes in gastrointestinal cancers, for which we predicted 10 HLA-A*02:01 and 27 HLA-B*07:02-restricted neoepitopes. To enable a multiplexed, high-throughput identification of the corresponding TCRs, we are currently implementing the principle of DNA-barcoding of the pMHCs, and also include a pMHC-TCR dissociation measurement to assess the TCR affinity directly within the TCR identification platform for preselection of the most promising TCR candidates worth of re-expression and in-depth characterization. In summary, we aim at mapping the frameshift-derived neoepitope landscape of gastrointestinal cancers to set up a library of well-characterized and highly functional TCRs for therapeutic use.

Helena Brezovec
Max Perutz Labs/ Medical University of Vienna
a11936802@unet.univie.ac.at

Human primary keratinocytes interaction with fungal pathogens and HDAC inhibitors as novel anti-fungal therapeutic

Brezovec H.[1,2], Penninger P.[1,2], Seiser S.[1,2], Phan-Canh T.[1,2], Elbe-Bürger A.[1,2], Kuchler K.[1,2]

1 Max Perutz Labs, Vienna Biocenter Campus (VBC), Dr.-Bohr-Gasse 9, 1030, Vienna, Austria
2 Medical University of Vienna, Center for Medical Biochemistry (Division of Molecular Genetics), Dr.-Bohr-Gasse 9, 1030, Vienna, Austria

Candida auris is a skin topic opportunistic human fungal pathogen, that recently emerged as urgent public health treat due to its rapid spread in health care settings and inherent multidrug resistance. To develop alternative treatment strategies, we thought out to better understand fungal interactions with human primary keratinocytes (pKC), primarily making up the outermost epidermal layer. Here, we show that human keratinocytes are subjected to time and dose-dependent extracellular association of C. auris. Moreover, we demonstrate that pKCs do not predominantly utilize the release of reactive oxygen species (ROS) as a primary defence mechanism, however actively inhibit overgrowth of C. auris in alignment with existing studies highlighting production of antimicrobial peptides as defence mechanism in keratinocytes. Additionally, our findings demonstrate that soluble factors released by infected pKCs (with live and heat-killed C. auris) limit C. auris growth. Additionally, KCs are in constant cross-talk with surrounding immune cells, including T cells. Interestingly, a T cell specific deletion of HDAC1 protects during experimental skin infection due to increased release of protective Th17 cytokines. Therefore, we further investigate the potential positive role of class-I HDAC inhibitors in limiting fungal spread by simple and combinatorial effects on C. auris, pKCs and human CD4+ T cells. These findings provide pivotal insights into the dynamic interplay between primary keratinocytes and C. auris. Especially, underlining potential defence mechanism employed by primary keratinocytes that produce antimicrobial factors to limit C. auris growth.

Madara Brice
Karolinska Institutet
madara.brice@ki.se

The Role of HIF Regulatory Pathways in CD8+ T Cell Metabolism

Madara Brice[1], David Bargiela[2], Pedro P. Cunha[2], Brennan J. Wadsworth[1], Randall S. Johnson[1]

1 Department of Cell and Molecular Biology, Karolinska Institute, Sweden
2 Department of Physiology, Development and Neuroscience, University of Cambridge, UK

CD8+ T cells are essential for adaptive immune responses across various microenvironments in the body. Often this involves nutrient and oxygen deprivation, requiring quick adaptation of metabolic pathways. This adaptation is regulated by the HIF transcription factors. The factor inhibiting HIF (FIH) and von Hippel-Lindau ubiquitin ligase (VHL) control HIF transcriptional activity in an oxygen-dependent manner. Previously, we have showed that when HIF is insensitive to FIH and VHL inhibition, it can lead to increased CD8+ T cell anti-tumour efficiency. Our hypothesis was that knocking out HIF inhibitors should influence the transcriptional profile of CD8+ T cells. To address this, we investigated OT-I CD8+ T cells derived from WT and T cell specific FIH, VHL and double FIH/VHL k/o mice. We performed RNAseq analysis of OT-I CD8+ T cells isolated from mouse spleen following 3 days of activation with anti-Cd3/Cd28 beads and IL2. Preliminary results show differential expression of genes involved in metabolism and cytokine signaling. We also investigate the effect of knock outs on markers of T cell activation and differentiation. The results of this study provide further insight into the contribution of different HIF regulatory pathways on metabolism and immune function in CD8+ T cells.

Marie Brinkmann
Medical University of Vienna
marie.brinkmann@meduniwien.ac.at

In-vitro drug screening reveals the potential of epigenetic compounds for the treatment of rheumatoid arthritis

Marie Brinkmann[1], Teresa Preglej[1], Anela Tosevska[1], Thomas Krausgruber[2], Wilfried Ellmeier[3], Nicole Boucheron[3], Iris Gratz[4], Bianca Luckerbauer[1], Günter Steiner[1], Daniel Aletaha[1], Lisa Göschl[1] and Michael Bonelli[1]

1 Division of Rheumatology, Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria
2 CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria
3 Division of Immunobiology, Medical University of Vienna, Vienna, Austria
4 Department of Biosciences, Paris Lodron University Salzburg, Salzburg, Austria

Introduction: Rheumatoid Arthritis (RA) is a systemic, chronic inflammation of joints, affecting up to 1% of the worldwide population. While existing treatments successfully enhance the quality of life for patients, not all respond to these therapeutic approaches. Inhibitors of proteins involved in chromatin modification can effectively modulate gene expression, holding the potential to modify disease-driving signatures. Consequently, they represent novel prospects as epigenetic therapeutics for autoimmune diseases.

Methods: PBMCs from healthy donors and RA patients were activated with Phytohemagglutinin (PHA) and treated for 24 hours with epigenetic inhibitors. Drug effects were analysed utilizing spectral cytometry (Cytek Aurora), enabling the discrimination of 26 cell types and 11 comprehensive activation markers. In addition, we employed bulk RNA sequencing to inspect compound-induced alterations at the transcriptional level. A bioinformatics pipeline was established, including quality control, dimensionality reduction methods, and multi-parameter analysis, allowing the integration of data on the cellular and transcriptional levels.

Results: 26 potential drugs were screened and characterized, demonstrating their potential as novel treatment approaches for RA. Flow cytometry revealed compound-specific effects on the cell subset distribution and cell activation status of PBMC subsets. We could show a clustering of the tested epigenetic compounds based on their targets, consistent with a clustering found in the RNA sequencing data. Enrichment analysis revealed the association of treatment-affected genes with immunological processes, cytokine signalling, and genes involved in autoimmune diseases like RA.

Conclusion: We established a high-throughput drug screening tool to characterize compound-specific effects that might predict treatment responses and underlying mechanisms. We could indeed provide evidence of potential therapeutic mechanisms, proving the remarkable potential of epigenetic inhibitors as novel treatment approach for RA.

Hsin-Hsiang Chen
Experimental Neuroimmunology Klinikum rechts der Isar Technical University Munich
hh.chen@tum.de

CD38-mediated Nicotinamide Adenine Dinucleotide Metabolism Sustains the Immunosuppressive Function of Regulatory T cells within the Central Nervous System

Hsin-Hsiang Chen , Andreas Muschaweckh, Thomas Korn

Foxp3-expressing regulatory T cells (Tregs) have been established to play an important role in orchestrating tissue homeostasis. During central nervous system (CNS) inflammation, Tregs migrate towards and accumulate within the CNS, ameliorating CNS inflammation and facilitating neuronal repair processes. However, the biological mechanisms of the retention of Tregs within the CNS remain unknown. Furthermore, the heterogeneity of the CNS Tregs and their associated effector functions have yet to be comprehensively elucidated. Our investigation revolves around the characterization of Tregs in the CNS using an experimental murine autoimmune encephalomyelitis (EAE) model. We observed a consistent accumulation in the CD38-positive (CD38+) Treg subset within the CNS, spanning from the acute to the chronic phase of the disease progression. CD38 is identified as one of the main NAD hydrolases, regulating nicotinamide adenine dinucleotide (NAD+) metabolism. Notably, the CD38+ Tregs exhibit elevated expression of immune suppressive markers and transcription factors such as Foxp3 and Blimp1. Through the transfer of either CD38+ or CD38-negative (CD38-) Tregs into Treg-depleted murine hosts, we have demonstrated that CD38+ Tregs are superior in ameliorating the disease progression. Molecularly, we postulate that the CD38-mediated NAD+ consumption inhibits NAD-dependent deacetylase SIRT1 activity. Therefore, it stabilizes the Foxp3 transcription and maintains the acetylated form of FOXP3 and EZH2, which has a crucial role in maintaining the immunosuppressive functionality of Tregs.

Apurva Dhavale
Institute for Immunology, Biomedical Center, LMU Munich
apurva.dhavale@med.uni-muenchen.de

Dendritic cells infected with Yellow Fever vaccine virus promote T follicular helper type-1 cell polarization

Apurva Dhavale, Linus Rinke, Elena Winheim, Ev-Marie Schuster, Kilian Schober, Anne Krug

Vaccination with the live-attenuated yellow fever vaccine virus (YF17D) is highly effective and provides long-lasting immunity mediated by neutralizing antibodies and T cells. It was previously shown T follicular helper (Tfh) cell activation and frequency of circulating activated Tfh1 cells (CXCR5+ CXCR3+) correlate with neutralizing antibody titres after vaccination with YF17D. DCs are one of the targets of yellow fever virus infection. Tfh cells are induced by DCs and then further differentiate during cognate interaction with B cells in germinal centres where they support B cell differentiation into antibody secreting cells. In the context of YFV infection and vaccination, the precise mechanism underlying the induction and regulation of Tfh cells by DCs remains incompletely understood. Tfh cell induction was investigated using cocultures of human allogeneic monocyte-derived DCs (moDCs) and naïve CD4+ T cells. DCs exposed to live but not inactivated YF17D virus promoted the differentiation of naïve CD4+ T cells into CXCR5+ CXCR3+ PD1+ T cells producing IFN-γ upon restimulation, resembling Tfh1 cells along with Th1 cells. This was in part mediated by cytokines released from the DCs upon infection with YF17D and further enhanced by direct DC-T cell interaction. For further investigation of Th and Tfh cell differentiation by cognate interaction with YF17D-infected DCs we generated CD4+ T cells specific for an NS3-derived peptide by orthotopic TCR replacement. T cells lacking endogenous TCR and expressing the engineered TCRs responded to stimulation with anti-CD3/CD28 beads and to HLA-matched peptide-pulsed antigen presenting cells demonstrating their functionality and specificity. In conclusion, allogenic moDCs exposed to live YF17D virus promote the induction of naïve Th cells into Tfh1-like cells. Questions pertaining to the mechanism of Th/Tfh polarization by cognate interaction with YF17D-infected DCs and the role of various DC subpopulations in the induction of these Th cell subsets in the context of YF17D vaccination remain to be answered.

Sandra Dirschl
Reseach Unit Type 1 Diabetes Immunology, Helmholtz Diabetes Center, Helmholtz Zentrum München, Munich, Germany
sandra.dirschl@helmholtz-munich.de

Impairments in Regulatory T Cells During Early Stage Type 1 Diabetes

Sandra M. Dirschl[1,2], Martin G. Scherm[1,2], Isabelle Serr[1,2], Carolin Daniel[1,2,3]

1 Reseach Unit Type 1 Diabetes Immunology, Helmholtz Diabetes Center, Helmholtz Zentrum München, Munich, Germany,
2 Deutsches Zentrum für Diabetesforschung, Neuherberg, Germany,
3 Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-Universität München, Munich, Germany

In autoimmune Type 1 Diabetes (T1D), impaired immunological self-tolerance promotes the immune-mediated destruction of insulin producing β-cells in the pancreas, resulting in insulin deficiency. Regulatory T cells (Tregs) are key mediators of immunological self-tolerance and have been shown to be essential in preventing autoimmunity and limiting chronic inflammatory diseases. Impairments in Treg function, induction and stability were shown to be present at later stages of islet autoimmunity and in overt clinical T1D. Especially, Treg instability is hypothesized to be a consequence of autoimmune activation and the proinflammatory environment during autoimmunity. In contrast to this hypothesis, we observed Treg impairments already before the onset of islet autoimmunity in autoimmune-prone NOD mice, which are a well-established model of human T1D. We showed impaired in vitro Treg induction in young NOD mice (age < 30 days) before and after the onset of islet autoimmunity, when compared to non-autoimmune-prone BALB/c mice ($p<0.001$). Furthermore, we found evidence of early onset Treg instability in these young NOD mice. Specifically, methylation of Foxp3 CNS2 region- a marker for Treg instability- was increased in NOD mice after onset of islet autoimmunity. In addition, Tregs from NOD mice with early onset islet autoimmunity secreted higher amounts of the pro-inflammatory cytokine IL-17a ($p<0.01$), which is indicative for Treg plasticity. These findings provide evidence that Treg impairments occur very early in T1D pathogenesis, indicating a possible causative role in disease initiation, autoimmune activation and progression. These results illustrate the importance of early immunological impairments for T1D pathogenesis and highlight the need for future preventive intervention strategies in children at risk for developing T1D.

Jan Dobeš
Charles University, Prague, Czech Republic
jan.dobes@natur.cuni.cz

Segmented filamentous bacteria–induced epithelial MHCII regulates cognate CD4+ IELs and epithelial turnover

Tomáš Brabec[1], Martin Schwarzer[2], Katarína Kováčová[1], Martina Dobešová[1,3], Dagmar Schierova[4], Jiří Březina[3], Iva Pacáková[1], Dagmar Šrůtková[2], Osher Ben-Nun[5], Yael Goldfarb[5], Iva Šplíchalová[3], Michal Kolář[6], Jakub Abramson[5], Dominik Filipp[3] and Jan Dobeš[1]

1 Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic
2 Laboratory of Gnotobiology, Institute of Microbiology of the Czech Academy of Sciences, Nový Hrádek, Czech Republic
3 Laboratory of Immunobiology, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic
4 Laboratory of Anaerobic Microbiology, Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Prague, Czech Republic
5 Department of Immunology and Regenerative Biology, Weizmann Institute of Science, Rehovot, Israel
6 Laboratory of Genomics and Bioinformatics, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic

Intestinal epithelial cells have the capacity to upregulate MHCII molecules in response to certain epithelial-adhesive microbes, such as segmented filamentous bacteria (SFB). However, the mechanism regulating MHCII expression as well as the impact of epithelial MHCII–mediated antigen presentation on T cell responses targeting those microbes remains elusive. Here, we identify the cellular network that regulates MHCII expression on the intestinal epithelium in response to SFB. Since MHCII on the intestinal epithelium is dispensable for SFB-induced Th17 response, we explored other CD4+ T cell–based responses induced by SFB. We found that SFB drive the conversion of cognate CD4+ T cells to granzyme+ CD8α+ intraepithelial lymphocytes. These cells accumulate in small intestinal intraepithelial space in response to SFB. Yet, their accumulation is abrogated by the ablation of MHCII on the intestinal epithelium. Finally, we show that this mechanism is indispensable for the SFB-driven increase in the turnover of epithelial cells in the ileum. This study identifies a previously uncharacterized immune response to SFB, which is dependent on the epithelial MHCII function.

Vinicius Duarte da Silva
Würzburg Institute of Systems Immunology & International Max Planck Research School IEM
vinicius.alves-duarte-da-silva@uni-wuerzburg.de

Expression of Tcf1 and Hobit identifies distinct populations of CD8 TRM cells across tissues

Vinicius Duarte da Silva[1,2,3]; Georg Gasteiger[1]

1 Würzburg Institute of Systems Immunology, Max Planck Research Group at the Julius-Maximilians-Universität Würzburg, Würzburg, Germany
2 International Max Planck Research School for Immunobiology, Epigenetics, and Metabolism (IMPRS-IEM), Freiburg, Germany
3 Faculty of Biology, University of Freiburg, Freiburg, Germany

T lymphocytes have the ability to form long-lived memory cells after priming, giving rise to either circulating or resident populations that are functionally distinct. Circulating memory cells comprise stem-like central memory (TCM) cells that are able to re-expand and form new cells in case of reactivation. Tissue-resident memory (TRM) cells, on the other hand, are epigenetically and metabolic wired to a more terminally differentiated phenotype, having more restricted expansion capacity. Stemness in TCM is associated with the expression of the Wnt pathway mediator T Cell Factor 1 (Tcf1), a transcriptional factor that promotes expression of memory-associated genes and chromatin remodeling. TRM cells, on the other hand, upregulate a specific transcriptional program led by Homolog of Blimp1 in T cells (Hobit) that prevents tissue exit pathways and promotes tissue adhesion. Hobit has been suggested as a direct repressor of Tcf7, and the upregulation of Hobit in TRM cells drive effector differentiation and loss of stemness. By transferring TCR-transgenic Tcf1- and Hobit-reporter T cells into C57Bl/6 mice, we have identified a Tcf-1hi Hobithi TRM subset of CD8 T cells after acute viral infection that shows a different phenotype of Tcf-1- Hobithi TRM cells. We hypothesize that this population could serve as a local reservoir of stem-like TRM cells across tissues, being able to replenish and maintain the TRM pool over time. We now aim to functionally characterize these cells and to identify their location and niches within different tissues.

Lydia Dyck
Max Delbrück Center for Molecular Medicine
lydia.dyck@mdc-berlin.de

Analysis of mechanisms promoting T cell tolerance against autochthonous cancer in aged mice

Lydia Dyck[1#], Kathleen Anders[1,#*], Miha Milek[2], Elmehdi Belbaraka[1], Maria Teresa Norcia[1], Benedikt Obermayer[2], Dieter Beule[2,3], Inmaculada Martínez-Reyes[1], Thomas Blankenstein[1]

1 Molecular Immunology and Gene Therapy, Max Delbrück Center for Molecular Medicine, Berlin, Germany
2 Core Unit Bioinformatics Berlin Institute of Health at the Charité, Berlin, Germany
3 Max Delbrück Center for Molecular Medicine, Berlin, Germany
# Equal contribution
* Current address: Department of Pediatric Oncology and Hematology, Charité - Universitätsmedizin Berlin.

Chronic antigen exposure in solid tumors leads to T cell dysfunction and cancer progression. Cancer immunotherapies have demonstrated the induction of functional T cell responses in specific tumor models and human cancer types. However, the efficacy observed in mouse models with transplanted tumors often does not translate to the same degree in humans. To address this disparity, we have developed a transgenic, autochthonous tumor model called TTC, in which the oncogene and T cell antigen SV40 large T antigen (Tag) is induced by doxycycline (dox) from birth in cells with (a history of) tyrosinase expression. This model allows us to study the interplay between tumor-reactive T cells and tumor development over time. After 4 months on dox, TTC mice failed to mount an immune response against transplanted Tag-expressing cancer cells, indicating the onset of T cell dysfunction. Between 6-12 months on dox, TTC mice developed singular tumors. Transfer of dysfunctional TTC T cells into Rag-ko mice, followed by immunization, induced polyclonal expansion of Tag-reactive T cells. However, these cells failed to reject Tag-expressing tumor cells in vivo. Single cell TCR sequencing revealed a notable proportion of Tag-specific T cells in tumor-bearing TTC donor mice that were undetectable by Tag-directed tetramer staining. This suggests that tumor-specific dysfunctional T cells downregulate their TCRs. Consistent with this, the expression of several genes associated with the TCR complex (Cd3, Zap70, Cd8) was significantly downregulated in tumor-reactive CD8 T cells in TTC mice. This downregulation was reversed upon transfer into Rag-ko mice. Tumor-reactive CD8 T cells in autochthonous cancer-bearing mice predominantly exhibited an exhausted or progenitor-exhausted state, a phenotype they maintained after transfer and immunization. These findings indicate that, in addition to upregulation of inhibitory receptors, tumor-directed T cells downregulate TCR expression, which hampers T cell activation and potentially undermines the efficacy of immunotherapy.

Tamara Ehm
Ludwig-Maximilians-Universität München
tamara.ehm@campus.lmu.de

Decoding the COVID-19 Molecular Mosaic: Integrated Analyses of Protein Expression Dynamics and Histone Post-Translational Modifications in Circulating Immune Cells

Tamara Ehm[1], Claire Burny[2], Moritz Völker-Albert[2], Anne B. Krug[1]

1 Institute for Immunology, Biomedical Center (BMC), LMU Munich, Großhaderner Str. 9, D-82152 Planegg-Martinsried, Germany
2 MOLEQLAR Analytics - Am Klopferspitz 19, 82152 Planegg, Germany

The global response to the COVID-19 pandemic has generated an extensive volume of research data characterizing the immune response to SARS-CoV-2 in patient cohorts. However, a significant challenge remains to integrate diversedata sets and data modalities to gain a more comprehensive understanding. Our study leverages proteome and histone post-translational modification (PTM) data from peripheral blood mononuclear cells (PBMC) as well as plasma proteomedata from two patient cohorts. The first cohort comprises 24 hospitalized patients with or without COVID-19. The second multicentric cohort comprises 21 COVID-19 patientsmonitored over a 3-month time course. Results from the first cohort reveal protein expression and histone modification patterns specific for COVID-19 and highlight the heterogeneity among individual patients. Analysis of the results from the second longitudinal COVID-19 cohort allowed us to identify proteins and histone PTMs with different temporal dynamics. This analysis highlights protein expression and histone PTMs coregulated over time in mild, moderate and severe disease. By integrating histone PTMs and protein expression data in a longitudinal analysis, our study contributes to a more nuanced understanding of the dynamic molecular landscape associated with the systemic immune response in COVID-19 and the clinical outcome.

Carlota Farre Diaz
Max Delbrück Center
carlota.farreidiaz@mdc-berlin.de

Using sequential mutagenesis to model human multiple myeloma subgroups in mice

Farre Diaz, C.[1,2,3,4], Winkler W.[1,2,3,4], Janz M.[2,3,4], Rajewsky K.[1]

1 Immune Regulation and Cancer, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin 13125, Germany
2 Biology of Malignant Lymphomas, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin 13125, Germany
3 Experimental and Clinical Research Center, a cooperation between the Max Delbrück Center for Molecular Medicine in the Helmholtz Association and the Charité – Universitätsmedizin Berlin, Berlin 13125, Germany
4 Hematology, Oncology and Cancer Immunology, Charité – Universitätsmedizin Berlin, Berlin 13125, Germany

Multiple myeloma (MM) is a malignancy of germinal center (GC)-derived plasma cells (PCs) that shows a stepwise disease progression, mediated by the sequential acquisition of genetic aberrations. Until today, MM remains incurable due to its high genetic heterogeneity, that results in different prognosis and treatment response rates among patients. In recent work, we have developed subgroup-specific MM mouse models by the GC B cell-specific coactivation of mouse Cyclin D1 or MMSET, representing primary oncogenic translocation defects, along with constitutive NF-kB signaling (Ikk2ca) as a secondary genetic defect. Aged mice developed typical MM-like pathologies, with the expanded transgenic PCs exhibiting MM-subtype specific gene expression signatures1. Nonetheless, due to the simultaneous activation of both primary and secondary genetic events, these models do not fully recapitulate certain features of the human myeloma, such as the monoclonal outgrowth or the stepwise progression of the disease. To overcome these limitations, I use a sequential mutagenesis approach to develop a second generation of subgroup-specific MM mouse models, combining two recombinase technologies: the Cre/lox and the Dre/rox systems. Whereas Cre activates a primary mutation (here: Cyclin D1 or MMSET) early during the GC reaction upon B cell activation, the secondary mutation (here: Ikk2ca, Myc or KRasG12D) is induced later during terminal PC differentiation, using a tamoxifen (TAM)-inducible Dre recombinase (DreERT2), hence mimicking the stepwise acquisition of oncogenic events seen in human MM pathogenesis. Here I show that the dual recombinase approach is tight and functional in vivo. Based on these results, I have set up cohorts of compound mutant mice for distinct genetically defined MM subgroups.

1. Winkler, W., Farré Díaz, C., et al. (2023). Mouse models of human multiple myeloma subgroups. Proc. Natl Acad. Sci. USA. 120(10):e3319439120.

Johannes Fessler
Medical University of Graz
johannes.fessler@medunigraz.at

Combined analysis of JAK/STAT pathway activity and cytokine profiles aids to determine disease endotypes in Rheumatoid Arthritis

Dreo B[1], Muralikrishnan AS[1], Husic R[1], Lackner A[1], Brügmann T[1], Haudum P[1], Bosch P[1], Thiel J[1], Stradner MH[1], Fessler J[2]

1 Department of Rheumatology and Immunology, Medical University of Graz
2 Division of Immunology, Medical University of Graz

Objective: Rheumatoid Arthritis (RA) is a systemic autoimmune disease involving pro-inflammatory cytokines such as IL-6 and TNF-alpha. Blockade of cytokine signaling by monoclonal antibodies or kinase inhibitors, however, lead to significant reduction of disease activity only in roughly 40% of RA patients. This study aims to provide insights into active signaling pathways in peripheral blood leucocytes of RA patients and its possible application towards patient stratification.

Methods: The study included 62 RA patients and 9 healthy controls (HC). Phosphorylation of STAT 1-6 in various immune cell subsets was determined via flow cytometry. Serum concentrations of IL-6, IL-10, IL-12p70, IL-17A, interferon gamma, and TNF-alpha in the same samples were measured using highly sensitive single molecule array (SIMOA).

Results: We determined three endotypes of RA patients with active disease (cDAI > 10): 1) those with active STAT5a/b signaling in T cells, 2) those with a low STAT activity, and 3) those with active STAT1 and STAT3 signaling mainly in myeloid cells. Integrating intracellular STAT activation and cytokine analysis we present evidence for diminished JAK/STAT signaling despite elevated serum cytokine concentrations in a subset of patients.

Conclusion: Our data gives a possible explanation for the unresponsiveness to therapy targeting cytokine signaling in a subset of RA patients. Analysis of JAK/STAT phosphorylation could be useful to identify patients likely to respond to these therapies in future studies.

Lavinia Flaskamp
Biomedical Center Munich, Institute for Immunology, Ludwig-Maximilian-Universität
lavinia.flaskamp@med.lmu.de

In vivo detection and characterisation of phosphatidylserine positive extracellular vesicles

Lavinia Flaskamp[1], Wenbo Hu[1], Christine Ried[1], Jan Kranich[1], Thomas Brocker[1]

1 Biomedical Center Munich, Institute for Immunology, Ludwig-Maximilian-Universität

Phosphatidylserine (PS) is a phospholipid normally retained on the inner leaflet of cellular membranes. However, it has been shown to be exposed on the surface of apoptotic cells, activated platelets and importantly also on extracellular vesicles (EV). EV detection based on PS, using for example fluorescent Annexin V, has been widely established in vitro, but Ca2+-dependence makes its application in vivo challenging. In contrast, Milk fat globule-EGF factor 8 (MFG-E8) represents a Ca2+-independent PS-binding protein. Previously, we have used fluorescent MFG-E8, or its C1-domain, to detect EV-associated cells in vivo by imaging flow cytometry (IFC). Using this technique we have shown that cell-bound PS+ EVs are increased and provide adjuvant effects to activated T cells during viral infections. At the same time, circulating EVs were found to be reduced but the underlying mechanism and significance of this finding is unclear. In the present study we extended the application of C1-reagents to bind and detect circulating PS+ EVs in vivo. Our EV-labelling method could demonstrate that the great majority of plasma EVs is PS+, by a combination of IFC and superresolution microscopy. Moreover, circulating PS+ EVs have a half-life of ~1 hour whereas intra- and extracellular EVs persist on splenic B cells and macrophages even after 2 hours. This analysis platform and the knowledge on steady state EV turnover is essential to further investigate alterations in plasma EV composition and kinetics during viral infections.

João Augusto Freitas
Institute for Immunology, BMC/LMU
joao.freitas@med.uni-muenchen.de

The role of thymic B cells in central T cell tolerance

João Augusto Freitas[1], Dario Zebčević[1], Christine Federle[1], Nadia Khouja[1], Amina Sayed[1], Ludger Klein[1], Iliana Siamishi[1]

1 Institute for Immunology, BMC/LMU

Central T cell tolerance is essential for maintaining immune homeostasis and preventing autoimmunity. Traditionally, medullary thymic epithelial cells and dendritic cells are considered the key thymic antigen presenting cells (APCs) that promote the deletion (negative selection) or Treg cell differentiation of autoreactive thymocytes. We previously showed that the thymus also harbours a distinct population of B cells expressing Aire and exhibiting characteristics akin to activated B cells such as elevated CD80 and MHCII levels. These potent APC features of thymic B cells suggest that they may serve a non-redundant function in central T cell tolerance. To elucidate how thymic B cells contribute to shaping the T cell repertoire, we performed TCR sequencing of mature CD4 thymocytes and thymic Treg cells from B cell-deficient and -sufficient mice. This unveiled a significant divergence of the TCR repertoire between the two groups, indicating the B cell dependence of a substantial number of TCR clones. We identified TCR clonotypes that are either deleted from the conventional CD4 T cell repertoire, but also others that are diverted to the Treg repertoire in the presence of B cells. This is indicative of an impact of thymic B cells on both clonal deletion and diversion. Our findings unravel a pivotal role of thymic B cells in shaping the T cell repertoire.

Laura Marie Gail
CeMM/MedUni Wien
lgail@cemm.oeaw.ac.at

Single-cell dissection of tissue-immune regulation in cutaneous acute graft-versus-host disease

Laura Gail1[2*], Florian Deckert[2,3*], Luisa Unterluggauer[2], Ruth Dingelmaier-Hovorka[2], Johanna Strobl[2], Anna Redl[2], Lisa Ellen Shaw[2], Sylvia Knapp[3] , Matthias Farlik[2], Werner Rabitsch[4], Georg Stary[1,2]

1 CeMM Research Center for Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria
2 Department of Dermatology, MedUni Vienna, Austria
3 Department of Medicine I, MedUni Vienna, Austria
4 Department of Internal Medicine I, MedUni Vienna, Austria
* equal contribution

The skin contains tissue-resident memory T cells (TRMs), important for skin homeostasis but implicated in inflammatory processes, such as Graft-versus-Host Disease (GVHD). To date, there is a lack of adequate human models to study tissue-instructed T cell reprogramming and TRM involvement in tissue-specific disease mechanisms. To investigate the role of TRM in GVHD pathology, we study patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) and with acute GVHD (aGVHD). Before transplantation, these patients undergo myeloablative conditioning to eliminate existing immune cells. Host skin TRM can survive this treatment, and during immune recovery, transplanted donor T cells form new skin TRM. Cells of host and donor can be distinguished by genetic differences. We hypothesize, that comparing existing host and developing donor TRM timely after transplantation and later during GVHD, allows to identify pathways involved in regulating tissue residency and pathogenic signaling, and that interactions with structural skin cells and other immune cells are crucial in this regulation. Here, we collected longitudinal skin and blood samples of HSCT patients and aGVHD patients at diagnosis and performed single-cell RNA and TCR sequencing to define host/donor origin, uncover transcriptional changes in immune and non-immune cells, characterize dynamics of T cell clonality and assess cell-cell interactions in the skin. Among recovered T cells, few donor cells were the skin on day 14, whereas they dominated during aGVHD. We detected shared donor clones in skin and blood at day 100, wherein skin clones displayed a TRM gene expression profile. Disturbed TRM homeostasis and increased cytotoxic and proinflammatory signaling of T cells in aGVHD was accompanied by profound transcriptional changes in structural skin cells, including decreased expression of genes involved in extracellular matrix organization and upregulation of chemotactic molecules and antigen presentation pathways. Conclusively, our study has the potential to uncover new principles of tissue-immune cell interactions regulating immune reconstitution after HSCT and in GVHD-related inflammation.

Georg Gasteiger
Würzburg Institute of Systems Immunology
georg.gasteiger@uni-wuerzburg.de

Differentiation of innate and adaptive tissue-resident lymphocytes during ontogeny, infection and allergy

Tommaso Torcellan[1], Christin Friedrich[1], Emilia Vendelova[1], Remi Doucet-Ladeveze[1], Thomas Ossner[1], Ye Oyuang[1], Nicola Gagliani[2], Richard A. Flavell[3], Wolfgang Kastenmüller[1], Georg Gasteiger[1]

1 Würzburg Institute of Systems Immunology, Max-Planck Research Group at the Julius-Maximilians-Universität Würzburg, Germany
2 Hamburg Center for Translational Immunology (HCTI), University Medical Center Hamburg-Eppendorf, Hamburg, Germany
3 Department of Immunobiology, School of Medicine, Yale University, New Haven, CT 06520, USA

Innate lymphoid cells (ILC) and adaptive T cells populate diverse tissues as resident cells and acquire distinct organ-specific phenotypes. While ILCs establish tissue-residency as part of their innate developmental program during ontogeny, T cells require priming in lymphatic organs and subsequent recruitment into inflamed tissues. Here, we asked how the local pool of resident innate lymphocytes is reorganized in response to local infection (skin) or allergen challenge (lung). We found that viral and bacterial infections of the skin triggered the recruitment of circulating NK (cNK) cells and their differentiation into Tcf1hiCD69hi tissue-resident (trNK) cells that share transcriptional similarity with CD56brightTCF1hi NK cells in human tissues. Skin trNK arose from IFN-g-producing effector cells and required restricted expression of the transcriptional regulator Blimp1 to optimize Tcf1-dependent trNK formation. Upon secondary infection, trNK cells rapidly gained effector function and mediated an accelerated NK cell response. Thus, cNK cells redistribute and acquire tissue-residency at sites of previous infection via a "memory-like" mechanism that is distinct from Hobit-dependent developmental paths of NK cells and ILC1 seeding tissues during ontogeny. We will discuss similarities and differences in the mechanisms regulating innate and adaptive tissue-resident lymphocytes, and the reorganization of their niches during infection, and also in response to allergen challenge in the lung.

Carmen Gerlach
Division of Rheumatology, Department of Medicine Solna, Karolinska Institutet, Karolinska University Hospital, 17176 Stockholm, Sweden | Center for Molecular Medicine, Karolinska University Hospital, 17176 Stockholm, Sweden
carmen.gerlach@ki.se

CD8+ T cell differentiation and activation represent distinct axes of diversification

Anthonie Johan Zwijnenburg[1,2,*], Adam Gayoso[3,*], Natalia Ramirez Comet[1,2], Maria-Nefeli Christakopoulou[1], Aaron Streets[3,4,5,#], Nir Yosef[3,6,7,#], Carmen Gerlach[1,2,#]

1 Division of Rheumatology, Department of Medicine Solna, Karolinska Institutet, Karolinska University Hospital, 17176 Stockholm, Sweden.
2 Center for Molecular Medicine, Karolinska University Hospital, 17176 Stockholm, Sweden.
3 Center for Computational Biology, University of California, Berkeley, Berkeley, CA, USA.
4 Department of Bioengineering, University of California, Berkeley, Berkeley, CA, USA.
5 Chan Zuckerberg Biohub, San Francisco, CA, USA.
6 Department of Electrical Engineering and Computer Sciences, University of California, Berkeley, Berkeley, CA, USA.
7 Department of Systems Immunology, Weizmann Institute of Science, Rehovot, Israel.
* equal contribution, # equal contribution

Acute infections lead to the generation of a gradient of functionally distinct CD8+ T cell differentiation states, ranging from stem-like to highly cytotoxic cells. While stem-like and cytotoxic CD8+ T cells are present during both effector and memory phases of the response, albeit at opposite relative frequencies, it is unclear whether the response phase transition is associated with cellular changes that are independent of a T cell's differentiation state. We mapped the full spectrum of CD8+ T cell differentiation states during both effector and memory phase of an acute viral infection using CITEseq. Marker-guided cell sorting before hashed CITEseq enabled capturing sufficient transcriptional information from underrepresented differentiation states. Virus-specific CD8+ T cells were sampled from blood, spleen and lymph nodes for representation of the entire circulatory CD8+ T cell compartment. We showed that CD8+ T cell differentiation and the transition between effector and memory phase are molecularly distinct axes of CD8+ T cell diversification. This explains previous confusion about the lineage relationship of effector and memory T cells. We identified a transcriptional signature that classifies CD8+ T cells according their graded differentiation state, irrespective of response phase. Additionally, we identified a signature that distinguishes CD8+ T cells in effector phase from those in memory phase, irrespective of their differentiation state. The phase signature measures T cell activation status and distinguishes resting (naïve & antigen-experienced memory-phase cells) from recently activated CD8+ T cells. Translation of the transcriptional signatures to protein signatures allowed simple flow cytometry-based distinction between recently activated, i.e. responding, from bystander T cells in an antigen-agnostic manner. Disentangling CD8+ T cell differentiation state and activation status with our signatures demonstrated that while all cells, including the stem-like cells passed through a high-activation effector phase state, cells that reached the high-activation state first, differentiated furthest and remained longest in the high-activation state.

Tae-Young Gil
Kyung Hee University
sophia14t@gmail.com

Comparative effects of HME-DDS-nanocapsule astaxanthin from Hematococcus pluvialis in skin inflammatory environmental keratinocytes

Tae-Young Gil; Suji Ryu; Ha Yeon Lee; Jong-Suep Baek; Hyo-Jin An

Skin is the outermost barrier consisting of an immune-rich tissue. Through the skin, people get continual interactions with external surroundings and protection against environmental antigens. Therefore, regulating the skin immunological balance is one of the way of pivotal health conditions keeping the skin barrier. Astaxanthin is a representative antioxidant and anti-inflammatory compound. It is found in various microorganism sources including microalgae being applied as a pigment in food, cosmetic materials, or pharmaceutical agents. However, the low stability and fastidious characteristics of astaxanthin unfortunately cause to difficult utilization despite of its different physiological functions. The authors applied a way of developed and specialized microalgae leading higher content of astaxanthin. Hot melt extrusion (HME)-drug delivery system (DDS)-processing was performed to make nanocapsule containing high-content of astaxanthin from microalgae such as Hematococcus pluvialis (HME-DDS-NAH). The process is accompanied with purpose of enhancing drug delivery targeting functional food keeping the pharmaceutical features. We investigated the effects of HME-DDS-NAH in two kinds of cell line; murine macrophages and human keratinocytes. The aim of the study was focused on the systemic and skin immunological responses. We figured out the suppressing effects of HME-DDS-NAH in the production of typical pro-inflammatory cytokines like interleukin (IL)-6 or tumor necrosis factor (TNF)-α in the cells. Also pivotal skin barrier components like involucrin or filaggrin were recovered by treatment of HME-DDS-NAH or non-processed one in keratinocytes. Above all, we compared the effects of HME-DDS-NAH with non-HME-DDS-treated microalgae material. With these results, the authors figured out the differences between processed one and non-processed one and their different results on the immunological responses.

This research supported by Korea Institute of Marine Science & Technology Promotion (KIMST) funded by the Ministry of Oceans and Fisheries (grant \# RS-2023-00254674).

Ari Glasner
Technion - Israel institute of technology
ari.glasner@technion.com

Conserved transcriptional connectivity of regulatory T cells in the tumor microenvironment informs new combination cancer therapy strategies

Ariella Glasner, Samuel A. Rose, Roshan Sharma, Herman Gudjonson, Tinyi Chu, Jesse A. Green, Sham Rampersaud, Izabella K. Valdez, Emma S. Andretta, Bahawar S. Dhillon, Michail Schizas, Stanislav Dikiy, Alejandra Mendoza, Wei Hu, Zhong-Min Wang, Ojasvi Chaudhary, Tianhao Xu, Linas Mazutis, Gabrielle Rizzuto, Alvaro Quintanal-Villalonga, Parvathy Manoj, Elisa de Stanchina, Charles M. Rudin, Dana Pe'er & Alexander Y. Rudensky

While regulatory T (Treg) cells are traditionally viewed as professional suppressors of antigen presenting cells and effector T cells in both autoimmunity and cancer, recent findings of distinct Treg cell functions in tissue maintenance suggest that their regulatory purview extends to a wider range of cells and is broader than previously assumed. To elucidate tumoral Treg cell 'connectivity' to diverse tumor-supporting accessory cell types, we explored immediate early changes in their single-cell transcriptomes upon punctual Treg cell depletion in experimental lung cancer and injury-induced inflammation. Before any notable T cell activation and inflammation, fibroblasts, endothelial and myeloid cells exhibited pronounced changes in their gene expression in both cancer and injury settings. Factor analysis revealed shared Treg cell-dependent gene programs, foremost, prominent upregulation of VEGF and CCR2 signaling-related genes upon Treg cell deprivation in either setting, as well as in Treg cell-poor versus Treg cell-rich human lung adenocarcinomas. Accordingly, punctual Treg cell depletion combined with short-term VEGF blockade showed markedly improved control of PD-1 blockade-resistant lung adenocarcinoma progression in mice compared to the corresponding monotherapies, highlighting a promising factor-based querying approach to elucidating new rational combination treatments of solid organ cancers.

Willian Gomes
University of Sao Paulo
williangomes@usp.br

Immune disturbances in short telomere syndromes include dysregulation of unconventional T cells

Willian Gomes[1], Shan Hama[2], Vinicius Carvalho[1], Mohammad Karimi[2], Giorgio Napolitani[2], Ghulam Mufti[2], Rodrigo Calado[1]

1 Department of Medical Imaging, Hematology, and Clinical Oncology, Ribeirão Preto School of Medicine, University of São Paulo (USP), Ribeirão Preto, SP, Brazil
2 Comprehensive Cancer Centre, King's College London, London, United Kingdom

Telomere-biology disorders (TBD) are characterized by short telomeres and dysfunctional telomere repair machinery, which cause bone marrow failure, liver and lung fibrosis. Previous studies have proposed that TBD patients may have CD4+ T cells immunodeficiency, but no comprehensive investigation of minor subsets has been published to date. The aim of the present study was to deep-phenotype PBMCs of patients with TBDs and characterize the subsets of conventional and unconventional T cells, B cells, monocytes and dendritic cells. For that, 20 individuals diagnosed with pathogenic variants in telomere-biology genes and 10 healthy volunteers were recruited. CyTOF was performed with a customized 39-marker panel, and data was analyzed by FlowSOM and viSNE. Results showed significantly lower frequency of CD4+ lymphocytes and decreased CD4/CD8 ratio in patients. B cells count was also reduced. CD95 expression was inversely correlated with CD4+ and B cells, but not with CD8+ T cells, indicating that an enhanced apoptosis may be favoring the decline of the first two subsets. All naïve subsets (CD4+, CD8+ and B) were decreased in patients. TH1, TH17 and TH17.1 CD4+ lymphocytes were reduced. Classical monocytes expressing CXCR3 and CCR4 were found in a higher frequency in patients. Gamma-delta T cells were present in a frequency below 0.5% in half of TBD samples, and was significantly lower in those with the shortest telomere lengths. Double-negative T cells were significantly enriched in patients, being almost five times higher than in healthy individuals. Mucosal-associated invariant T cells (MAIT) were very reduced in patients, being found in a frequency below 0.05% in 55% of TBD samples. Additional flow-cytometry was then performed in new samples, and revealed that the remaining MAIT cells were highly activated and exhausted in patients, with an overexpression of HLA-DR and CD38. Our data indicate immune disturbances in several subsets of cells, which could contribute to the clinical onset of telomeropathies and increase the risk of infection, fibrosis and solid cancers.

Pablo Gonzalez-Garcia
University of Cádiz
pablo.gonzalezgarcia@alum.uca.es

Chimeric Antigen Receptors (CAR) Discordant Signaling Potentially Impacts Therapeutical Outcomes: ACE2-Based CAR-Like T Cells as Model of Study

Pablo Gonzalez-Garcia[1], Noelia Moares[1], Juan P. Muñoz-Miranda[1], Ricardo Fernandez-Cisnal[1], Antonio Gabucio[1], Rosa Luna-Espejo[1], Javier Ocaña-Cuestas[1], Lucia Olvera[1], Cecilia Fernandez-Ponce[1] and Francisco Garcia-Cozar[1]

1 University of Cádiz

The development of Chimeric Antigen Receptor (CAR)-T cells rapidly emerged as one of the major assets in the field of immunotherapy against malignant cancers. Despite the great success of the therapy against hematological tumors, achieving equivalent results in the treatment of solid tumors or infectious diseases remains beyond the reach of T cell engineering due to the inability of CAR-T cells to fully mimic TCR signaling, a direct consequence of the primitive architecture of these chimeric constructs. Recent studies highlight that the disorganization of the immune synapse given by CAR-antigen engagement, delayed early phosphorylation and calcium release events and sustained promoter activities are some of the early manifestations of these signaling mismatches, which in turn could be the explanation for some of the alterations observed in CAR-T cells, such as Cytokine Release Syndrome (CRS) and T-cell exhaustion. In this line, we have recently designed a CAR-like receptor based on the human ACE2 receptor for the recognition of the SARS-CoV-2 trimeric Spike protein and, upon stimulation, we have observed that it exhibits delayed and/or sustained activation compared to that of endogenous TCR-CD28 costimulation, highlighting these discordances. Therefore, our study aims to lay the groundwork for future studies in the CAR manufacturing process in order to obtain more physiological responses that will broaden the spectrum of applications of this therapy towards solid tumors and infectious diseases such as COVID-19.

Daniel Greenwood
Institute of Molecular Systems Biology, ETH Zurich
dgreenwood@ethz.ch

Mapping the heterogeneity of NF-kB response in primary human immune cells with deep-learning assisted high content microscopy

Dr. Daniel Greenwood, Dr. Yannik Severin, Ellen Arts, Yasmin Festl, Prof. Dr. Berend Snijder

Stimulation of immune cells causes NF-kB family transcription factors (TFs) to rapidly translocate to the nucleus, a process that can be observed by fluorescence microscopy. NF-kB induces the expression of both inflammatory response genes and its own inhibitors, leading to a similarly rapid loss of nuclear NF-kB. Despite the diverse functions of NF-kB signaling across cell types, we lack an understanding of NF-kB dynamics in mixed populations of interacting primary human immune cells, in part because their diverse sizes and morphologies make accurate image quantification difficult. We therefore trained a convolutional neural network (CNN) to quantify the nuclear localisation of immunofluorescently labeled (TFs) in high-content microscopy. This CNN achieved 98.2% accuracy across diverse peripheral blood mononuclear cell (PBMC) subsets - compared with only 81.6% accuracy using a standard cell segmentation technique. Notably, this classifier maintains high accuracy across cell types regardless of morphology or treatment. Combining this CNN-based transcription factor activity readout with lineage markers, we profiled the NF-kB response of PBMCs to LPS and a panel of cytokines in a cohort of healthy donors with single-cell resolution. Our analysis revealed significant donor-dependent variability in the magnitude and duration of NF-kB responses, strongly correlating with age. Furthermore, we found that the peak of NF-kB activation strongly predicts activation-induced physical cell-cell interactions both among monocytes and among T cells; phenotypes that persist after NF-kB localisation returns to baseline. Leveraging monocyte-monocyte interactions as an indicator of NF-kB response, we explored existing gene expression and protein-protein interaction datasets to identify the surface receptors that influence monocyte sensitivity to LPS. This work provides a novel methodology to study NF-kB dynamics in heterogeneous cell populations and uncovers inter-individual differences in immune response, with potential implications for personalized medicine.

Thomas Herrmann
Institute for Virology and Immunobiology, Julius-Maximilians-Universität Würzburg
thomas.herrmann@uni-wuerzburg.de

Division of labor and cooperation between different butyrophilin proteins controls phosphoantigen-mediated activation of human γδ T cells

Herrmann T[1], Subramanian H[2,3], Jin Y[4], Mohammed F[5], Kimmel B[6], Juraske C[7–10], Starick L[1], Nöhren A[1], Länder N[1], Willcox CR[5], Singh R[4], Schamel WW[7-10], Nikolaev VO[2,3], Kunzmann V[6], Wiemer AJ[4], Willcox BE[5], Karunakaran MM[1]

1 Institute for Virology und Immunobiology, University of Würzburg, Würzburg, Germany
2 Institute of Experimental Cardiovascular Research, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
3 DZHK (German Centre for Cardiovascular Research), partner site Hamburg/Kiel/Lübeck, Germany
4 Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269, USA; Institute for Systems Genomics, University of Connecticut, Storrs, CT 06269, USA
5 Institute of Immunology and Immunotherapy, University of Birmingham, UK
6 University Hospital Wuerzburg, Department of Internal Medicine II and Comprehensive Cancer Center (CCC) Mainfranken Wuerzburg, Wuerzburg Germany
7 Signaling Research Centers BIOSS and CIBSS, University of Freiburg, Freiburg, Germany
8 Department of Immunology, Faculty of Biology, University of Freiburg, Freiburg, Germany
9 Centre for Chronic Immunodeficiency (CCI), Faculty of Medicine, University of Freiburg, Freiburg, Germany
10 Spemann Graduate School of Biology and Medicine (SGBM), University of Freiburg, Freiburg, Germany

Butyrophilin (BTN)–3A and BTN2A1 molecules control the activation of human Vγ9Vδ2 T cells during T cell receptor (TCR)-mediated sensing of phosphoantigens (PAg) derived from microbes and tumors. However, the molecular rules governing PAg sensing remain largely unknown. Based on comparison of Vγ9Vδ2 TCR and BTN molecules of humans and alpaca – the so far only known non-primate species with PAg-responding Vγ9Vδ2 T cells, we analyzed the contribution of the human PAg-binding BTN3A1-moleule and its PAg-non-binding homologues BTN3A2 and BTN3A3 by reconstituting BTN3A deficient cells with various combination of BTN molecules, deletion variants and mutants. Apart from confirming and extending the crucial role for cooperation between the different BTN3A molecules for induction of a PAg-response, we established three mechanistic principles of PAg-mediated γδ T cell activation. First, in humans, following PAg binding to the intracellular BTN3A1-B30.2 domain, Vγ9Vδ2 TCR triggering involves the extracellular V-domain of BTN3A2/BTN3A3. Moreover, the localization of both protein domains on different chains of the BTN3A homo-or heteromers is essential for efficient PAg-mediated activation. Second, the formation of BTN3A homo-or heteromers, which differ in intracellular trafficking and conformation, is controlled by molecular interactions between the juxtamembrane regions of the BTN3A chains. Finally, the ability of PAg not simply to bind BTN3A-B30.2, but to promote its subsequent interaction with the BTN2A1-B30.2 domain, is essential for T-cell activation. Defining these determinants of cooperation and the division of labor in BTN proteins improves our understanding of PAg sensing and elucidates a mode of action that may apply to other BTN family members. Future experiments will show to which extend the different BTN domains and the interaction between BTN3A and BTN2A1 molecules are involved in other recently described features of BTN3 molecules such as suppression of peptide specific αβ T cell responses by BTN3A1 or cell autonomous resistance to avian influenza by BTN3A3.

Hannah Hipp
Helmholtz Munich
hannah.hipp@helmholtz-munich.de

Inhibition of pyrimidine de novo synthesis fosters Treg cells and reduces islet autoimmunity in models of Type 1 Diabetes utor einfügen: Hanna Gmehling1,2

Hannah Hipp[1,2], Lena Münch[3], Camilla Tondello[3], Hanna Gmehling[1,2] Evelyn Peelen[4], Hella Kohlhof[4], Urs Christen[3], Carolin Daniel[1,2,5*]

1 Reseach Unit Type 1 Diabetes Immunology, Helmholtz Diabetes Center, Helmholtz Munich, Munich, Germany,
2 Deutsches Zentrum für Diabetesforschung, Neuherberg, Germany,
3 Pharmazentrum Frankfurt, Goethe University Frankfurt, Frankfurt am Main, Germany,
4 Immunic AG, Lochhamer Schlag 21, Gräfelfing, Germany,
5 Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-Universität München, Munich, Germany.
* Corresponding author

In autoimmune Type 1 diabetes (T1D), a loss of immune tolerance and aberrant immune activation promotes the progressive destruction of insulin-producing beta cells in the pancreas. Regulatory T cells (Tregs) are critical cellular mediators of peripheral tolerance. We and others have identified critical Treg impairments during ongoing islet autoimmunity which highlights the challenge for the development of efficacious immunotherapeutic concepts. Regulatory mechanisms are hindered, among others, by the strong immune activation during islet autoimmunity and T1D. Reducing immune activation might represent a relevant strategy to efficiently target Tregs by broadening the window of opportunity to induce and strengthen Tregs. Therefore, we study the drug candidates IMU-838 and IMU-935 (Immunic AG, Germany). IMU-838 is a dihydroorotate dehydrogenase (DHODH) inhibitor, an enzyme which catalyzes the *de novo* pyrimidine synthesis and is essential for activated lymphocytes. IMU-935 is a potent RORγt inverse agonist, while it can also inhibit, to a lower extent, DHODH. We show that under challenging conditions that resemble autoimmune activation both compounds enhanced Treg induction (% Tregs: vehicle 3.0±1.8%, IMU-935 5.1±2.2%; vehicle 2.7±1.6%, IMU-838 6.2±3.1%). More detailed analyses showed that the modulation of *in vitro* Treg induction by the drug candidates was dependent on DHODH inhibition. In a model of accelerated T1D induced by adoptive transfer treatment with IMU-838 resulted in reduced incidences of T1D (hyperglycemia by day 10-12: vehicle 100%, IMU-828 7.1%) with reduced frequencies of activated T cells (vehicle 95.1±2.3%, IMU-838 82.8±13.3%) while Treg frequencies were increased in pancreatic LN (vehicle 0.5±0.4%, IMU-838 1.1±1%). Reduced incidences of T1D were confirmed in a virus-induced T1D model using RIP-GP mice (hyperglycemia by day 28: vehicle 81.0%, IMU-838 23.5%). Overall, these results show that DHODH inhibition can reduce immune activation while fostering Tregs during islet autoimmunity and overt T1D.

Tzu-Sheng Hsu
Institute of Molecular & Cellular Biology, National Tsing Hua University, Hsinchu, Taiwan
tshsu@life.nthu.edu.tw

Hypoxia-inducible factor-2α (HIF-2α) facilitates regulatory T cell suppressive capacity through HIF-1α inhibition

Tzu-Sheng Hsu[1], Yen-Lin Lin[2], Su-Ting Mo[2], Yu-An Wang[2] and Ming-Zong Lai[2]

1 Institute of Molecular & Cellular Biology, National Tsing Hua University, Hsinchu, Taiwan
2 Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan

Hypoxia-inducible factor 1α (HIF-1α) and HIF-2α are pivotal transcription factors in cellular adaptation to hypoxic conditions specifically focusing on their impact within regulatory T (Treg) cells. Our findings reveal that the development of Treg cells proceeds normally in mice engineered to lack either HIF-1α or HIF-2α in the Foxp3-positive cells. However, a critical functional disparity emerges in Treg cells deficient in HIF-2α. These cells demonstrate a marked deficiency in mitigating effector T cell-triggered colitis and in curtailing airway hypersensitivity, in contrast to the HIF-1α-deficient counterparts. We observe an intriguing propensity of HIF-2α-deficient Treg cells to undergo reprogramming into IL-17-secreting cells. Interestingly, in the absence of HIF-2α, there is an upregulation of HIF-1α in Treg cells, and the subsequent removal of HIF-1α from these cells reinstates their suppressive capabilities. Mice with a conditional Foxp3-specific knockout of HIF-2α exhibit enhanced resistance to the progression of MC38 colon adenocarcinoma and the metastasis of B16F10 melanoma. These insights collectively underscore the pivotal role of HIF-2α in the functional dynamics of Treg cells and its potential as a target in cancer therapeutics.

Franc Hysenaj
Helmholtz Zentrum München
franc.hysenaj@helmholtz-munich.de

Functional role of T-cell specific inceptor expression in metabolic tissues

Franc Hysenaj[1,2,3], Emily Schardey[1,2],Maike Becker[1,2], Isabelle Serr[1,2] and Carolin Daniel[1,2,3]

1 Type 1 Diabetes Immunology, Helmholtz Diabetes Center at Helmholtz Zentrum München, Munich, Germany
2 Deutsches Zentrum für Diabetesforschung, Neuherberg, Germany
3 Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-Universität München, Munich

The role of insulin signaling in T cells extends beyond basic regulation of cellular metabolism, and it has been shown that T cell-specific deletion of the insulin receptor results in decreased cytokine production and effector differentiation of T cells. The insulin inhibitory receptor (inceptor) has recently been identified as a negative regulator of insulin signaling on pancreatic β-cells. Using Cre/LoxP technology to generate T cell-specific inceptor knockout (TKO) mice and high-throughput single-cell RNA sequencing (scRNA-seq), this study investigates the role of inceptor in the differentiation of T cells into specific effector subtypes. First, we observed a significant increase in effector memory T cells in the pancreas of TKO mice compared to control mice (TKO vs. flox: 61.1±12.0% vs. 48.8±11.2%, p=0.035). FACS sorted pancreatic CD45+ immune cells from inceptor TKO and floxed mice were subsequently sequenced. ScRNA-seq analysis revealed a prominent Th2-like cluster present only in inceptor TKO mice. These Th2 cells displayed a broad cytokine profile characterized by the simultaneous production of Il4, Il5 and Il13. In addition, the partial or complete loss of Cd27, Cd28 and Ctla4 observed in these Th2 cells indicated a mature, highly differentiated state with potentially altered functional capacities. Notably, these Th2 cells expressed Irs2, a signaling molecule involved in the Il4 pathway. This upregulation of Irs2 upon inceptor knockout was consistent with the original inceptor publication. Il4-mediated signaling, a critical driver for the differentiation of naive Cd4+ T cells into Cd4+ Th2 effector cells, is significantly influenced by the presence of Irs2. In the absence of Irs2, the number of Il4 producing cells is reduced and Il5 production is impaired, highlighting the essential role of Irs2 in Th2 cell differentiation and cytokine production. With these initial findings, we aim to deepen our understanding of how insulin-related signaling impinges on the control of phenotypic adaptations of T-cells in metabolic tissues.

Borja Jimenez Lasheras
CIC Biogune
bjimenez@cicbiogune.es

Neddylation as a relevant agent in metabolism and antitumor capacity of CD8+ T cells

Borja Jimenez-Lasheras[1], Leire Egia-Mendikute[1], Asier Antoñana-Vildosola[1], So Young Lee[1], Samanta Romina Zanetti[1], Paloma Velasco Beltrán[1], Ander de Blas Martín[1], Endika Prieto-Fernández[1], Ana García Del Río[1], Alexandre Bosch[1], Lorena Pérez-Gutiérrez[1], Jone Etxaniz-Díaz de Durana[1], Marina Serrano-Maciá[2], Naroa Goikoetxea[2], María Luz Martínez-Chantar[2], Sebastian Peer[3], Thomas Gruber[3], Gottfried Baier[3], Asís Palazón[1,4,*]

1 Cancer Immunology and Immunotherapy Lab, Centre for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Bizkaia Technology Park, Building 801A, Derio, Spain
2 Liver Disease Laboratory, Center for Cooperative Research in Biosciences (CIC bioGUNE, Basque Research and Technology Alliance (BRTA), Bizkaia Technology Park, Derio, Spain
3 Division of Translational Cell Genetics, Department for Pharmacology and Genetics, Medical University Innsbruck, Innsbruck, Austria
4 Ikerbasque, Basque Foundation for Science, Bilbao, Spain
* Corresponding author: Asis Palazon (apalazon@cicbiogune.es)

Introduction: Neddylation is a post-translational modification (PTM) by which the ubiquitin-like protein Nedd8 is covalently attached to target proteins. This pathway participates in the regulation of protein homeostasis, cell stress and survival. Of interest, neddylation is often upregulated in tumor cells, offering opportunities for the treatment of cancer with neddylation inhibitors (e.g. mln4924). However, the relevance of neddylation in CD8+ T cell-mediated anti-tumor responses remains largely unexplored.

Methods: Analysis of publicly available scRNA-seq datasets included tumor-infiltrating CD8+ T cells (TILs) from four distinct tumor types. We generated a conditional knockout mouse lacking Nedd8 activating enzyme (Nae1-KO) exclusively in T cells, and proteomic analysis was performed. Phenotype of CD8+ TILs and mice survival was assessed in in vivo tumor assays. Pull down of neddylated proteins was examined by proteomic analysis and targets were validated by immunoprecipitation (IP).

Results: Single cell transcriptomic analyses revealed that Nedd8 expression is upregulated in TILs during differentiation into effector cells. In vitro, neddylation was increased upon activation of mouse and human CD8+ T cells. Neddylation was strongly inhibited in Nae1-KO CD8+ T cells. Proteomic analysis of Nae1-KO CD8+ T cells showed a decrease in glycolytic capacity and T cell receptor signalling pathway. In vivo tumor studies reported that Nae1-KO CD8+ TILs are less activated and differentiated, which resulted in impaired mice survival after tumor challenge. IP of neddylated proteins in human CD8+ T cells unravelled several novel Nedd8 interactors, all of which are relevant metabolic enzymes.

Conclusion: Neddylation is required for the differentiation of CD8+ TILs into effector cells and for their antitumoral function. Moreover, CD8+ T cells with impaired neddylation have decreased glycolytic function. We identified important glycolytic enzymes as novel Nedd8 targets. In this context, we propose neddylation as an emerging modulator of CD8+ T cell metabolism and function.

Katarzyna Jobin
University of Wuerzburg
katarzyna.jobin@uni-wuerzburg.de

Post priming long-term T cell interaction niches scale CD8 and CAR Teff formation

Katarzyna Jobin[1*], Deeksha Seetharama[1*], Lennart Rüttger[1], Chloe Fenton[1], Annerose Wirsching[1], Anfei Huang[1], Tsuneyasu Kaisho[2], Konrad Knöpper[1], Antoine-Emmanuel Saliba[3], Georg Gasteiger[1], Frederick Graw[4], Alain Pulfer[5], Santiago Gonzalez[5], Milas Ugur[1] and Wolfgang Kastenmüller[1]

1 Würzburg Institute of Systems Immunology, Max Planck Research Group at the Julius-Maximilians-Universität Würzburg; Würzburg, Germany
2 Department of Immunology, Institute of Advanced Medicine, Wakayama Medical University; Wakayama, Japan
3 Helmholtz Institute for RNA-based Infection Research (HIRI), Helmholtz-Center for Infection Research (HZI); Würzburg, Germany
4 Department of Internal Medicine , Hematology and Oncology, Friedrich Alexander University Erlangen-Nuremberg (FAU) and Universitätsklinikum Erlangen, Erlangen, Germany
5 Institute for Research in Biomedicine, Faculty of Biomedical Sciences, USI—Switzerland

T cell priming is characterized by three distinct phases. A search phase with short-term interactions with DC, the activation phase with long-term interactions and finally disengagement from DC which is followed by T cell proliferation. During this third phase T cells recommence their migratory activity and lose their TCR responsiveness based on altered Ca2+ signaling. However, following viral infection, we suspected that additional T cell activation events could take place at later time points in the deep paracortex. Indeed, we found that activated CD8 T cells re-engaged and formed stable clusters with DC-networks for several hours on d3 after infection in deep paracortex. Consistently, these CD8 T cells maintained their TCR responsiveness and Ca2+ signaling capacity. Access to these post-priming niches required the chemokine receptor CXCR3, allowing activated CD8 T cells to sense CD4 T cell derived IL2. Accordingly, activated CD4 T cells were also found in these niches, but their migratory behavior differed from that of CD8 T cells. In particular, CD4 T cells showed a more dynamic behavior with shorter DC interactions, allowing them to interact with multiple DC and to provide IL2 at several DC-interfaces. This dynamic behavior further contrasted with Treg cells that did not arrest and did not require CXCR3 to access the post-priming niche, but nevertheless controlled CD8 T cell differentiation by limiting IL2. Together, we identified a post-priming niche that regulates the scaling of CD8 Teff cell differentiation with an unexpected and divergent migratory behavior of the involved T cell populations. Importantly, we found that CD19-CAR T cells failed to fully differentiate into Teff cells but could be rescued by active recruitment into these niches. Our results underscore the notion of a tight spatial regulation of critical signals during the developing cellular immune response. Optimization for their access can be exploited for cellular therapies.

Till Johannsmann
Type 1 Diabetes Immunology Helmholtz Munich (TDI)
till.johannsmann@helmholtz-munich.de

Novel inducers of regulatory T cells to control autoimmune Type 1 Diabetes

Till Johannsmann[1,2], Maike Becker[1,2], Kenji Schorpp[3], Kamyar Hadian[3], Carolin Daniel[1,2,4]

1 Type 1 Diabetes Immunology (TDI), Helmholtz Diabetes Center at Helmholtz Munich, Munich, Germany.
2 German Center for Diabetes Research (DZD), Munich, Germany.
3 Drug Screening and Assay Development Platform, Helmholtz Munich, Munich, Germany.
4 Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-Universität München, Munich, Germany.

Novel intervention strategies are needed to counteract the rising incidences of autoimmune diseases such as Type 1 Diabetes (T1D). An imbalance between autoimmune activation and tolerance results in the immune-mediated destruction of insulin-producing β-cells in the pancreas and the progression to clinical T1D. Regulatory T cells (Tregs), key cellular mediators of immune tolerance, are critically impaired during islet autoimmunity. Pharmacologically increasing Tregs could counteract the observed impaired Treg induction and overshooting T cell activation in T1D, offering a promising approach for future interventions. In a high-throughput screening of 25,000 compounds using an antigen-specific Treg induction assay, we identified 54 hits that significantly increased Treg frequencies. Seven compounds were independently confirmed using polyclonal Treg induction. In subsequent assays, we challenged the Treg induction potential of these compounds by mimicking strong immune activation *in vitro*. Notably, the known Treg inducer everolimus was ineffective (2-fold increase), whereas the newly identified compounds, especially our top-candidate G, demonstrated superior Treg induction potential (10-fold increase). In murine models of T1D, top-candidate G enhanced antigen-specific Treg induction *in vitro* from T cells recognizing a mimotope of the T1D-relevant autoantigen Chromogranin A 29-42 (5-fold increase, $p<0.05$). Strikingly, even under T_H1 (4-fold increase, $p<0.01$) and T_H17 (6-fold increase, $p<0.0001$) polarizing conditions, top-candidate G significantly fostered Treg induction *in vitro*. A first *in vivo* trial of the top-candidate G in non-obese diabetic mice indicated higher Treg frequencies in the pancreas. Phenotypically, these pancreatic Tregs showed trends towards increased tissue-residency and activation. To increase potency, the target identification and hit-to-lead optimization for the top-candidate G are the critical next steps. All these findings indicate that an optimized novel compound could function as a next-generation Treg inducer suppressing aberrant immune activation in T1D.

Eleni Kabrani
Max Delbrück Center for Molecular Medicine
eleni.kabrani@mdc-berlin.de

High-order oligomerization of 53BP1 is essential for productive V(D)J Recombination and CSR

Kabrani E.[1*], Saha T.[1*], Ebeling S.[1], Rüster T.[1], Keller L.[1], Shang Y.[2], Meng FL.[2] and Di Virgilio M.[1,3]

1 Laboratory of Genome Diversification and Integrity, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin 13125, Germany
2 State Key Laboratory of Molecular Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, China
3 Charité-Universitätsmedizin Berlin, Berlin 10117, Germany
* These authors contributed equally

53BP1 is a multidomain protein well studied for its role in DNA double-strand break (DSB) end protection. Following ATM-mediated phosphorylation, 53BP1 recruits RIF1, the Shieldin and CST protein complexes to DSBs, where they counteract nucleolytic end resection, hence channeling the repair process through the non-homologous end joining (NHEJ) pathway. B lymphocytes depend on NHEJ to repair DSBs formed during V(D)J recombination and Class Switch Recombination (CSR). While previous studies have shown that 53BP1's oligomerization domain is essential for successful CSR, its role during V(D)J recombination remains elusive. Here, we describe a novel mouse model, where the core of five aminoacid (ITDVY) motif of 53BP1's oligomerization domain is deleted (Trp53bp1 Delta (Δ) core allele). Despite the fact that Trp53bp1 $^{\Delta core/\Delta core}$ mice express 53BP1 at physiological levels, they exhibit a mild arrest at the pro-B cell stage in the bone marrow and DN3-T cell stage in the thymus. Interestingly, the combination of the Dcore mutation with a NHEJ-deficient background (Trp53bp1 $^{\Delta core/\Delta core}$; Xlf$^{-/-}$) results in a severely immunocompromised phenotype, similar to the complete loss of 53BP1 protein (Trp53bp1$^{-/-}$; Xlf$^{-/-}$). We conclude, that high-order oligomerization of 53BP1 is essential to exert its function during both V(D)J recombination and CSR.

Julia Kargl
Medical University of Graz
julia.kargl@medunigraz.at

Myeloperoxidase creates an immunosuppressive tumor microenvironment in non-small cell lung cancer by altering T cell function

Anna Lueger[1], Paulina Valadez-Cosmes[1], Kathrin Maitz[1], Nejra Cosic Mujkanovic[1], Oliver Kindler[1], Julia Kargl[1]

1 Medical University of Graz

Background: The tumor microenvironment (TME) of non-small cell lung cancer (NSCLC) involves high infiltration of immune cells, including neutrophils. These neutrophils contribute to the complexity of the TME by releasing myeloperoxidase (MPO) upon activation and degranulation. In the presence of H2O2, MPO generates HOCl, a highly reactive molecule that can cause damage to proteins, lipids and DNA. In this study, we investigated the functional role of MPO in the NSCLC and its effect on T cells within the TME. We hypothesize that MPO in the TME may alter T cell activation and function, ultimately leading to an immunosuppressive TME.

Methods: We studied MPO knock-out mice in a flank tumor mouse model. Additionally, we conducted in vitro experiments using recombinant MPO treatments to analyze the impact of MPO on T cells.

Results: MPO knock-out mice exhibited reduced tumor growth compared to WT controls. This decrease in tumor growth was accompanied by an increase in lymphocyte populations, including natural killer cells (NKs) and CD8+ T cells. Furthermore, MPO knock-out mice demonstrated enhanced expression of IFN-γ by T cells. In vitro experiments also revealed that CD8+ T cells treated with MPO exhibited reduced proliferation and production of IFN-γ.

Conclusion: Our findings indicate that the deletion of MPO promotes an anti-tumorigenic immune environment in a mouse tumor model, characterized by an increase in CD8+ T cells and heightened expression of IFN-γ. Additionally, MPO negatively affects function of anti-tumor T cells, supported by in vitro experiments demonstrating decreased proliferation and IFN-γ expression of CD8+ T cells after MPO treatment. These results suggest that MPO contributes to tumor growth and exhibits an immunosuppressive role in NSCLC. Consequently, MPO might serve as a potential target for lung cancer therapies, aiming to counteract its immunosuppressive effects in NSCLC.

Janine Kemming
DTU
jsoke@dtu.dk

Deciphering TCR independent, KIR-HLA-C interactions on KIR+CD8+ T cells

Janine Kemming[1], Annie Borch[1], Andreas Kongsgaard[1], Sunil Kumar Saini[1], Sine Reker Hadrup[1]

1 Department of Health Technology, Technical University of Denmark, Lyngby, Denmark

While the role of Killer immunoglobulin-like receptors (KIRs) as important receptors for antigen recognition and function of Natural killer (NK) cells has long been established, their expression on a subset of CD8+ T cells has only recently been described and increased percentages of these cells were found in a variety of autoimmune and infectious diseases. However, little is known about the antigens these CD8+ T cells recognize via their KIR and consequently the phenotype, functionality and therapeutic potential these cells may harbor, warranting future research efforts in this direction. Here, we comprehensively analyzed 10 healthy donors for KIR expression on CD8+ T cells and the respective phenotype, specificity and function of these cells using DNA-barcoded pHLA-multimer screenings, multi-parametric flow cytometry and functional assays, comparing virus-specific HLA-A and B restricted CD8+KIR- T cell responses with CD8+KIR+ T cell populations that bind to HLA-C via their KIR. We also used single-cell analyses to decipher differences in clonality and gene expression profiles between these cell populations. We found that KIR+CD8+ T cells resemble a distinct CD8+ T cell population, with no overlap into the virus-specific HLA-A and -B restricted CD8+ T cell populations. Binding of pHLA-C multimers could be prevented by prior blocking of KIRs with antibodies, confirming TCR independent, KIR-mediated multimer binding. HLA-C binding to KIR seemed to be partially independent of the specificity of the bound peptide as at least 2 different bound HLA-C peptide ligands identified the same KIR+CD8+ T cell population. These cells were mainly of a terminally differentiated CD45RA+CCR7- phenotype, with gene expression patterns and TCR clonalities distinct from that of TCR-dependent virus-specific CD8+KIR- T cell populations. Here we show that KIR+CD8+ T cells are a distinct cell population identified by TCR independent HLA-C-KIR interaction and unique phenotypic, functional and clonal properties that distinguish them from TCR-driven virus-specific CD8+KIR- T cell responses.

Michael Kern
Kern_M4@ukw.de
Universitätsklinikum Würzburg, Medizinische Klinik und Poliklinik II, AG Beilhack

Host intestinal perivascular macrophages protect against murine acute graft-versus-host disease via PD-L1

Michael AG Kern[1,2,*], Duc Dung Le[1,2,*], Ana-Laura Jordán Garrote[1,2,*], Haroon Shaikh[1,2,*], Maria Ranecky[1,2], Katja Jarick[1,2], Juan Gamboa[1,2], Rémi Doucet Ladevèze[6], Musga Qureischi[1,2], Lukas Scheller[1,2], Tim Steinfatt[1,2], Andreas Brandl[1,2], Julia Hartweg[1,2], Christian Brede[1,2], Daniela Simao Vaz[1,2], Carina A. Bäuerlein[1,2], Simone S. Riedel[1,2], Tata Nageswara Rao[3], Anja Mottok[4], Ehsan Vafadarnejad[5], Antoine Emmanuel Saliba[5], Amy J. Wagers[3], Natalio Garbi[6], Juan Prada[7], Chunguang Liang[7], Thomas Dandekar[7], Rebecca Gentek[8], Marc Bajenoff[8], Ernst Holler[9], Mercedes Gomez de Agüero[6], Hermann Einsele[1], Heiko Bruns[3], Manfred B. Lutz[10], Andreas Beilhack[1,2]

1 Department of Medicine II, Würzburg University Clinics, Würzburg, Germany
2 Interdisciplinary Center for Clinical Research (IZKF) Würzburg, Germany
3 Department of Internal Medicine 5, Friedrich Alexander University Erlangen-Nuremberg, Erlangen, Germany
4 Department of Stem Cell and Regenerative Biology, Harvard University, Harvard 4Institute of Pathology, Würzburg University, Germany
5 Helmholtz Institute for RNA-based Infection Research (HIRI), Würzburg, Germany
6 Institute for Systems Immunology, Würzburg, Germany
7 Stem Cell Institute and Joslin Diabetes Center, Cambridge, USA
8 Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany
9 Department of Bioinformatics, Biocenter, University of Würzburg, Würzburg, Germany
10 Aix Marseille Univ, CNRS, INSERM, CIML, Marseille, France
11 Department of Internal Medicine III, University Medical Centre, Regensburg, Germany
12 Institute for Virology and Immunobiology, Würzburg, Germany

Acute graft versus host disease (aGvHD) remains a major complication in patients undergoing allogeneic hematopoietic cell transplantation (allo-HCT). We observed that alloreactive effector T cells infiltrating the lamina propria of the small and large intestines closely interact with intestinal myeloid cells of host origin. Here we investigated whether these intimate interactions regulate alloreactive effector T cell responses and how they impact intestinal aGvHD. To this end, we employed non-invasive bioluminescence imaging, light sheet fluorescence and confocal microscopy, clinical and histopathologic scoring, flow cytometry, single cell RNA sequencing and fate mapping in murine models of myeloablative MHC-mismatched allo-HCT. In the lamina propria, allogeneic T cells closely interacted with CD11b+CD11c+CD103- radio-resistant host type myeloid antigen presenting cells. Single cell RNA-Seq, flow cytometric analysis, lineage reporter-, and defined knockout mice identified these cells to be CSF-1R dependent MHC II, CD11c, F4/80 and CXC3CR1 expressing non-migratory macrophages. Fate mapping analysis unveiled the presence of both embryonic and adult-derived macrophage subsets within intestinal macrophages, which exhibited a perivascular gene expression profile and strategic localization around the lamina propria vasculature, facilitating direct interactions with incoming donor T cells. Notably, host macrophages but not stroma or other hematopoietic cells displayed high PD-L1 expression that proved decisive for improved survival in mice after allo-HCT. The loss of PD-L1 expression on intestinal macrophages correlated with the onset of intestinal aGvHD in human patients. In conclusion, we report a distinct and enduring subpopulation of host tissue-resident macrophages exerting potent suppression of alloreactive T cells in the lamina propria of the intestinal tract.

Hye-Min Kim
Department of Rehabilitative Medicine of Korean Medicine and Neuropsychiatry, College of Korean Medicine, Sangji University, Wonju, Gangwon-state 26339, Republic of Korea
mins7576@daum.net

Down-regulating effects of processed curcuma longa rhizome on differentiated adipocytes

Hye-Min Kim[1], Yea-Jin Park[1,2], Hyo-Jung Kim[2], Tae-Young Gil[2], Yun-Yeop Cha[1] and Hyo-Jin An[2,3*]

1 Department of Rehabilitative Medicine of Korean Medicine and Neuropsychiatry, College of Korean Medicine, Sangji University, Wonju, Gangwon-state 26339, Republic of Korea
2 Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea
3 Department of Integrated Drug Development and Natural Products, Graduate School, Kyung Hee University, Seoul 02447, Republic of Korea

Imbalance energy between excessive intake and insufficient expenditure causes obesity, a condition associated with various metabolic diseases or negatively-interacting immune functions. It is obvious that obesity is deeply linked to impair immune function and increase the risk of severe complex diseases such as COVID-19-related high hospitalizations. Since there are no clear therapeutic agents to treat obesity, multiple approaches to suppress obesity are necessary. One of them is to beige white adipose tissue or activate brown adipose tissue. We investigated whether a processed *Curcuma longa rhizome* (PC) activates the adipose tissues. In this study, two kinds of cells were applied to estimate the activation of PC. Preadipocytes 3T3-L1 and UCP1-lucifease were differentiated with fetal bovine serum included a mixture of differentiating adipogenic inducers such as dexamethasone and insulin. The number and size of lipid droplets were measured with Oil-Red O staining assay. PC inhibited lipid accumulation with reduced expression. And, we evaluated the effects of PC on high-fat diet-induced obesity mouse model. 6 weeks old male C57BL/6N mice were fed high-fat diet (45 kcal % fat) or normal diet. The obese mice were orally administered PC (100 mg/kg) 5 times per a week. Mice's white adipose tissues were separated and analyzed adipogenic markers. Mice fed high-fat diet and PC showed decreased body weight and adipose tissue mass compared to the ones fed only high-fat diet. A representative adipogenetic regulator, peroxisome proliferator-activated receptor gamma (PPAR-γ) was evaluated in the differentiated cells and subcutaneous adipose tissues from high-fat diet-induced mice at protein level. PC worked on the suppressive expression of PPAR-γ on obese-induced white adipose tissue. With these results, it is shown that the possibilities of PC on profound research as a therapeutic agent as regulating adipocytes.

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government Ministry of Science and ICT (MSIT) (#NRF-2021R1A2C3011862).

Hyo-Jung Kim
Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea
hyojung_95@naver.com

Anti-benign prostatic hyperplasia effect of Fucoidan through regulation of prostatic cell proliferation and inflammation mediated by transforming growth factor-β

Hyo-Jung Kim, Bo-Ram Jin, and Hyo-Jin An

Benign prostatic hyperplasia (BPH), accompanied by lower urinary tract symptoms (LUTS) that impair quality of life, is emerging as a major public health problem for men. Since indiscriminate cell proliferation and inflammatory responses exacerbate LUTS, controlling them becomes an important therapeutic strategy for BPH. Fucoidan (FU) is a natural sulfated polysaccharide predominantly found in brown algae, which has extensive biological benefits. Although the efficacy of FU on prostate diseases (e.g., prostatitis and prostate cancer) has been partially described, the effect of FU on BPH has still not been investigated. Therefore, the purpose of this study is to investigate whether FU alleviates BPH by improving the proliferation and inflammatory environment within prostatic cells. To confirm the anti-BPH effect of FU in vivo, animal experiments were conducted as follows: testosterone (10 mg/kg) was subcutaneously injected into rats for 4 weeks, while finasteride (5 mg/kg) and FU (70 or 140 mg/kg) were administered by gavage. A well-established BPH-1 cell line was used to elucidate human prostate growth and physiology in vitro. As a result, FU inhibited prostatic cell proliferation by suppressing the expression of androgen receptor-related markers, which are key factors in the pathogenesis of BPH. In addition, FU reduced the expression of inflammatory mediator and cytokines. Furthermore, FU blocked the activation of transforming growth factor-β 1 (TGF-β1) signaling pathway known to mediate cell proliferation and inflammatory responses. In summary, the current study suggests that FU alleviates BPH by improving the prostatic cell proliferation and inflammation through inhibition of TGF-β1 activation.

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI21C2537).

Andrew Koh
University of Chicago
akoh@uchicago.edu

Thymic epithelia destabilize chromatin via p53 repression for AIRE-mediated immune tolerance

Noah Gamble[1,2], Jason A. Caldwell[1], Caroline Kaiser[1,3], Joshua McKeever[1,4], Alexandra Bradu[1], Sandy Klemm[5], William J. Greenleaf[5,6], Narutoshi Hibino[7], Aaron R. Dinner[8], and Andrew S. Koh[1,8]

1 Department of Pathology, University of Chicago, Chicago, IL, USA
2 Graduate Program in Biophysical Sciences, University of Chicago, Chicago, IL, USA
3 Department of Human Genetics, University of Chicago, Chicago, IL, USA
4 Committee on Molecular Metabolism and Nutrition, University of Chicago, Chicago, IL, USA
5 Department of Genetics, Stanford University, Stanford, CA, USA
6 Chan Zuckerberg Biohub, San Francisco, CA, USA
7 Department of Surgery, University of Chicago, Chicago, IL, USA
8 Institute for Biophysical Dynamics, University of Chicago, Chicago, IL, USA

To select a self-discriminating T cell repertoire for adaptive immunity, each developing T cell clone is tested for self-reactivity in the thymus with high-affinity interactions triggering cell death or functional deviation. Epithelial cells of the thymic medulla (mTECs) are central to this selection as they ectopically express nearly the entire coding genome to expand the scope of self-antigens presented in the thymus. How cells of a defined epithelial lineage express genes highly restricted to other specialized tissues remains largely unknown. Using multi-modal genomics at single cell resolution, we aimed to characterize the cis-regulatory landscape that facilitates the activation of tissue-specific genes in mTECs. Surprisingly, we could not identify differentially accessible enhancers at tissue-specific loci between mTECs that transcribe these loci vs. those that do not. Rather, we find that mTECs destabilize chromatin barriers and amplify noise in accessibility dynamics at tissue-specific loci, a process that is not dependent on the transcriptional regulator AIRE. Epigenomic feature analysis implicated p53 repression as a linchpin of this chromatin instability. Augmenting p53 activity in mTECs via an inducible hypermorph allele stabilized chromatin barriers at tissue-specific genes and inhibited their ectopic expression, especially those that are AIRE-regulated. Moreover, this p53 perturbation caused systemic T cell hyperactivity and lymphocytic infiltration in multiple peripheral organs. Taken together, we uncover key modes by which mTECs establish a permissive epigenetic landscape that provides the chromatin context that AIRE can act on to promote expression of tissue-specific genes and immunological tolerance.

Saskia Kolb
Institute of medical microbiology, immunology and hygiene (MIH), TUM
saskia.kolb@tum.de

SATB1 differentially regulates proinflammatory cytokine and cell activation profiles in human Treg and Teff cells

S. Kolb[1], L. Warmuth[1], I. Serr[3], C. Daniel[3], D. H. Busch[1], Ch. Schmidl[4], K. Schumann[1,2]

1 Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München (TUM), Munich, Germany
2 Institute for Advanced Study, TUM, Munich, Germany
3 Helmholtz Diabetes Center (HDC), Helmholtz Munich, Germany
4 RCI Regensburg Centre for Interventional Immunology, Regensburg, Germany

Human Treg and CD4+ Teff cells have opposite functions in autoimmunity and in cancer. A better understanding of transcriptional signatures in these subsets can accelerate the identification of new modulators in immunotherapies. The impact of the chromatin organizer SATB1 on T cell development has been studied. However, SATB1 function in mature human Treg and CD4+ Teff cells in the steady state and during inflammation is less clear. Therefore, we ablated SATB1 in ex vivo expanded Treg and Teff cells using CRISPR/Cas9. Loss of SATB1 results in Treg phenotypic destabilization indicated by reduced expression of Treg-specific markers such as CTLA-4 and FOXP3 and increased levels of proinflammatory IL-2 and IL-4. Further destabilization was observed during proinflammatory IL-12 conditioning mimicking an inflammatory microenvironment. Interestingly, SATB1 KO in Teff cells resulted in the opposite cytokine profile with reduced IL-2 and IL-4 expression levels and upregulation of FOXP3 and Helios. RNA-seq and ATAC-seq analysis further confirmed subset-specific modulation of cytokine production, activation, and proliferation in SATB1-deficient Treg and CD4+ Teff cells. SATB1 KO Treg cells were functionally validated in suppression assay showing reduced suppressive capacity. In a more complex setup – transferring SATB1-deficient Treg cells with PBMCs in NSGS mice - we found reduced numbers of Treg cells in comparison to the control mice. Next, we generated SATB1 KO Teff cells expressing an aCD19-CAR and tested them in in vitro and in vivo tumor models. Massive proliferation of CAR+ SATB1-KO Teff cells upon strong activation through tumor cells was observed in vitro and in vivo. SATB1-KO CAR+ T cell resolved thereby CD19-expressing tumors faster than control cells in a humanized NSGS mouse model. Dissection of differentially regulated transcriptional patterns in human T cell subsets can potentially lead to the identification of novel targets for immunotherapies against autoimmune diseases or cancer.

Victor Linus Kreiner
Institute for Experimental Immunology, University of Zurich
vic.kreiner@live.de

Ontogenies, phenotypes, and functions of regulatory T cells in fetomaternal tolerance

Victor Kreiner, Burkhard Becher

Pregnancy represents an immunological paradox as the maternal immune system accommodates the implantation and growth of a semi-allogenic fetus, which shares half of its genetic material with the father. The success of pregnancy thereby relies on a complex interplay of mechanisms that allow for tolerance of the invasion of the uterine wall. A multitude of studies have identified cellular and molecular players, most of which concern the direct maternal-fetal interface. It has become clear, that regulatory T cells (Tregs) are enriched at the maternal fetal interface and are required for maternal tolerance. However, both their ontogeny, effector mechanisms and antigen specificity, are still unknown. Concerning their ontogeny, conflicting evidence was found for both thymic (tTregs) and locally induced (pTregs) being essential for successful pregnancy. In case of tTregs, several cytokines are thought to attract circulating Tregs, but their relevance has so far not been proven in-vivo. For the case of pTregs, few signals have been identified to drive Treg induction, but their contribution to total Treg numbers at the maternal-fetal interface is unclear. Concerning antigen specificity, while several groups have reported expansion of Tregs specific for paternal antigens, no study has shown evidence for the necessity of these for successful pregnancy. Concerning effector mechanisms, we found uterine Tregs to exhibit a tissue resident effector phenotype including transcripts such as CXCR6, GZMA and LAYN. Despite this, is it unclear to what degree Tregs contribute to tissue remodelling during placentation or killing of fetus-reactive T cells. We aim to address these queries by tracking and characterizing T lymphocytes from pregnant and non-pregnant reporter or TCR-transgenic mice in-vivo and in-vitro by high dimensional flow cytometry and single cell transcriptome and immune receptor sequencing. Overall understanding unraveling the contributions of uterine Tregs and other tolerogenic mechanism in pregnancy can further drive our understanding of scenarios where tissue-specific tolerance is not given, such as autoimmune conditions and transplantation.

Andreas Krueger
Justus Liebig University Giessen
andreas.krueger@immu.bio.uni-giessen.de

High-resolution mapping of cell-cycle dynamics during steady-state T-cell development and regeneration in vivo

Heike Kunze-Schumacher, Nikita A. Verheyden, Zoe Grewers, Michael Meyer-Hermann, Victor Greiff, Philippe A. Robert, Andreas Krueger

Control of cell proliferation is critical for the lymphocyte life cycle. However, little is known on how stage-specific alterations in cell-cycle behavior drive proliferation dynamics during T-cell development. Here, we employed in vivo dual-nucleoside pulse labeling combined with determination of DNA replication over time as well as fluorescent ubiquitination-based cell-cycle indicator mice to establish a quantitative high-resolution map of cell-cycle kinetics of thymocytes. We developed an agent-based mathematical model of T-cell developmental dynamics. To generate the capacity for proliferative bursts, cell-cycle acceleration followed a 'stretch model', characterized by simultaneous and proportional contraction of both G1 and S phase. Analysis of cell-cycle phase dynamics during regeneration showed tailored adjustments of cell-cycle phase dynamics. Taken together, our results highlight intrathymic cell-cycle regulation as an adjustable system to maintain physiologic tissue homeostasis and foster our understanding of dysregulation of the T-cell developmental program.

Mirela Kuka
Vita-Salute San Raffaele University
kuka.mirela@hsr.it

IFN-γ Suppresses T Follicular Helper Cell Differentiation and Antibody Responses

Eleonora Sala[1,2], Chiara Laura[1,2,3,*], Maria Nelli[1,2,*], Marta Mangione[1,2], Pietro Di Lucia[1,2], Elisa B. Bono[2], Leonardo Giustini[2], Giuliana Furiato[1,2], Eleonora Consolo[1,2], Cristian Gabriel Beccaria[2], Eyal David[4], Merav Cohen[4], Amir Giladi[4], Ido Amit[4], Matteo Iannacone[1,2,5,§], and Mirela Kuka[1,2,§]

1 School of Medicine, Vita-Salute San Raffaele University, Milan, Italy
2 Division of Immunology, Transplantation, and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milan, Italy
3 Center for Omics Sciences, IRCCS San Raffaele Scientific Institute, Milan, Italy
4 Department of Immunology, Weizmann Institute of Science, Rehovot, Israel
5 Experimental Imaging Centre, IRCCS San Raffaele Scientific Institute, Milan, Italy
* co-second authors
§ co-last authors

Humoral and cellular immune responses typically co-exist during viral infections. However, there are instances where one response predominates, determining the primary antiviral activity. For example, lymphocytic choriomeningitis virus (LCMV) infection elicits a pronounced cellular response, yet exhibits a suboptimal neutralizing antibody (nAb) response. This deficiency in nAb response hinders its clearance and facilitates persistent infection. Recent findings indicate that this preference for cellular immunity over humoral immunity is significantly regulated at the $CD4^+$ T cell differentiation stage. Specifically, subcutaneous LCMV infection predominantly induces T_H1 differentiation, which augments cellular immunity, while largely neglecting T_{FH} differentiation, a key driver of humoral immunity. Here, we investigated the mechanisms responsible for this inhibited T_{FH} differentiation. We found that the T_H1 cells induced by subcutaneous LCMV infection are heterogeneous. They encompass a terminally differentiated T_H1 subset expressing Granzyme-B (Gzmb) and a Tcf-1^+subset that retains the potential for T_{FH} differentiation. While IL-12 appeared to be non-essential for this differentiation, T cell-derived IFN-γ facilitated the proliferation of the $GzmB^+$ subset and inhibited the Tcf-1^+ cells' progression into T_{FH}. Consistently, inhibition of IFN-γ enabled robust T_{FH} differentiation, leading to the formation of germinal centers and increased antibody production. Our study provides novel insights into the mechanisms inhibiting nAb production in response to specific viruses and offers a foundation for the development of advanced vaccine strategies.

Taku Kureha
Ludwig-Maximilians-Universität München
Taku.Kureha@med.uni-muenchen.de

Functional importance of m6A mRNA modification and the m6A reader protein YTHDC1 in T cells

Taku Ito-Kureha[1], Vigo Heissmeyer[1,2]

1 Institute for Immunology, Ludwig-Maximilians-Universität München
2 Research Unit Molecular Immune Regulation, Helmholtz Zentrum München

Upon antigen recognition, T cells experience a rapidly increasing demand for energy and biosynthesis, forcing them to enhance their metabolic capabilities. A dynamic change in energy metabolism and the concomitant induction of genes related to activation, proliferation and differentiation allow T cells to undergo clonal expansion and acquire effector function. Our understanding of the gene regulation leading to metabolic reprogramming and acquisition of effector function is still incomplete. Post-transcriptional gene regulation (PTR) by RNA-binding proteins (RBPs) constitutes an essential layer of gene regulation. In this program, the mRNA-encoded information is "read" by RBPs, and the fate of mRNAs is controlled by many different RBPs. Recently, the new field of "epitranscriptomics" has emerged and we now ask how this type of PTR affects T cell development and function. N6-methyladenosine (m6A), which is the most prevalent mRNA modification, generates unique RBP-binding sites on mRNA to which m6A reader proteins (m6A-BPs) bind. Our previous research highlighted a decisive role of m6A modification in T cell signaling and survival. m6A modifications are required for thymocyte differentiation, they control activation-induced death of peripheral conventional T cells and prevent colitis by enabling gut regulatory T cell differentiation and function. While the global loss of m6A modification and associated profound changes in T cells have been extensively studied, analyses of deletion of m6A-BPs will help to define specific regulatory mechanisms during T cell development and homeostasis. To begin to dissect the role of m6A-BPs expressed by T cells, we first inactivated the nuclear m6A-BP Ythdc1. We found that Ythdc1 was required for the maturation of SP thymocytes in the thymus, and that was involved in peripheral T cell proliferation by controlling metabolic programming. Our ongoing analyses aim to define how Ythdc1 regulates gene expression and affects energy metabolism of T cells. Together, we aim to provide a comprehensive phenotypic study and to uncover novel molecular mechanisms underlying m6A-BPs function in T cell development and immune responses.

R. Jacob Labios
Würzburg Institute of Systems Immunology
reuben.labios@uni-wuerzburg.de

Development and integrin-dependent migration of cDC1 in a distinct perivascular niche of the splenic red pulp

R. Jacob Labios, Defne Akkar, Anika Grafen, Sanjiv Luther, Ronen Alon, Tal Arnon, Marc Bajénoff, Milas Ugur, Wolfgang Kastenmüller

Precursor of dendritic cells (preDCs) develop in the bone marrow and continuously seed tissues where preDCs further differentiate and migrate to and within secondary lymphoid organs to orchestrate adaptive immunity. Where preDC seed the splenic red pulp, locally develop, and migrate to the white pulp following their maturation is unresolved. By applying cDC1 depletion and reconstitution approaches and analyzing CCR7-deficient cDC1 we found that they develop in the perivascular niche of capillaries in the red pulp. Following systemic Poly I:C treatment to induce an accelerated and collective cDC1 migration, we identified a migratory path along the continuation of the capillary perivascular niche towards CD31-positive, Gpr182-negative arterioles. Thereby, we identified a scaffold that supports cDC1 development and serves as a guiding structure for the migration of cDC1 to the bridging channel. Efficient CCR7-dependent migration required both ligands CCL19 and CCL21a as well as the scavenging receptor ACKR4. This long-ranged migration fully depended on ICAM1 on stromal cells within the perivascular niche, highlighting fundamental differences to DC migration to and within lymph nodes. We are currently investigating how this gradient is formed over several hundred µm along the perivascular niche as well as the potential impact of cDC1 localisation on T cell activation.

Alexander Leithner
Kennedy Institute of Rheumatology, University of Oxford
alexander.leithner@kennedy.ox.ac.uk

A second-generation supported lipid bilayer system to study the effects of ligand lateral mobility on T cell activation

Alexander Leithner, Michael L. Dustin

Reductionist model systems, such as supported lipid bilayers (SLBs), have been indispensable tools for the investigation of the molecular and cellular processes on the T cell side of the immunological synapse (IS). However, recent evidence underscores the active role of antigen presenting cells (APCs), in shaping the IS. For instance, APCs regulate the lateral mobility of surface proteins through their underlying cytoskeleton, with implications for the ability of ligands to trigger T cell-expressed mechanosensitive receptors, as well as their accessibility and clustering. Importantly, these aspects are not captured by current SLB systems. Here, we introduce a 'second-generation' SLB system that allows for the simultaneous presentation of mobile and immobile ligands, closely mimicking the situation on APCs while preserving the superior optical accessibility of 'classic' SLB platforms. Using the integrin ligand intercellular adhesion molecule 1 (ICAM1) as a proof-of-principle candidate, we show that immobilisation of ICAM1 induces profound morphological changes in interacting T cells. Notably, we demonstrate that ICAM1 immobilisation abrogates centripetal F-actin flow, leading to a conversion of the IS from a monofocal to a multifocal configuration where the T cell receptor remains in the periphery in discrete microclusters. Importantly, this is accompanied by an increase in integrin-related signalling, enhanced signalling by other co-stimulatory receptors and an overall increase in T cell activation. Strikingly, immobilisation of ICAM1 correlates with an increase in Perforin 1 secretion by CD8+ T cells, suggesting an enhancement in T cell-mediated cytotoxicity and the adoption of effector functions. Our findings underscore the dynamic interplay between ligand mobility and T cell activation, shedding light on the intricate levels of immune regulation at the IS. The second-generation SLB system will serve as foundational platform for further developments, aimed at emulating key aspects of APCs in a fully controlled environment in order to unravel the nuanced complexities of APC-T cell interactions.

Jule Müschen
Technical University of Munich (TUM), School of Medicine and Health, Department of Preclinical Medicine, Institute for Medical Microbiology, Immunology and Hygiene, Munich 81675, Germany
jule.mueschen@tum.de

Regulation of DNA-deletion sizes and aneuploidy in CRISPR-edited human T cells by the control of T cell stimulation and pifithrin-alpha

Laurenz T. Ursch[1,8], Jule S. Müschen[1,8], Julia Ritter[1], Julia Klermund[2,3], Bettina E. Bernard[1], Saskia Kolb[1], Geoffrey Andrieux[4,5], Melanie Boerries[4,5,6], Toni Cathomen[2,3,5], Kathrin Schumann[1,7]

1 Technical University of Munich (TUM), School of Medicine and Health, Department of Preclinical Medicine, Institute for Medical Microbiology, Immunology and Hygiene, Munich 81675, Germany
2 Institute for Transfusion Medicine and Gene Therapy, Medical Center – University of Freiburg, 79106 Freiburg, Germany
3 Center for Chronic Immunodeficiency (CCI), Medical Center – University of Freiburg, 79106 Freiburg, Germany
4 Institute of Medical Bioinformatics and Systems Medicine, Medical Center – University of Freiburg, Freiburg, Germany
5 Faculty of Medicine, University of Freiburg, 79106 Freiburg, Germany
6 German Cancer Consortium (DKTK), Partner site Freiburg, a partnership between DKFZ and Medical Center - University of Freiburg, 79106 Freiburg, Germany
7 TUM, Institute for Advanced Study, Garching 85748, Germany
8 equal contribution
Corresponding author: Kathrin Schumann

Genome editing in human T cells using CRISPR/Cas9 has shown great promise to improve T cell effector functions for adoptive T cell therapies. Several tools, like optimized gRNAs, novel Cas9 variants or off-target prediction algorithms have been developed to increase the safety of Cas9 editing. Nevertheless, there is evidence that induction of double strand breaks by the Cas9 system leads to large deletions of DNA fragments up to several kbs, chromosomal translocations and aneuploidy (Stadtmauer et al 2020). Edited T cells with chromosomal abnormalities can withstand ex vivo expansion and are, therefore, an ongoing concern for CRISPR/Cas9-engineered T cell products for therapy (Nahmad et al. 2022). To develop safer Cas9-engineering strategies for human T cells, we need a better understanding of the T cell-intrinsic mechanisms that control deletion sizes and chromosomal aberrations. Here, we describe driving forces of indel formation in primary human T cells. Increased proliferation speed and stronger T cell activation result in larger deletions. Using non-activated T cells for editing minimizes the risk of large deletions, with the downside of reduced knock-out efficiencies. An alternative strategy for the reduction of large deletions is the addition of the small molecule pifithrin-alpha (PFT-a), supposedly a p53 inhibitor. PFT-a treatment causes smaller more defined deletions and reduces the risk of CRISPR-induced chromosomal translocations and aneuploidy in a p53-independent manner. The functionality of engineered T cells is sustained during CRISPR editing in the presence of PFT-a. Both strategies, controlling T cell activation and the addition of PFT-a directly after genome editing, are easily adaptable for safer CRISPR/Cas9 engineering for adoptive T cell therapies.

Inmaculada Martínez Reyes
Max Delbrück Center for Molecular Medicine, Berlin
imreyes@mdc-berlin.de

Unraveling the dynamics of immunosurveillance: T cell responses and dysfunction in tumor development

Inmaculada Martínez-Reyes[1], Marta F. Nogueira[1], Katrin Hönig[1], Ana Milojkovic[1], Kathleen Anders[1], Benedikt Obermayer[2], Elmehdi Belbaraka[1], Maria Teresa Norcia[1], Arunraj Dhamodaran[1], Dieter Beule[2], Thomas Blankenstein[1]

1 Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Molecular Immunology and Gene Therapy, Berlin, Germany.
2 Berlin Institute of Health at Charité - Universitätsmedizin Berlin, Core Unit Bioinformatics, Charitéplatz 1, 10117 Berlin, Germany.

Immunosurveillance, the ability of the immune system to detect and destroy malignant cells, is well-established for virus-associated tumors. However, immunosurveillance of spontaneous tumors is still controversial. A limitation in addressing this question has been the lack of cancer mouse models resembling the sporadic nature of human cancer. To fill this gap, we developed a model that involves inoculating cancer cells in which antigenic oncogene expression and proliferation are triggered once artificial transplantation-induced inflammation subsides. We used cancer cells with doxycycline (dox)-inducible expression of the tumor antigen and tumor driver gene SV40 large T antigen (Tag) fused to luciferase (TagLuc). Dox deprivation leads to loss of TagLuc expression and growth arrest in vitro. To investigate T cell responses against de novo-expressed Tag under non-acute inflammatory conditions, cells were deprived of dox and subsequently inoculated into mice. After a resting period, mice were administered dox to induce TagLuc expression and proliferation. While tumors progressively grew in immunodeficient mice, Tag-expressing cancer cells were rejected in T cell competent mice. Single-cell analysis of tumors at various stages revealed expanded TCR clonotypes with an effector memory phenotype in rejected tumors. These results demonstrate immunosurveillance under resting conditions using a potent antigen with multiple MHC-I epitopes. However, most neoantigens result from a single amino acid exchange. Cancer cells expressing the neoantigen mutant p68 were rejected if dox was never withdrawn (inflammatory conditions), but in sharp contrast to TagLuc, not anymore under resting conditions. These results suggest that CD8 T cells might have encountered mut-p68 but become dysfunctional. Overall, our model resembles pathophysiologic conditions, allowing us to study the immune response to clinically relevant antigens. Our results provide a better understanding of the mechanisms underlying immunosurveillance or the inability of the immune system to control nascent tumors.

Eleanor Minogue
University of Cambridge
eam84@cam.ac.uk

Glutarate regulates T cell metabolism and anti-tumour immunity

Eleanor Minogue, Pedro P Cunha, Brennan Wadsworth, Shiv Sah Teli, Guinevere L. Grice, Rob Hughes, Alessandro Quaranta, Javier Zurita, Pedro Velica, Laura Barbieri, David Bargiela, Petros Tyrakis, Craig Wheelock, Peppi Karppinen, Iosifina P Foskolou, James A. Nathan, Randall S Johnson

T cell function and fate can be determined by a range of metabolites; these include succinate, fumarate, and 2-hydroxyglutarate, acting both through enzymatic inhibition of alpha-ketoglutarate dependent dioxygenases (αKGDD's) and in the case of succinate through post-translational modification of lysines in metabolically important targets. We show here that glutarate, a key product of amino acid catabolism, is increased in T cells post activation, in a HIF dependent manner. This results in potent effects on T cell function and differentiation, both through αKGDD inhibition, and through direct regulation of T cell metabolism via the post-translational modification glutarylation. Cytotoxic T cells show significant, oxygen-dependent shifts in glutarate levels during differentiation, and these are correlated with shifts in glutarylation of pyruvate dehydrogenase E2. Administration of a cell-permeable form of glutarate, diethyl-glutarate, increases cytotoxic activity in CD8+ T cells and in vivo administration of the compound reduces tumor growth, correlated with increased levels of both peripheral and intratumoral T cells. These results demonstrate that glutarate is both a potent and effective regulator of T cell metabolism and differentiation, and that it is an important potential means for the improvement of immunotherapy.

Noelia del Carmen Moares Fernández
University of Cádiz
noelia.moares@uca.es

A step forward in the rational design of Chimeric Antigen Receptors using AlphaFold

Noelia Moares[1], Pablo Gonzalez-Garcia[1], Juan P. Muñoz-Miranda[1], Ricardo Fernandez-Cisnal[1], Antonio Gabucio[1], Rosa Luna-Espejo[1], Javier Ocaña-Cuestas[1], Lucia Olvera[1], Cecilia Fernandez-Ponce[1] and Francisco Garcia-Cozar[1]

1 University of Cádiz

Although CAR-T cell therapy has made great advances in treating certain hematological malignancies such as multiple myeloma or B-cell acute lymphoblastic leukemia, it still needs to overcome many optimization and safety issues. Since most CAR-T cells use scFvs for antigen recognition, scFV aggregation is a concerning issue that needs to be addressed, as it can lead to tonic signaling and non-specific CAR-T cell exhaustion. In addition, choosing the length and nature of the hinge domain in order to enhance or reduce affinity with the targeted epitope for optimal T-cell activation upon antigen recognition, as well as decrease related tonic signaling with the aim of improving overall antitumor activity and safety is another challenge that is faced during CAR-T design. Since the potential of powerful structure prediction tools such as AlphaFold-Multimer for rationally designing CAR-T cells has barely been explored, in this study we use AlphaFold2 based protein modelling methods together with in silico protein and binding energies predictions based on the Rosetta Score Function to optimize the design chimeric antigen receptors targeting BCMA and SLAMF7,which have already been shown to elicit strong antitumor responses when directed against multiple myeloma (MM) cells .

Philipp Nawrath
Institute for Research in Biomedicine (IRB) & ETH Zürich
philipp.nawrath@irb.usi.ch

Cross-reactive peptides: A mechanistic link between multiple sclerosis and EBV

Philipp Nawrath, Valentina Ferrari, Antonio Lanzavecchia, Federica Sallusto

Multiple sclerosis (MS) is an autoimmune disorder characterized by the demyelination of the central nervous system. While certain environmental and genetic risk factors, such as specific HLA alleles and prior Epstein-Barr virus (EBV) infection, are known, the etiology remains elusive. Conventional emphasis in MS research lies on CD4 T cells; however, CD8 T cells have been found to be the predominant T cell population in MS lesions and could play an important role. Moreover, a large-scale patient study recently demonstrated a strong association between EBV infection and MS onset, suggesting cross-reactivity of CD8 T cells after an EBV infection as a potential driving force behind MS pathogenesis. Our computational analyses identified over 1300 EBV/Myelin cross-reactive peptide candidates, with a notable enrichment in binding to the MS risk allele HLA-B*07:02. Further scrutiny of the HLA-B*07:02 binding peptides unveiled an O-Glycosylation motif present in both EBV and myelin peptides, but not in other Herpesviruses. We aim to validate these cross-reactive peptide candidates in vitro and identify specific T-cell receptors (TCRs) which recognize both EBV and myelin antigens. Concurrently, we plan to confirm their processing and presentation on HLA-B*07:02 alleles through immunopeptidomic analysis. Furthermore, we intend to identify potentially cross-reactive TCRs by screening patients with recent symptom onset. In summary, this project aims to enhance our understanding of the role of CD8 T cells in autoimmunity, opening new avenues for therapeutic interventions in MS.

Thomas O'Neill
Signaling and Translation (SAT)
thomas.oneill@helmholtz-munich.de

Constitutive MALT1 paracaspase activity in T lymphocytes or dendritic cells drives autoimmune inflammation

Thomas J. O'Neill[1], Andreas Gewies[1], Thomas Seeholzer[1], Maria Szente-Pasztoi[2], Caspar Ohnmacht[2] and Daniel Krappmann[1]

1 Research Unit Signaling and Translation, Molecular Targets and Therapeutics Center, Helmholtz Zentrum, München, Germany
2 Institute of Allergy Research - Mucosal Immunology, Helmholtz Zentrum, München, Germany

MALT1 is a multifunctional protein which is essential for adaptive immunity and cellular homeostasis. Within the CARD11-BCL10-MALT1 (CBM) complex downstream of antigen receptors, MALT1 adopts a complex role as both a scaffold, binding to TRAF6 to drive downstream NF-kappaB activation, and as the only mammalian paracaspase, cleaving a diverse set of substrates involved in post-translational and post-transcriptional regulation. Recently, we found that abrogation of MALT1-TRAF6 interaction in mice with loss-of-function mutations in the TRAF6 binding motifs of MALT1 (Malt1 TBM mice) yields constitutive MALT1 protease activity and subsequent fatal autoimmune activation, establishing TRAF6 as a negative regulator of MALT1 enzyme activity in the absence of acute antigen receptor ligation. Further, we found that mice with T cell-dependent MALT1 TBM mutations (Malt1 TBM-T) are phenotypically analogous to mice lacking TRAF6 in T cells (Traf6-deltaT), and that loss of TRAF6 similarly drives constitutive MALT1 activity and autoimmune inflammation. Indeed, genetic or pharmacological abrogation of MALT1 activity in either Malt1 TBM or Traf6-deltaT mice reverts autoimmune inflammation. Here, we show that expression of MALT1 TBM mutations in non-T cell compartments drives autoimmune inflammation in a manner phenotypically discrete from that of Malt1 TBM-T mice. Mice lacking MALT1-TRAF6 interaction in all hematopoietic cells have a more severe phenotype than T cell-dependent TBM mice, with thickening and inflammation of the small intestine, mesenteric lymphadenopathy and expansion of activated lymphocyte populations. This phenotype persists in a milder form upon crossing of TBM and CD11c-Cre mice and resembles that of mice with loss of TRAF6 in dendritic cells, indicating a novel, essential role of MALT1 paracaspase activity in dendritic cell homeostasis. These finding yield a new avenue for research on the role of MALT1 activity in non-T cell populations and its potential contribution to the development of diseases such as inflammatory and autoimmune diseases or lymphoma.

Philipp Penninger
Medical University of Vienna
philipp.penninger@univie.ac.at

Histone deacetylase 1 modulates pro-inflammatory cytokine release in T cell-mediated antifungal immunity

Helena Brezovec, Magdalena Teufl, Irina Tsymala, Phan-Canh Trinh, Christian Seiser, Karl Kuchler

Life-threatening invasive fungal infections by commensal Candida spp. in immunocompromised individuals represent high healthcare burdens, especially owing to the worrisome rise of antifungal resistance. Although innate immune responses are pivotal to control fungal infections, antifungal immunity also relies on protective T and B cell responses. Of note, the molecular knowledge about how epigenetic mechanisms mediate or contribute to T cell-mediated immunity, polarization and plasticity upon fungal challenges remain scarce. Here, we use a conditional CD4+ T cell-specific knock-out mouse model lacking histone deacetylase 1 (HDAC1-/-). HDAC1 is responsible for reversible chromatin modifications and involved in many biological functions, including inflammatory responses and T helper (Th) subset activation. We show here that HDAC1-/- CD4+ T cells exhibit cell-intrinsic dysregulated cytokine expression profiles, leading to increased release of pro-inflammatory Th17 cytokines IL-17A, GM-CSF and IL-22. Hence, HDAC1-deficient mice display increased susceptibility following systemic fungal infections accompanied by severe immunopathology of infected organs. Further, analysis of isolated CD4+ T cells from infected organs and secondary lymphatic tissue revealed HDAC1-dependent alterations of cytokine receptors gp130 and TgfbrII causing increased Th17 polarization. Our data highlights the crucial role HDAC1 exerts on the control of cytokine release and T cell polarization during the onset of the adaptive response to pathogenic fungal challenge.

Anneli Peters
Institute of Clinical Neuroimmunology, LMU Hospital and BMC
Anneli.Peters@med.uni-muenchen.de

T:B cell cooperation in ectopic lymphoid follicles in CNS autoimmunity

Anna Kolz[1,2], Isabel J. Bauer[1,2], Clara de la Rosa[1,2], Vladyslav Kavaka[1,2], Anna S. Thomann[1,2], Rosa Schmitz[1,2], Martin Kerschensteiner[1,2,3], Eduardo Beltran[1,2,3], Naoto Kawakami[1,2], Anneli Peters[1,2]

1 Institute of Clinical Neuroimmunology, University Hospital Ludwig-Maximilians-Universität München, Munich, Germany
2 Biomedical Center (BMC), Faculty of Medicine, Ludwig-Maximilians-Universität München, Martinsried, Germany
3 Munich Cluster for Systems Neurology (SyNergy), Munich, Germany

Meningeal ectopic lymphoid follicle-like structures (eLFs) have been described in multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE), but their role in CNS autoimmunity is unclear. To analyze the cellular phenotypes and interactions within these structures, we employed a Th17 adoptive transfer EAE model featuring formation of large, numerous eLFs. Single-cell transcriptomic analysis revealed that clusters of activated B cells and B1/Marginal Zone-like B cells are overrepresented in the CNS and identified B cells poised for undergoing antigen-driven germinal center (GC) reactions and clonal expansion in the CNS. Furthermore, CNS B cells showed enhanced capacity for antigen presentation and immunological synapse formation compared to peripheral B cells. To directly visualize Th17:B cell cooperation in eLFs, we labeled Th17 cells with a ratiometric calcium sensor, and tracked their interactions with tdTomato-labeled B cells in real-time. Thereby, we demonstrated for the first time that T and B cells form long-lasting antigen-specific contacts in meningeal eLFs that result in reactivation of autoreactive T cells. Consistent with these findings, autoreactive T cells depended on CNS B cells to maintain a pro-inflammatory cytokine profile in the CNS. Collectively, our study reveals that extensive T:B cell cooperation occurs in meningeal eLFs in our model promoting differentiation and clonal expansion of B cells, as well as reactivation of CNS T cells and thereby supporting smoldering inflammatory processes within the CNS compartment. Our results provide valuable insights into the function of eLFs and may provide a direction for future research in MS.

Georg Petkau
The Babraham Institute
georg.petkau@babraham.ac.uk

RNA binding proteins compartmentalize T cell effector differentiation, clonal expansion and memory formation

Georg Petkau, Twm J. Mitchell, Krishnendu Chakraborty, Marian Jones Evans, Sarah E. Bell, Vanessa D´Angeli, Louise Matheson, David J. Turner, Alexander Saveliev, Ozge Gizlenci, Fiamma Salerno, Peter D. Katsikis and Martin Turner

Activation of T cells induces rapid transcriptional activity. This results in the production of multiple mRNAs, encoding genes which regulate many processes like differentiation, metabolism or cell cycle. Upon antigen encounter, T cells need to rapidly incorporate environmental cues to program the magnitude and quality of their response. As a result of successful priming, at the peak of the T cell response, high affinity T cells outcompete their lower affinity counterparts and comprise most of the cytotoxic effector pool. How individual T cells compete for and respond to environmental signals and, consequently, how differentiation of effector and memory cells is connected to selection and expansion at the molecular level is poorly understood. We find that this process is critically regulated by the RNA binding protein (RBP) ZFP36 and its paralogue ZFP36L1. We find that both RBPs have roles in limiting the costimulation dependent effector T cell differentiation and cytotoxic function by suppressing a network of transcription factors and cytokines early during activation. At the same time, as part of an incoherent feed forward loop ZFP36L1 has a non-redundant role in suppressing negative regulators of cytokine signalling and mediating a selection mechanism based on competition for IL2. Zfp36l1 acts as a sensor of antigen affinity and establishes dominance of high affinity T cells by installing a hierarchical response to IL2 and enabling formation of protective memory, while Zfp36 limits long lived effector memory formation. In summary we identify the ZFP36 family of RNA binding proteins to disconnect effector differentiation and function from clonal expansion and memory formation on a posttranscriptional level.

Elisabetta Petrozziello
BioMedicalCenter of the LMU Munich, Institute for Immunology
elisabetta.petrozziello@med.uni-muenchen.de

The role of cathepsin L in shaping a functional CD4+ T cell repertoire

E. Petrozziello[1], C. Federle[1], J. Nedjic[1], S. Daley[2], S. Ravens[3], M. Schmidt-Supprian[4], T. Reinheckel[5], J. Kisielow[6], I. Prinz[3] and L. Klein[1]

1 Institute for Immunology, Ludwig-Maximilians University of Munich, Germany
2 Center for Immunology and Infection Control, Queensland University, Brisbane, Australia
3 Institute for Immunology, Hannover Medical School, Germany
4 Hematology and Oncology, Klinikum rechts der Isar, Technical University of Munich, Germany
5 Institute of Molecular Medicine and Cell Research, Albert-Ludwigs-University, Freiburg, Germany
6 Institute for Molecular Health Sciences, ETH, Zurich, Switzerland

The generation of the fittest T cell repertoire is accomplished through positive selection, a process by which cortical thymic epithelial cells (cTECs) ensure the survival of useful T cell receptors (TCRs), both in terms of diversity and functionality. While the nature of self-peptides presented to developing T cells is still extremely challenging to investigate, solid evidence points at the uniqueness of antigen-processing pathways adopted by cTECs. With our project, we focused on how cathepsin L (Ctsl), a cTEC-specific protease involved in MHCII-antigen processing, shapes the selected CD4+ T cell compartment. To do so, we generated a conditional knock out model, in which the expression of Ctsl is ablated from the thymic epithelium, so that antigen presentation to CD4+ T cells is altered exclusively during positive selection. In this scenario CD4+ T cell development is severely impaired, with regards to both cell numbers and TCR diversity, although we could identify some TCRs that are selected regardless of Ctsl expression. Furthermore, polyclonal TCR stimulation and immunization with the listeriolysin O antigen revealed defective T cell responses, which could derive from a decreased functionality of TCRs per se or from the lack of some specific clones. To test if the very same TCR – selected in the presence or absence of Ctsl – is equally functional, we used the identified "Ctsl-independent" TCRs to generate TCR transgenic mice on both genetic backgrounds. Interestingly, our data show that CD4+ T cells bearing the same TCR, but selected in the absence of Ctsl, are less fit in homeostatic conditions, yet get hyperactivated when they encounter their cognate antigen. In summary, we propose that the positive-selecting peptides produced by Ctsl in the thymus influence not only the size and diversity, but also the functionality of the peripheral CD4+ T cell compartment by fine-tuning the levels of CD5, CD6 and other TCR-associated molecules on developing T cells.

Immo Prinz
University Medical Center Hamburg-Eppendorf, Institute of Systems Immunology
i.prinz@uke.de

Resident γδ and αβ T cells in human lung and associated lymph nodes adapt into complementary regulatory and cytotoxic phenotypes

Likai Tan, Evgeny Chichelnitskiy, Joana de Barros Martins, Inga Sandrock, Elena Bruni, Danny Jonigk, Christine Falk, Immo Prinz

γδ T cells are prototype tissue-resident T cells (Trm), but only little is known about their functional plasticity and specific adaptation to human tissues. Here, we investigated the phenotype and function of circulating and tissue-resident αβ T and γδ T cells in paired samples from explant lung parenchyma and associated lymph nodes from seven end-stage emphysema patients. Sorted αβ T cells and γδ T cells were tagged and analyzed via sc-RNA-seq, CITEseq, and concomitant sc-αβTCR-seq or sc-γδTCR-seq, analysis to barcode individual T cell clones. Trm were identified based on the protein and RNA expression of CD103 and CD49a. T cell differentiation networks were inferred from TCR repertoire signatures and subsequently validated through transcriptome analysis. Our results revealed that the phenotype of many αβ T cells and γδ T cells segregated according to tissue origin and thus were strongly influenced by their microenvironment. Interestingly, some circulating CD4+ αβ, CD8+ αβ, and γδ T cells adopted highly overlapping cytotoxic phenotypes. In contrast, CD103+CD49a+ Trm displayed a clear distinction between γδ T cells and CD4+, CD8+ αβ T cells. While CD8+ αβ Trm were NK cell-like cytotoxic effectors, the CD4+ αβ Trm and γδ Trm showed rather immune-regulating phenotypes. All Trm cell subsets exhibited more focused clonal TCR repertoires compared to their circulating counterparts. However, similarity analysis demonstrated more distinct TCR repertoires of γδ Trm and CD4+ Trm, suggesting that these may have resided in lung tissue for a longer time than CD8+ Trm. Furthermore, TCR-guided trajectory analysis placed γδ Trm and circulating γδ T cells on different lineage branches. In sum, we demonstrate that the tissue microenvironment shapes the phenotype of αβ T cells and γδ T cells in end-stage emphysema patients. Side by side analysis of αβ T cells lung and associated lymph nodes revealed distinct migration and differentiation trajectories of CD4+, CD8+ and γδ T cells.

Franziska Prummer
Institute for Immunology, Biomedical Center, Ludwig Maximilian University Munich, Germany
franziska.prummer@med.uni-muenchen.de

Binding of Extracellular Vesicles by T Cells during Chronic Infection

Franziska Prummer, Tijana Kandić, Christine Ried, Jan Kranich and Thomas Brocker

During acute viral infection with lymphocytic choriomeningitis virus (LCMV), lymphocytes are decorated with phosphatidylserine (PS)-exposing extracellular vesicles (EVs). These EVs have antigen-specific adjuvant effects on activated CD8 T cells in vivo. In cancer, programmed cell death ligand-1 (PDL1)-carrying EVs induce T cell exhaustion; however, it is not known whether the same applies for chronic infection. Previous experiments from our lab revealed that lymphocyte–EV interactions peak at day 5 and decline until day 15 during acute viral infection. During chronic infection, we hypothesized that if EVs persist longer in the spleen, they might contribute to T cell function or exhaustion. This might be caused by sustained T cell–EV interactions, involving continuous presentation of viral antigens or other molecules, like PDL1, to T cells. To address this question, we infected mice with the LCMV strains Armstrong (Arm) or Clone 13 (Cl13) leading to acute or chronic infection, respectively. Subsequently, we analyzed T cell–EV interactions by imaging flow cytometry and changes of the splenic microarchitecture that could affect EV availability by immunohistochemistry. Indeed, we found preliminary evidence that PDL1 might be expressed on EVs 8 days post infection (dpi) with LCMV-Cl13 on EV+ CD8 effector T cells. Furthermore, higher frequencies of EV+ CD8 T cell subsets (naïve, central memory and effector) were found in LCMV-Cl13-infected mice compared to LCMV-Arm infection. Taken together, our data show that EVs persist longer in the spleen during chronic infection and indicate that PDL1+ EVs potentially contribute to CD8 T cell exhaustion.

Marine Pujol
Technical University of Munich
Marine.pujol@tum.de

Mapping the origins of adaptive-like NK cells responses

Marine Pujol, Sophie Flommersfeld, Weixu Wang , Anton Mühlbauer, Fabiola Curion,Sebastian Jarosch, Simon Grassmann, Dietmar Zehn, Veit R. Buchholz

Advances over the past decades have revealed that NK cells mediate memory-like responses after viral infection, specific hapten exposure, or cytokine activation (Min-Oo et al, 2013). Immunological memory is a hallmark of adaptive immunity and is characterized by the long-term persistence of memory cells that rapidly undergo clonal expansion and present enhanced effector functions in response to secondary challenge. The first evidence of NK cell memory was demonstrated in the well-established model of murine cytomegalovirus (MCMV) infection (Sun et al, 2009). Murine NK cells harboring the activating receptor Ly49H which specifically interacts with the m157 protein encoded by MCMV, were found to reside in lymphoid and non-lymphoid organs for several months; their functions were enhanced upon reinfection; and they produced a robust secondary expansion upon second viral challenge. By fate mapping of single NK cells, we previously described that single Ly49H+ NK cells clones substantially expand in response to primary MCMV infection (Grassmann et al., 2019). Moreover, Ly49H+ NK cell clones adopt two phenotypically distinct response patterns during MCMV infection. While one of these patterns emerges from conventional NK (cNK) cells the other was generated by a subset of NK cells that where transcriptionally similar to type 1 innate lymphoid cells (ILC1s). These ILC1-like NK cells strongly produced cytokines; induced clustering of conventional type 1 dendritic cells; and facilitated antigen-specific T cell priming early during MCMV infection (Flommersfeld et al, 2021). We could show that cNK and ILC1-like NK cells remained transcriptionally distinct during acute and memory phase of MCMV infection while only ILC1-like NK cells restored the expression of potential memory-precursor markers in a subset of cells. Further experiments indicated that ILC1-like NK cells were preferentially maintained during contraction phase and could therefore play a predominant role in the generation of NK cell-mediated immunological memory.

Timsse Raj
LMU Munich
timsse.raj@med.uni-muenchen.de

Roquin-deficient T cells drive pancreatitis and tumorigenesis through IL-17 and Th17-inappropriate cytokine production

Timsse Raj, Desheng Hu, Francesco Gualdrini, Sara Polletti, Jessica Zoeller, Elaine H. Wong, Sebastian Mueller, Gaja Bianco, Katja Steiger, Maike Becker, Sophie E. Isay, Jürgen Ruland, Danijela Heide, Carolin Daniel, Jana Schor, Roland Rad, Gioacchino Natoli, Mathias Heikenwälder and Vigo Heissmeyer

Pancreatitis can trigger pancreatic ductal adenocarcinoma (PDAC), however, the underlying origins can be diverse and the cellular and molecular mechanisms remain elusive. We show that combined genetic inactivation of the RNA-binding proteins Roquin-1 and Roquin-2 in T cells induced pancreatitis, pancreatic neoplasia or accelerated PDAC formation, if KrasG12D was expressed in acinar cells. In the pancreas, Roquin-deficient T cells were activated, recruited proinflammatory neutrophils via IL-17A secretion, and inappropriately produced G-CSF that, in a feed-forward loop, further induced and mobilized neutrophils. Consistently, neutralization of IL-17A or G-CSF ameliorated pancreas pathology. Roquin repressed G-CSF in two ways. It directly inhibited Csf3 mRNA expression through its 3'-UTR, and indirectly, because Roquin loss-of-function imposed transcriptional reprogramming by NF-kB and established an active enhancer at the Csf3 locus in Th17 cells. Together, we identified critical cellular and humoral components and epigenetic, transcriptional and post-transcriptional mechanisms within a regulatory circuit that prevents pancreatic cancer formation.

Julie Ribot
Instituto de Medicina Molecular, University of Lisbon
julie.ribot@gmail.com

IL-17 regulates peripheral nerve regeneration

André Bombeiro, Rodrigo Fernandes, Vladimir Ghilas, Julie Darrigues, Bruno Silva-Santos and Julie Ribot

Peripheral nervous system (PNS) injury is a major clinical and economical concern that leads to chronic pain and motor dysfunction. The immune response is critical to clear the site of injury and secrete growth factors for axonal regeneration. While the role of macrophages and neutrophils is well known, it remains poorly understood whether and how T cells influence this process. Recently, a cross-talk between T cells producing IL-17A (henceforth IL-17) and the gut microbiota has been established, with IL-17 modulating the microbiome composition, which in turn regulates IL-17 levels in tissue. Here, we hypothesize that IL-17 influences the regeneration of the PNS by shaping the microbiota. Using a mouse model of sciatic nerve crush, we observed an accumulation of immune cells expressing IL-17 in the crushed nerve, throughout the recovery process. Interestingly, when injured mice were treated with a broad spectrum antibiotic cocktail, the numbers of IL-17+ cells were significantly reduced in the nerve and draining popliteal lymph node. To test the role of these cells, we submitted mice deficient in this cytokine (Il17-/-) and their wild-type littermate controls (Il17+/+) to a walking track test. Of note, animals were either co-housed or separated according to their genotype. We observed that when separated, il17-/- mice did not fully recover their sciatic nerve function - contrary to their littermate controls -, showing that motor recovery relies on a mechanism involving IL-17. Noteworthy, the impaired motor function observed in il17-/- mice was rescued when the animals were cohoused with their wild-type littermates, suggesting a critical implication of the microbiome in this process. Mechanistically, we demonstrated that separated Il17-/- mice have a dysregulated expression of the regeneration marker growth associated protein 43 (GAP43), which was restored when the animals were co-housed with their wild-type littermates, or when treated with antibiotics. Overall, our data reveals a bidirectional pathway of the IL-17-microbiota crosstalk in regulating PNS regeneration.

Sandra Riemer
LMU Munich, BMC, Institute for Immunology
sandra.riemer@med.uni-muenchen.de

Effects of a live viral vaccine on innate immune cells at transcriptomic and epigenomic level

Sandra Riemer, Elena Winheim, Rika Mack, Linus Rinke, Antonia Eicher, Johanna Klughammer, Simon Rothenfusser, Anne Krug

The life-attenuated yellow fever virus vaccine YF17D is a highly effective vaccine. Adjuvanted vaccines, in addition to the eliciting adaptive immunity, have been shown to induce longer lasting effects in innate immune cells such as monocytes, DCs and NK cells via epigenetic mechanisms leading to altered innate responses to subsequent infections. This has not yet been studied in the setting of a live viral vaccine. We therefore investigated the response of circulating innate immune cells to YF17D vaccination on the transcriptomic and epigenomic level. Bulk RNAseq results showed that DC and monocyte subsets displayed differentially expressed genes up to day 28, with a peak on day 7 after YF17D vaccination. These transcriptomic changes beyond the innate immune response phase were found most prominently in monocytes, but also in DC subsets. As a potential mechanism we hypothesized that epigenetic changes are elicited in these cells or their precursors by YF17D vaccination leading to differential gene expression at later time points. Therefore, we performed scATAC and RNAseq on DCs, monocytes, and NK cells isolated from PBMC of vaccinees at different time points before and after YF17D vaccination. Our analysis shows successful data generation for all DC, monocyte and NK cell subpopulations, including rare subtypes such as transitional DC and cDC1. Further analysis will reveal whether long-lasting changes in chromatin accessibility are associated with altered gene expression. To determine whether the vaccine evokes innate immune training or tolerance as a consequence of such epigenetic imprinting, we are currently establishing a restimulation assay with PBMCs from before and 28 days after YF17D vaccination. The PBMCs are stimulated with various viral and bacterial stimuli, as well as with a RIG-I ligand and the concentrations of secreted cytokines are measured in the supernatants. The approaches above will aid in better understanding the role of the innate immune response to YF17D and provide insights into the presumed training or tolerance effects of this live viral vaccine.

Sonja Rittchen
Medical University of Graz
sonja.rittchen@medunigraz.at

Exploring functional modulation of T cell subsets by extracellular nucleotides: Differential response in the aged immune system?

Sonja Rittchen, Anja Zdouc, Stefano Angiari, Johannes Fessler

Age-related changes in immune function include a greater susceptibility to infections, autoimmune disorders, cardiovascular disease and even cancer. Cellular aging known as senescence is associated with functional changes in T cells, which contribute to chronic low-grade inflammation in the elderly. Cell activation or tissue damage leads to release of nucleotides that bind P2 receptors and modulate T cell function in various settings. The aim of this study is to characterize whether alterations in the aged immune system lead to a differential response to extracellular nucleotides. PBMC of young (<30 years) and elderly (>55 years) healthy donors were isolated from buffy coats. Flow cytometry was used to investigate the differential effects on T cell populations including naïve, central and effector memory, senescent and Th1, Th2, Th17 subsets as well as subset-specific expression levels of P2Y receptors. Acute activation with 1 μM to 100 μM of various nucleotide ligands on cytosolic calcium-flux, cellular reactive oxygen species (ROS) and mitochondrial mass, potential and ROS was investigated. T cell migration towards nucleotides was quantified with a transwell migration assay. P2Y receptors are differentially expressed in T cell subsets, while some are lost in senescent populations. At the concentrations tested, cellular ROS and mitochondrial parameters showed only minor differences after nucleotide activation. Ligands alone did not induce a detectable calcium flux in resting T cells, however, pre-treatment with ATP strongly potentiated the ionomycin-induced response in young donors. In contrast, T cells from aged donors displayed a higher response to ionomycin, while losing sensitivity to ATP. In line, T cells migrated towards ATP, an effect even more pronounced in aged donors. These data suggest a differential response of circulating T cell subsets to extracellular nucleotides depending on (i) variations in receptor expression, (ii) state of cellular senescence as well as (iii) biological age of the donor.

Ahmed Saadawi
ETH Zurich
ahmed.saadawi@biol.ethz.ch

Deciphering CD4+ T Cell Specificity to the Intestinal microbiota

Ahmed Saadawi, Manfred Kopf

Fundamental to understanding host-microbiota alliance is deciphering CD4+T-cell antigenic specificity. However, our knowledge of microbiota-derived antigens is limited. Here, we leveraged the MCR system to identify immunogenic peptide epitopes from two prominent members of the human gut microbiota, specifically Akkermansia muciniphila and Bacteroides thetaiotaomicron. Both bacteria play pivotal and beneficial roles in diverse biological contexts, including cancer immunotherapy, diabetes, allergy, obesity, and metabolism. A total of 27 peptides were identified from these bacteria, with a preferential enrichment of the tyrosine amino acid at position one—a critical anchor residue for MHC II binding. Subsequently, seven peptides were validated through in vivo mouse immunizations. CD4+T cells responsive to these seven peptides elicited dominant IL-17a response. Our ongoing investigations are aimed at elucidating the protective efficacy of select peptides across various pathological disease models.

Shinya Sakaguchi
Division of Immunobiology, Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria
shinya.sakaguchi@meduniwien.ac.at

Role of histone deacetylases for CD8+ T cell-mediated immunity

Ramona Rica, Monika Waldherr, Marlene Schülein, Emi Miyakoda, Lisa Sandner, Valentina Stolz, Darina Waltenberger, Thomas Krausgruber, Christoph Bock, Nicole Boucheron, Wilfried Ellmeier and Shinya Sakaguchi

CD8+ T cell exhaustion is orchestrated by a complex differentiation process of functionally distinct cell subsets; however, the underlying epigenetic and transcriptional mechanisms remain largely unknown. Here, we investigated the role of histone deacetylase 1 (HDAC1), a key epigenetic regulator, in the differentiation of exhausted T (Tex) cells during chronic viral infection. We uncovered that HDAC1 controls the generation as well as maintenance of CX3CR1+ Tex cell subset, known to retain certain effector-like function, in a CD8 T cell-intrinsic manner. Deletion of HDAC1 led to the enlargement of an alternative Tex cell subset enriched in exhaustion markers, accompanied with elevated viremia. Mechanistically, HDAC1 shapes the chromatin landscape at effector-like signature gene loci in progenitor Tex cells, thereby contributing to their cell fate specification process towards the CX3CR1+ Tex cell subset. Thus, our study demonstrates that HDAC1 serves as a gatekeeper controlling viral load by ensuring CX3CR1+ Tex cell subset differentiation.

Julia Scheffler
Gothenburg University, Dept. of Rheumatology and inflammation research
julia.scheffler@gu.se

Blood Th2 cells at baseline associate negatively with cortical bone density after 48 weeks of biological treatment in early rheumatoid arthritis

Julia M. Scheffler, Christina Drevinge, Catharina Lindholm, Inger Gjertsson, Hans Carlsten, Mattias Lorentzon, Anna-Karin Hultgård Ekwall, Anna Rudin, and Ulrika Islander

Background: The high prevalence of osteoporosis in rheumatoid arthritis (RA) is due to an inflammatory environment that stimulates the differentiation and activity of osteoclasts, a process that involves circulating monocytes and T-cell derived factors. So far, no studies have evaluated associations between circulating monocytes, T cell subsets and bone characteristics in RA patients before and after treatment with biological disease-modifying antirheumatic drugs (bDMARDs).

Methods: Thirty patients with untreated early (ue) RA fulfilling the ACR/EULAR 2010 criteria were included and data was obtained before the start of treatment and after 48 weeks of treatment with methotrexate (MTX) and one of the following bDMARDs: CTLA-4Ig (abatacept), anti-IL6R (tocilizumab) or anti-TNF (certolizumab-pegol). Disease activity was measured using CDAI, swollen joint counts, tender joint counts, CRP, and ESR. The proportions of monocyte subsets and CD4+ T helper cell subsets were analyzed in peripheral blood by flow cytometry. Bone densitometry was performed using high resolution peripheral quantitative CT (HR-pQCT).

Results: HR-pQCT measurements revealed a decrease in cortical and trabecular bone density after 48 weeks of treatment. The level of reduction was not associated with age, sex, or disease activity determined at baseline and 48 weeks. High proportions of circulating Th2 cells at baseline associated independently with low cortical volumetric bone mineral density at 48 weeks of treatment, while high proportions of baseline Th1 cells associated independently with high total volumetric bone density at 48 weeks.

Conclusions: Treatment with MTX and bDMARDs does not prevent disease related bone loss in ueRA patients during the first 48 weeks of treatment. The association of baseline Th2 cell with cortical bone loss after 48 weeks of treatment indicates a potential novel role for these cells in RA-induced bone metabolism.

Mika Schneider
Department of Biomedicine, University of Basel, University Hospital Basel
mika.schneider@unibas.ch

Brain viral infection unleashes latent autoimmune response

Mika Schneider, Hyein Kim, Nicholas Sanderson, Tobias Derfuss

Multiple Sclerosis (MS) is an autoimmune disease characterized by central nervous system (CNS) demyelinated lesions with perivascular immune cell accumulation. The efficacy of anti-CD20 therapy suggests that a likely pathomechanism involves single autoreactive B cells within the CNS that secrete demyelinating antibodies, whose action is limited to the adjacent parenchyma. This model requires several tolerance failures: (i) CNS-antigen-reactive B cells must escape central tolerance; (ii) these B cells must enter the CNS; and (iii) they must either obtain T cell help, or differentiate into antibody secreting cells without it. We have examined each of these checkpoints. (i) Screening the blood of normal humans reveals a frequency of B cells specific for the CNS antigen myelin oligodendrocyte glycoprotein (MOG) of around 1 per million. (ii) B cells are largely excluded from the healthy CNS, but focal infection of the mouse striatum with vesicular stomatitis virus induces robust infiltration of B cells regardless of their specificity or activation status, and leads to formation of perivascular lymphocyte cuffs as seen in MS lesions, but without demyelination. Such infections in mice whose B cell receptor is specific for MOG similarly induces B cell infiltration, but still has no impact on myelin. B cells retrieved from the brains of these animals can activate MOG-specific T cells, demonstrating antigen capture and presentation, but at later time points, flow cytometry and single cell RNA sequencing reveal that the MOG-reactive B cells have been eliminated, possibly by activation-induced cell death. If brain-infiltrating, MOG-reactive B cells get exogenous CD40 stimulation, they escape activation-induced death, differentiate into brain-resident plasma cells, and induce demyelinated lesions similar to those seen in MS. In vitro experiments demonstrate that expression of the EBV protein LMP1 in B cells provides this survival signal. These results suggest that the only requirements for developing MS are infection of CNS and infection with EBV, which is epidemiologically near-universal in people with MS.

Barbara Schraml
LMU Munich Biomedical Center
barbara.schraml@bmc.med.lmu.de

RORγt-expressing DC-like cells are antigen presenting cells distinct from cDCs and ILC3s conserved across age, tissues and species

Hamsa Narasimhan, Maria Richter, Ramin Shakiba, Kaushikk Ravi, Nikolaos Papaioannou, Isabel Ulmert, Christina Stehle, Vanessa Küntzel, Ludger Klein, Diana Dudziak, Katharina Lahl, Chiara Romagnani, Maria Colomé-Tatché, Barbara U. Schraml

Antigen presenting cells (APCs) orchestrate T cell-mediated tissue immunity and pathogen defense with maintaining tolerance to benign antigens. To ensure situation adapted immunity, APCs, such as conventional dendritic cells (cDCs), exist as functionally spezialised subtypes. Recently, Retinoic acid receptor-related orphan receptor-γt (RORγt) expressing APCs have emerged as regulators of T cell mediated inflammation and tolerance to self and commensals, however, their subsets, developmental origins and functional specializations are incompletely understood. Here we report a unique RORγt-expressing APC in murine spleen that is ontogenetically and transcriptionally distinct from cDCs, RORγt+ type 3 innate lymphocytes (ILC3s) and extrathymic autoimmune regulator (AIRE)-expressing cells (eTACs). These cells appear conserved across murine lymphoid and non-lymphoid tissues and can also be found in human spleen, lymph nodes and intestine. Collectively, our data indicate that RORγt+ DC-like cells represent a unique type of APC, present across organs and responsive to inflammation. Our work suggests that these cells may exhibit distinct roles in T cell mediated immunity and therefore opens new avenues for harnessing the ontogenetic diversity and functional specialization of APC subtypes to understand and specifically manipulate immune responses.

Isabelle Serr
Helmholtz Munich
isabelle.serr@helmholtz-munich.de

miR150 targets Akt signaling to shape Th9 differentiation in models of type 1 diabetes

Isabelle Serr[1,2], Alexey Surnov[1,2] and Carolin Daniel[1,2,3]

1 Type 1 Diabetes Immunology, Helmholtz Diabetes Center at Helmholtz Zentrum München, Munich, Germany,
2 Deutsches Zentrum für Diabetesforschung, Neuherberg, Germany,
3 Division of Clinical Pharmacology, Department of Medicine IV, Ludwig-Maximilians-Universität München, Munich

Type 1 diabetes (T1D) has been linked to critical Treg impairments, however, the mechanisms and cellular contributors are insufficiently understood. In NOD mice overshooting PI3K-Akt signaling was shown to reduce Treg induction, function and stability. PI3K-Akt signaling also regulates Th9 cells, a CD4+T cell subtype that secretes Il9, a cytokine with context-dependent pro- or anti-inflammatory properties. However, the relevance of these pleitropic functions has not been studied in T1D before. Strikingly, in vitro Th9 induction is strongly impaired in T cells from NOD vs. non-autoimmune-prone Balbc mice (5.6+/-0.3% vs. 42.8+/-2.7%, p<0.0001), which can be partly restored by AKT inhibition (vehicle vs AKT inhibitor: 4.6+/-0.7% vs 7.5+/-1.4%, p=0.0051). Among possible molecular regulators involved in this impaired Th9 induction microRNAs (miRs) come into play, being regulators of complex cellular states. From RNA sequencing experiments, one candidate miR possibly involved in regulating the PI3K-AKT pathway, is miR150-5p, which we found to be downregulated in T cells from children and mice with islet autoimmunity. miR target prediction and HITS-CLIP analysis indicate that miR150-5p targets components of PI3K-AKT signalling, and specifically Akt3, which we confirmed as a direct target in luciferase assays. Interestingly, siRNA-mediated silencing of Akt3 or the delivery of a miR150-5p mimic, improves in vitro Th9 induction from NOD mice (control vs Akt3 siRNA: 4.2+/-0.2% vs 7.3+/-0.5%, p<0.0001; control vs miR150-5p mimic: 4.7+/-1.2% vs 6.7+/-1.3%, p=0.0025). Il9 can excert anti-inflmmatory functions by fostering Tregs. Accordingly, we demonstrate a reduction in in vitro Treg induction, effector Treg frequencies in the pancreas and Treg suppression in vitro in Il9 deficient mice. Importantly, the treatment of NOD mice with recombinant Il9 in vivo resulted in signifcantly increased frequencies of insulin-specific Tregs in the pancreas (vehicle vs Il9: 18,8+/-2,1% vs 41,8+/-2,2%, p=0,030) and reduced insulits scores. Overall, our data highlight Th9 cells and the miR150-AKT3 signaling pathway as potential novel targets for immune intervention to foster Tregs in T1D.

Margarita Shnipova
LMU Munich, BMC, Institute for Immunology
margarita.shnipova@med.uni-muenchen.de

Labeling endosomal compartments in live dendritic cells to investigate viral entry and fusion

Margarita Shnipova, Linus Rinke, Helen Stirling, Max Baker, Giovanna Barba-Spaeth, Anne Krug

Yellow fever virus (YFV) is a single stranded RNA virus belonging to the flavivirus family. Life-long immunity against yellow fever can be induced by the live-attenuated vaccine YF17D. Despite being derived almost a century ago, the exact molecular mechanisms governing its immunogenicity still remain to be completely elucidated. Previous studies have demonstrated that YF17D infects dendritic cells (DCs), yet viral replication is highly restricted by type I interferons (IFNs). However, the precise infection mechanisms of YF17D in DCs including endocytic trafficking and fusion which influence virus sensing and viral replication in DCs remain unclear. To explore endocytosis and fusion of YF17D we plan to perform live cell imaging experiments using labeled virus particles. Our objective is to conduct co-localization analysis of viral fusion events with endosomal compartments in DCs. This will be achieved by labeling early and late endosomes by expressing fluorescently tagged endosomal proteins in DCs and by labeling virus particles with a lipophilic dye that dequenches during fusion. To overcome challenges of transfecting moDC with plasmid DNA, we aimed to develop a method to label endosomal compartments in DCs by introducing in vitro transcribed mRNAs through electroporation. Thus far, we were able to generate mRNAs encoding fusion proteins of early GFP-tagged endosomal markers Rab5 and EEA1 and late markers Rab7a and Rab9a fused with mCherry. These mRNAs were successfully transfected and expressed in human monocyte-derived DCs, as verified by flow cytometry and confocal microscopy. Confocal imaging showed distinct vesicular staining patterns likely reflecting correct labeling of early and late endosomes by the respective endosomal fusion proteins. Our work establishes a basis for investigations of viral endocytosis and fusion in human DCs in the future.

Dimitrios Starfas
Institute for Immunology, Biomedical Center LMU
Dimitrios.Starfas@med.uni-muenchen.de

Influence of type I IFNs on cDC2 heterogeneity and regeneration from precursors in acute LCMV infection

Dimitrios Starfas, Konstantin Lutz, Barbara Schraml, Jan Kranich, Anne Krug

Dendritic cells (DCs) can be classified into conventional DC (cDCs) and plasmacytoid DC (pDCs based on function, ontogeny and differential expression of surface proteins and transcription factors. While cDCs are highly potent antigen presenting cells, pDCs play a major role in antiviral defense, by secreting type I IFNs. Recently, transitional DCs (tDCs), which share characteristics of pDCs and cDCs, were described, which differentiate into type 2 cDCs (cDC2) in steady state, while at the same time being able to respond to microbial stimulation and to activate T cells. Within the CD11c+ SiglecH+ B220lo Ly6D+ Zbtb46– fraction of murine bone marrow (BM) cells we identified immediate precursors of pDCs and cDCs. The latter differentiated into mostly cDC2, via a Zbtb46+ Ly6D+ intermediate stage, when cultured in vitro or adoptively transferred. These intermediate cells are related to tDCs described in spleen. Interestingly, when Ly6D+ Zbtb46– precursors were cultured in vitro in the presence of type I IFN, they were arrested in the Ly6D+ Zbtb46+ intermediate stage and their cDC potential was abrogated. To further investigate this in vivo, a systemic type I IFN response was induced in Zbtb46-eGFP and Zbtb46-eGFP/IFNAR-KO mice by injection of PolyI:C or by acute LCMV infection. Our results indicate that type I IFNs lead to rapid activation and temporary depletion of all splenic DC subsets including tDCs, with different sensitivity of the subsets. For example, ESAM+ cDC2A were more frequent than ESAM– CX3CR1+ cDC2B and within the tDCs CD11clo Ly6C+ tDCs were reduced compared to CD11chi Ly6C– tDCs. These effects were abrogated in IFNAR-KO mice treated with Poly I:C and partially reduced in IFNAR-KO mice infected with LCMV, indicating that type I IFN skews the balance between subsets of cDC2 and tDCs. In the BM, DC progenitors were reduced on day 3 and partially replenished on day 5 after LCMV infection with a faster recovery in IFNAR-KO mice. Our data indicate that DC frequencies, subset composition and regeneration from precursors are temporarily altered during acute viral infection mediated largely by type I IFNs.

Maria Stigler
PLUS Salzburg
mariaantonia.stigler@plus.ac.at

HDAC1 as a regulator of CD4+ T cell expansion in skin autoimmunity

Jensen M., Taliento A. E., Stigler M.A., Ellmeier W., Gratz I.K.

The skin constitutes the first line of defense against pathogens and toxins derived from the environment. It mounts host-protective responses while maintaining immune homeostasis. These functions are fulfilled by immune cells, including CD4+ T cells. Histone deacetylases (HDACs) regulate the acetylation status of histones and non-histone proteins by removing acetylation marks on lysine residues. Hence, HDACs control several biological processes, such as differentiation and function of CD4+ T cells. However, the exact role of HDAC1 in CD4+ T cells, specifically in skin autoimmune diseases, is not well understood. To assess the role of HDAC1 in the regulation of cutaneous CD4+ T cells, we utilized a well-established mouse model of experimental skin autoimmune disease (K5/TGO) in which ovalbumin (Ova) is expressed by keratinocytes in a tetracycline-dependent manner. We adoptively transferred HDAC1-WT or HDAC1-deficient (HDAC1-cKO) naïve Ova-specific CD4+ OTII T cells in K5/TGO/TCRα-/- recipient mice. Transferred OTII cells responded to the neo-self-antigen Ova in the skin and elicited inflammation. Recipients of HDAC1-cKO T cells displayed increased skin inflammation. This was in line with a decreased fraction of peripherally induced Foxp3+ Treg in HDAC1-cKO OTII recovered from K5/TGO recipients and with increased numbers of HDAC1-cKO T cells in the skin-draining lymph nodes (sdLNs). Surprisingly, in a competitive setting in which HDAC1-WT and HDAC1-cKO T cells were mixed in a 1:1 ratio with congenically labeled naïve CD4+ OTII T cells, respectively, before adoptive transfer, HDAC1-cKO T cells showed a disadvantage for expansion both in sdLNs and skin as well as reduced activation and skin-homing, which might indicate that HDAC1 impacts the potential of OTII to interact with antigen-presenting cells. To test this, we will study early events of T cell activation and proliferation and chemokine receptor regulation of HDAC1-cKO versus WT CD4+ T cells. With the help of already obtained scRNA-sequencing data, we hope to further understand transcriptional changes and determine HDAC1-dependent regulators of T cell expansion and TCR signaling in skin autoimmunity.

Patrizia Stoitzner
Medical University of Innsbruck
patrizia.stoitzner@i-med.ac.at

Tumor-targeted therapy with BRAF-inhibitor remodels the myeloid landscape to promote tumor immunity in melanoma

Florian Hornsteiner, Janine Vierthaler, Helen Strandt, Antonia Resag, Zhe Fu, Markus Ausserhofer, Christoph H Tripp, Sophie Dieckmann, Kathryn Farrand, Sarah Bregar, Niloofar Nemati, Natascha Hermann-Kleiter, Athanasios Seretis, Markus Kanduth, Sudhir Morla, David W Mullins, Francesca Finotello, Zlatko Trajanoski, Guido Wollmann, Franca Ronchese, Marc Schmitz, Ian F Hermans, Patrizia Stoitzner

Tumor-targeted therapy causes impressive tumor regression, but the emergence of resistance mechanisms results in limited long-term survival benefits in patients. Little information is available on the role of the myeloid cell network, especially dendritic cells (DC), during tumor-targeted therapy. Therefore, we investigated therapy mediated immunological alterations using the D4M.3A preclinical melanoma model harboring the BRAFV600E mutation. Our findings reveal that BRAF-inhibitor (BRAFi) therapy increased tumor immunogenicity, reflected by a distinct transient inflammatory tumor milieu. We observed a T cell-inflamed tumor microenvironment (TME) linked to an upregulation of genes associated with immune activation in treated tumors. The inflamed TME contained higher numbers of activated cDC1 and cDC2 but also inflammatory CCR2-expressing monocytes. At the same time, tumor-targeted therapy enhanced the frequency of migratory, activated DC subsets in tumor-draining lymph nodes (LN). Even more, we identified a cDC2 population expressing the FcγRI/CD64 in tumors and tumor-draining LN that displayed high levels of CD40 and CCR7 indicating involvement in T cellmediated tumor immunity. The importance of cDC2 is underlined by just partial loss of therapy response in a cDC1-deficient mouse model. Both CD4+ and CD8+ T cells were essential for therapy response as their respective depletion impaired therapy success. Upon resistance development, the tumors reverted to an immunologically inert state with a loss of DC and inflammatory monocytes together with the accumulation of regulatory T cells (Treg). Moreover, tumor antigen-specific CD8+ T cells were compromised in proliferation rate and IFNγ-production. Our findings give novel insights into the remodeling of the immune landscape by tumor-targeted therapy. This knowledge has important implications for the development of future combinatorial therapies.

Adrian Straub
Technische Universität München, Institut für medizinische Mikrobiologie, Immunologie und Hygiene
adrian.straub@tum.de

Unraveling T-cell-receptor (TCR) specificity through generation of ultra-deep antigen-reactive TCR libraries

Adrian Straub[1,†], Felix Drost[2,†], Yang An[2], Sabrina Wagner[1], Jack Barton[1], Linda Warmuth[1], Anton Mühlbauer[1], Julian Hönninger[1], Monika Hammel[1], Kilian Schober[3,4], Dirk H. Busch[1,5]

1 Institute for Medical Microbiology, Immunology, and Hygiene, Technical University of Munich, Munich, Germany
2 Institute of Computational Biology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
3 Mikrobiologisches Institut–Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
4 Medical Immunology Campus Erlangen, Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg, Schlossplatz 1, Erlangen, Germany
5 German Center for Infection Research, Deutschen Zentrum für Infektionsforschung (DZIF), Partner Site Munich, Munich, Germany
† These authors contributed equally to this work

T cells express a diverse repertoire of antigen-reactive T-cell-receptors (TCR) which lay the foundation for adaptive T-cell-mediated immunity. The TCR encodes not only the specificity to its target but also the functionality of a T cell. Nevertheless, to reliably link a TCR sequence to its cognate antigens remains a challenge. So far, only limited amounts of paired TCR-epitope interactions are publicly available. We generated an ultra-deep paired TCR library against the H-2Kb-restricted model epitope SIINFEKL comprised of more than 10.000 unique TCRs from 360 individual repertoires. Additionally, we constructed intermediate sized TCR libraries against frequently used model antigens derived from murine cytomegalovirus (MCMV) and lymphocytic choriomeningitis virus (LCMV). We found that TCRs are limited in their structural diversity to convey a defined specificity and share public TCR sequence motifs which allow for binding to its epitope. In addition, we trained a large language model to predict TCR-epitope interactions based on our own sequencing data consisting of more than 10.000 annotated TCR-epitope interactions and more than 50.000 unique T-cell-receptors derived from naïve repertoires. We achieved a strong prediction performance in-between an epitope specificity and are expanding to evaluate performance across specificities including functional evaluation. The capability to reliably predict antigen-reactive and at same time highly functional TCRs in-silico, is highly relevant e.g. for adoptive T cell therapies and will significantly increase the speed of target discovery and TCR evaluation.

Robin Thiele
German Cancer Research Center (DKFZ)
robin.thiele@dkfz-heidelberg.de

Constrained B-cell development promotes malignant transformation

Robin Thiele[1,3], Verena Körber[2], Csilla Kongsaysak-Lengyel[1], Immanuel Rode[1], Thorsten B. Feyerabend[1], Thomas Höfer[2], Hans-Reimer Rodewald[1]

1 Division of Cellular Immunology German Cancer Research Center
2 Division of Theoretical Systems Biology German Cancer Research Center
3 Faculty of Biosciences Heidelberg University

Cancer is generally thought to be initiated by oncogenic mutations. However, cancer driver mutations are found frequently also in normal tissues, leading to cell clones with minor selective advantages that normally fail to progress to cancer. This discrepancy suggests that additional promoting conditions are required for malignant transformation. In the past, our lab has demonstrated that the absence of cell competition among early T-cell progenitors in the thymus promotes spontaneous development of T-cell acute lymphoblastic leukemia (T-ALL). These T-ALL exhibit recurrent genomic lesions that mirror the human disease. Here, we provide evidence for a second model of tumor promotion by a similar perturbation, now yielding B-cell lymphomas by limiting stem/progenitor contribution to the B-cell lineage. These transplantable B-cell lymphomas display recurrent genetic lesions, including chromosome 18 trisomy and a deletion in the Kmt2c locus. In conclusion, our novel B-cell lymphoma model supports the concept that in hematological malignancies stochastically acquired driver mutations become 'unleashed' for malignant transformation by extended progenitor dwell times. Hence, perturbation of cell competition among B-cell progenitors may promote malignancy.

Stephen Todryk
Northumbria University, Newcastle upon Tyne, UK; & Cellular Technology Ltd, Cleveland, USA
stephen.todryk@northumbria.ac.uk

Antiviral immunity revealed by B cell ImmunoSpot is informative for immunity to future viral exposures

Stephen Todryk[1,2], Zhigang Liu[2], Yong Gao[2], Noémi Becza[2], Jack Chepke[2], Xing-Huang Gao[2], Paul Lehmann[2], Greg Kirchenbaum[2]

1 Northumbria University, Newcastle upon Tyne, UK
2 Cellular Technology Ltd, Cleveland, USA

Viruses that circulate in the human population are subject to immune-mediated selection pressure (naturally-acquired or vaccine-mediated) that drives the emergence of antigenic variants. Such variants can cause reinfections owing to their ability to evade neutralizing antibody activity. Pre-existing memory B cells (Bmem) serve as a secondary wall of humoral defence. While the Bmem may initially possess a reduced affinity for the viral variant, they are at an elevated precursor frequency. Moreover, such Bmem can be re-recruited into germinal center reactions where they undergo further rounds of proliferation and acquire somatic mutations to refine both the specificity and affinity of their antigen receptors and ultimately their secreted antibodies. Identification of such cross-reactive Bmem, together with a measure of their affinity, indicates a subject's ability to mount an effective recall response upon viral re-encounter and might inform re-vaccination regimens. Owing to the scarcity of antigen-specific Bmem and technical difficulties associated with their identification, assessment of Bmem reactivity and affinity against emerging SARS-CoV-2 variants in large donor cohorts has been largely neglected. Using recent innovations in the B cell ImmunoSpot® platform, this work evaluated Bmem reactivity in subjects infected early in the pandemic with the prototype Wuhan virus (Hu-1) for recognition of Receptor-Binding Domain antigens representing the Delta (B.1.617.2) and Omicron (BA.1) variants of concern (VOC). Fluorescent spots in the assay revealed Bmem secreting specific antibodies (following culture) of varying affinity. This study was thus able to demonstrate B cell cross-reactivity against key antigens from VOCs. Collectively, this effort demonstrates the feasibility of performing B cell ImmunoSpot® assays to efficiently, and with limiting cell input numbers, identify cross-reactive Bmem and their affinity at single-cell resolution. This assay is therefore a useful addition to the current immune monitoring toolbox.

Janine Trombke
Max Delbrück Center Berlin-Buch
janine.trombke@gmail.com

Occurrence of T-ALLs in mice reconstituted with hematopoietic stem and progenitor cells (HSPCs) carrying activating mutations in the PEST domain of Notch1

Trombke J[1], Graf R[2], Thiele R[3], Kongsaysak-Lengyel C[3], Rodewald HR[3], Chu VT[1,4] Rajewsky K[1]

1 Immune Regulation and Cancer, Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany
2 Muscle Research Unit, Experimental and Clinical Research Center (ECRC) of MDC and Charité, Berlin, Germany
3 Cellular Immunology, German Cancer Research Center (DKFZ), Heidelberg, Germany
4 Genome Engineering & Disease Models, Max Delbrück Center for Molecular Medicine (MDC) in the Helmholtz Association, Berlin, Germany

Introduction: Signaling through the Notch1 receptor plays a critical role in T lymphocyte development, and its dysregulation is implicated in T cell acute lymphoblastic leukemia (T-ALL) in humans. 50% of T-ALL patients harbor recurrent activating mutations in the heterodimerization domain or the PEST domain of the NOTCH1 gene. PEST domain mutations are thought to have a weaker activating effect than heterodimerization domain mutations.

Objective: This study aims to test whether mutations introduced into the PEST domain of Notch1 in HSPCs initiate T cell leukemogenesis in mice.

Methods: Using CRISPR/Cas9 mutagenesis, we introduced truncating point mutations and deletions into the PEST domain of the mouse Notch1 gene into HSPCs in vitro and used these cells to repopulate the hematopoietic system of immunodeficient recipients.

Results: T-ALLs developed 17-50 weeks post transplantation in 14 out of 30 (47%) transplanted Rag2-/-gc -/- mice. The observed T-ALLs were monoclonal, developed in the thymus and infiltrated the periphery, resembling human disease. 13 of 14 T-ALLs carried activating mutations in the Notch1 PEST domain.

Conclusion: Our study suggests that activating mutations in the Notch1 PEST domain arising in HSPCs can initiate T-ALL development. A possible contribution of Cas9-induced genome instability remains to be excluded.

Irina Tsymala
Medical University of Vienna
irina.tsymala@meduniwien.ac.at

Innate lymphoid cells regulate inflammation in acute fungal lung infection

Irina Tsymala[1], Philipp Penninger[1], Filomena Nogueira[1,2], Sabrina Jenull[3], Hossein Arzani[1], Dagmar Gotthardt[4], Veronika Sexl[4], Karl Kuchler[1]

1 Department for Medical Biochemistry, Max Perutz Labs, Campus Vienna Biocenter, Medical University Vienna, Vienna, Austria
2 CCRI—St. Anna Children's Cancer Research Institute, Vienna, Austria
3 Institute of Microbiology, Department of Pathobiology, University of Veterinary Medicine Vienna, Vienna, Austria
4 Institute of Pharmacology and Toxicology, Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Vienna, Austria

Innate Lymphoid Cells (ILCs) are part of the first-line immune defense protecting mucosal barriers from pathogens. ILCs support mucosal barrier integrity, but can also promote inflammation by contributing to tissue hyperreactivity and allergy. Yet, factors guiding their pro-inflammatory or protective functions in acute fungal infections remain to be resolved. Therefore, we explored how pulmonary ILCs respond to fungal pathogens in vitro and in the context of acute infections by two of the most clinically relevant fungal pathogens, *Candida albicans* and *Aspergillus fumigatus*. We analyzed the secretory activity, downstream pathways and short-term plasticity of ex vivo isolated ILCs and compared the activation and subset polarization of ILCs in mouse models of acute systemic and lung infection. Flow cytometry analysis revealed a pathogen-dependent plasticity of ILCs as reflected by a steady increase in IL-17A production. To assess the effect of pulmonary ILCs on disease progression, we performed adoptive transfer of ex vivo expanded ILC lineages into lymphocyte-deficient mice. The presence of ILCs was associated with attenuated inflammation and sustained tissue integrity, but led to increased fungal burden in acute lung infection. Overall, our data indicates that pulmonary ILCs are essential for maintaining tissue homeostasis during fungal infection and influence early antifungal immunity.

Gleb Turchinovich
University of Basel
gleb.turchinovich@unibas.ch

Power Of Two: New Binary Cre Mouse Model Specifically Targeting Type 3 Innate Lymphoid Cells

Franziska Bosch[1], Yagmur Farsakoglu,[1] Pawel Pelczar[1], Daniela Finke[1] and Gleb Turchinovich[1]

1 University of Basel

Type 3 innate lymphoid cells (ILC3s) are a subset of tissue resident lymphocytes that share functional programmes with IL-17/IL-22 secreting T cells. While being a clearly distinct lineage both developmentally and functionally, the precise role of ILC3s in many immune processes has not been clearly elucidated. The main obstacle to this is the aforementioned similarity to T cells. To date, no single promoter or gene has been shown to be uniquely specific for ILC3s. As a consequence of that, there is no suitable Cre mouse line for conditional mutagenesis in ILC3s. We have been able to circumvent this by using a "split" Cre approach, where Cre is expressed as two non-functional polypeptides fused to parts of trans-splicing intein protein. This system allows us to use two promoters to achieve reconstitution of Cre activity specifically in ILC3s. We chose RORgT as a promoter driving the expression N-terminal part of Cre. The second promoter was chosen based on the analysis of publicly available transcriptomic datasets. When crossed to ROSA26-STOP-YFP recombination reporter split-Cre activity was exclusively detected in ILC3s with no detectable activity in any other cell type. This reporter strain already allowed us to specifically trace ILC3s microscopically in organs, where their detection had been previously impossible. In sum, this approach finally enables conditional mutagenesis in ILC3s and opens new opportunities of functional genetic studies of ILC3s in vivo.

Sofia Tyystjärvi
Experimental Neuroimmunology, Klinikum rechts der Isar, Technical University Munich
sofia.tyystjaervi@tum.de

Tissue imprinting of CD4+ T cells in central nervous system autoimmunity

Sofia Tyystjärvi, Michael Hiltensperger, Thomas Korn

Multiple sclerosis (MS) is a chronic demyelinating autoimmune disease of the central nervous system (CNS). It is the most common acquired disabling neurological condition among young adults and therefore introduces a great burden not only on the affected individuals but also on the society. In the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis, autoreactive CD4+ T cells infiltrate the CNS and trigger inflammatory demyelination. Before infiltrating the CNS, T cells undergo a priming process in the secondary lymphoid organs. However, it is not well understood if the site of priming, e.g skin- versus gut-draining lymph node, will imprint functional differences in T cells. Using a mouse line bearing a green-to-red photoconvertible fluorescent protein to label T cells at different priming sites, we recently found that T cells primed in inguinal (i-T cells) versus mesenteric lymph nodes (m-T cells) have distinct transcriptomes, surface marker expression and infiltration patterns in the CNS. Here, we developed a simple surface marker-based strategy to distinguish signature subsets of i-T and m-T cells, thereby eliminating the need for using a labor-intensive photoconversion labelling system. Moreover, we found that i-T and m-T signature subsets differ by their activation status, Treg identity and memory potential.

Emilia Vendelova
Würzburg Institute of Systems Immunology (WüSI), Max Planck Research Group at the Julius-Maximilians-Universität Würzburg, Germany
emilia.vendelova@uni-wuerzburg.de

Stem-like memory cells give rise to pathogenic Th2 effector cells in allergic asthma

Emilia Vendelova, Ye Oyuang, Remi Doucet-Ladeveze, Christin Friedrich, Georg Gasteiger

Th2 cells represent a major pathogenic cell population driving allergic asthma. Using the house dust mite (HDM) challenge model of allergic asthma it has been shown that memory Th2 cells persist as circulatory cells as well as tissue-resident cells in the lung where they drive pathology upon allergen re-exposure. Here, we explored HDM-induced effector and memory Th2 cell heterogeneity in the spatiotemporal context, using intravascular staining, multiplex single-cell mRNA-sequencing, multicolor spectral flow cytometry and confocal microscopy. We show that diverse T helper responses are induced upon acute lung inflammation but only Th2 cells are efficiently maintained. Lung tissue was enriched for ILC2-like Th2 cells, which constitutively expressed IL5 and populated diverse niches, including adventitial spaces around vessels and bronchi. In addition, we identified a population of cells with features of stem-like memory cells. Adoptive transfer and genetic fate-mapping studies revealed that these cells can differentiate and give rise to pathogenic effector Th2 cells. Our study reveals unappreciated heterogeneity of memory and effector Th2 cells with implications for self-renewal and pathogenic effector function. Similarities and differences of ILC2 and resident Th2 cells will be discussed.

David Vermijlen
Université Libre de Bruxelles (ULB)
david.vermijlen@ulb.be

Invariant gammadeltaTCR natural killer-like effector T cells in the naked mole-rat

Guillem Sanchez Sanchez[1,2,3,4], Stephan Emmrich[5], Maria Georga[6], Ariadni Papadaki[6], Sofia Kossida[6], Andrei Seluanov[5], Vera Gorbunova[5] and David Vermijlen[1,2,3,4,#]

1 Department of Pharmacotherapy and Pharmaceutics, Université Libre de Bruxelles (ULB), Brussels, Belgium
2 Institute for Medical Immunology, Université Libre de Bruxelles (ULB), Gosselies, Belgium
3 ULB Center for Research in Immunology (U-CRI), Université Libre de Bruxelles (ULB), Belgium
4 WELBIO Department, WEL Research Institute, Wavre, Belgium
5 Department of Biology and Medicine, University of Rochester, Rochester, NY, USA
6 IMGT®, the international ImMunoGenetics information system®, Institut de Génétique Humaine (IGH), Centre National de la Recherche Scientifique (CNRS), Université de Montpellier (UM), Montpellier, France

The naked mole-rat (Heterocephalus glaber) is a long-lived rodent species, with a distinct set of unique biological traits conferring resistance to the spontaneous development of cancer. While, unexpectedly, it was reported that they lack natural killer (NK) cells, it has been recently suggested that naked mole-rats possess a gammadelta T cell-based immunity, that could form a main arm of the immune system involved in immunosurveillance and anticancer responses. Therefore, we investigated the biology of these unconventional T cells in peripheral tissues (blood, spleen) and thymus of the naked mole-rat at different ages by TCR repertoire profiling and single-cell (sc) gene expression analysis. Following a new TCR annotation in the naked mole-rat genome, we found that the gammadelta TCR repertoire is dominated by a public invariant Vgamma4-2/Vdelta1-4 TCR, of which the complementary-determining-region-3 (CDR3) sequences are likely generated by short homology repeat-driven DNA rearrangements. This invariant TCR is specifically expressed by gammadelta T cells possessing NK killing machinery, while more diverse gammadelta TCRs are associated with other functional phenotypes. Finally, the invariant Vgamma4-2/Vdelta1-4 gammadelta T cells are generated in both the thoracic and cervical thymus of the naked mole-rat until adult life. Our results indicate that invariant Vgamma4-2/Vdelta1-4 natural killer-like effector T cells in the naked mole-rat are continuously produced and can contribute to cancer immunosurveillance by gammadelta TCR-mediated recognition of a common molecular signal.

Key words: *gammadelta; naked mole-rat; invariant; short homology repeat; microhomology; butyrophilin; NK; iNKT; MAIT*

Linda Warmuth
Institute for Medical Microbiology, Immunology and Hygiene, Technical University Munich, Germany
linda.warmuth@tum.de

Optimization of chimeric antigen receptor (CAR)-T cells for adoptive T cell therapy

Linda Warmuth, Sarah Dötsch, Manuel Trebo, Wenzel Manlik, Anna Hochholzer, Monika Hammel, Jacqueline Seigner, Michael Traxlmayr, Sebastian Jarosch, Sabrina Wagner, Stanley Riddell, Elvira D'Ippolito and Dirk H. Busch

Adoptive transfer T cell therapy with chimeric antigen receptor (CAR)-T cells targeting the CD19 surface marker has shown impressive clinical success in the treatment of B cell malignancies. Despite this success, patients still suffer from potentially life-threatening side effects in a form of cytokine release syndrome (CRS) and neurotoxicity. Furthermore, strong and persistent stimulation of CAR-T cells resulting from high receptor binding affinity towards the CD19 antigen impairs long-term functionality of transferred cells, which is one of the main causes for disease relapses. Thus, fine-tuning of the receptor binding affinity shows potential to maintain clinical efficacy, while circumventing severity of side effects as well as CAR-T cell exhaustion. Therefore, we compared the efficacy of two anti-CD19 CAR constructs with a 40-fold difference in antigen binding affinity. Both, the high and low affinity binder demonstrated comparable sensitivity and anti-tumor functionality in in vitro assays. In contrast, low affinity CAR-T cells failed to control tumor growth in a mouse xenograft model. Moreover, we observed a strong decrease in common treatment-related side effects in a humanized CRS mouse model treated with low affinity CAR-T cells, revealing a clear correlation between CRS and antigen binding kinetics. Based on these findings we followed a combinatorial treatment approach by administration of mixed doses of high and low affinity CAR constructs. Intriguingly, we found that this combination mediated a faster tumor clearance, and induced less expansion and exhaustion of the high-affinity CAR-T cells. In summary our data show a dependency of CAR-T cell functionality from the receptor affinity. The combination of high and low affinity CARs can improve the anti-tumor efficacy, with the potential to overcome the limitations of current CAR-T cell products.

Louise Webb
The Babraham Institute, Babraham, Cambridge, UK
louise.webb@babraham.ac.uk

Rejuvenation of the Germinal Center Reaction and Affinity Maturation Following Depletion of T Follicular Helper Cells

Louise M.C. Webb, Emily Watson, Silvia Innocentin, Sigrid Fra-Bido, Stephane Guillaume, Sam Woolliscroft, and Michelle Linterman

The Germinal Center (GC) reaction is mounted in response to infection or immunisation. It culminates in the generation of long-lived humoral immunity with B cells that have evolved their B cell receptors to fine tune antigen binding. During the GC reaction B cells undergo somatic hypermutation and migrate between distinct zones of the GC. The signalling and antigen presenting capacity of their newly mutated B cell receptors is thought to determine how much help they receive, resulting in a higher survival and proliferative capacity of those with high affinity B cell receptors. T Follicular Helper (Tfh) cells are a subset of CD4+ T cells found in the B cell follicle. They provide critical help to B cells during the GC reaction. There is a widely held view that competition by B cells for Tfh cell help drives the selection of B cells and the evolution of the GC reaction. The lack of tools that enable genetic manipulation of established Tfh cells has hampered our understanding of their biology and progress within the field.

We have generated an in vivo model where we can track and/or delete Tfh cells at the peak of the GC reaction. Importantly, this model leaves T Follicular Regulatory cells (Tfr) untouched. This is important as Tfr cells provides opposing signals to Tfh cells and can shut down the GC. We have used this model to ask what happens when Tfh cells are deleted in an established GC. We show that deletion of Tfh cells results in the demise of the GC. This confirms the widely held assumption that Tfh cells provide essential signals to GC B cells. However, we find that this is a temporary demise. Our data shows that Tfh cells have the capacity to re-new and re-populate the GC following their deletion. This rejuvenates the GC and the return of affinity maturation.

This work unambiguously shows the importance of Tfh cells in ongoing GC reactions. Interestingly, it shows that Tfh cells have the capacity to self-renew, enabling recovery of the GC response. Our data also suggests that competition for Tfh cell-derived signals may not be the main driver of affinity maturation.

Riekje Winzer
Institute of Immunology, University Medical Center Hamburg-Eppendorf (UKE)
r.winzer@uke.de

Role of CD73 in the specification of human memory CD8 T cells

Riekje Winzer, Niklas Bach, Hauke Wasielewski, Enja Schneider, Anne Rissiek, Nathalie Richter, Laura Glau, Filippo Cortesi, Christa E. Müller, Friedrich Koch-Nolte, Nicola Gagliani, Eva Tolosa

The stepwise hydrolysis of extracellular ATP by the ectonucleotidases CD39 and CD73 generates adenosine. During immune cell activation, adenosine is an important immune regulator, controlling T cell effector responses and preventing immune pathology. In the human T cell compartment, CD73 is mainly expressed on naïve CD8 T lymphocytes, and these cells release enzymatically active CD73 in extracellular vesicles (EVs) upon activation. CD73-expressing EVs are able to generate adenosine, which leads to immunosuppression of target cells. At the same time, their release prevents self-inflicted suppression of the recently activated donor cell. Thus, T cell activation goes along with the loss of CD73 from the plasma membrane, and effector T cells do not express CD73 on the cell surface. However, a small subset of memory CD8 T cells will re-express CD73, and these cells are characterized by a central memory, non-effector phenotype with elevated expression of IL7R, TCF7, and tissue homing markers. So far, the function of CD73 on these cells remains unclear. We found that human CD73-expressing CD8 memory T cells have an enhanced survival compared to CD73-negative cells both with and without IL-7 treatment. This, however, does not seem to be dependent on their CD73-mediated AMPase activity. To unravel the function of CD73 on CD8 memory cells, we either eliminated CD73 by CRISPR/Cas9, or induced the constitutive presence of CD73 on the plasma membrane using a nanobody-based anchor. Our work aims to elucidate the role of CD73 in CD8 memory T cells, shedding light on the mechanisms underlying their enhanced survival and a potential effect of CD73 on differentiation in tissue-resident or circulating memory cells.

Johannes Woelk
Medical University of Innsbruck
Johanneswoelk@i-med.ac.at

Loss of the nuclear receptor NR2F6 enhances NK cell-mediated anti-tumor responses

Johannes Woelk, Florian Hornsteiner, Veronika Sexl, Patrizia Stoitzner, Gottfried Baier and Natascha Hermann-Kleiter

Natural killer (NK) cells are essential for killing infected and transformed cells and are critical for preventing metastasis formation [N.K. Wolf, Nat Rev Immunol., 2023]. The orphan nuclear receptor subfamily 2, group F, member 6 (NR2F6) belongs to the nuclear receptor (NR) family, that regulates both pro-and anti-inflammatory processes [CK. Glass, Nat Rev Immunol, 2010]. We have previously investigated the functional role of NR2F6 in T lymphocytes and established it as an intracellular immune checkpoint during cancer immune surveillance [N. Hermann-Kleiter, Cell Rep., 2015]. However, depletion of NK cells in a subcutaneous EL-4 tumor model completely abolished the benefits observed in Nr2f6-deficient mice, suggesting a significant role of NR2F6 in NK cell mediated anti tumor response. Lung metastasis formation in the MHC-I-deficient B16 F10 tumor model, primarily dependent on NK cell killing, is significantly reduced in Nr2f6 deficient mice. RNA-Seq analysis of healthy, splenic Nr2f6-deficient NK cells revealed strongly enhanced Ncr1 (NKp46) and Ccr5 levels. Consistent with this observation, loss of NR2F6 significantly increases NKp46 expression at both the transcriptional and surface receptor levels in splenic, blood and tumor-derived NK cells. Recently, NKp46 recognition of ecto-calreticulin was shown to control murine B16 melanoma and RAS-driven lung cancer by enhancing tumor-infiltrating NK cell degranulation of cytotoxic granules and cytokine secretion [S. Sen Santara, Nature, 2023]. Nr2f6 deficient NK cells exhibit increased TNFα and IFNγ when ex vivo co-cultured with B16-F10 cells compared to wild type controls. Mechanistically, NR2F6 harbors two putative binding sites at the basal promoter of Ncr1. Preliminary data from chromatin immunoprecipitation suggest direct binding of NR2F6 to one site, which could facilitate the repression of NKp46 expression. In conclusion, our findings provide insight into NR2F6-regulated anti-tumor NK cell responses. Therapeutic targeting of NR2F6 may be a promising strategy for boosting NK cell-mediated tumor surveillance and metastasis.

Elaine Wong
Ludwig-Maximilians-University München, Institute for Immunology
elaine.wong@med.uni-muenchen.de

Roquin-1 and Regnase-1 binding to the T cell transcriptome defines evolutionary conservation, modes of regulation and uncovers novel roles in lymphocyte differentiation

Elaine H Wong, Giulia Cantini, Taku Ito-Kureha, Lambert Moyon, Timsse Raj, Annalisa Marsico, Vigo Heissmeyer

The RNA-binding proteins (RBPs) Roquin-1 and Regnase-1 are instrumental for post-transcriptional gene regulation in T cells. The cooperative regulation of proinflammatory target mRNA is essential for maintaining immune homeostasis. Mutations that impair the interaction between Roquin-1 and Regnase-1 induce spontaneous T cell activation, T follicular helper cell differentiation and autoantibody formation, ultimately leading to autoimmunity in mice. At the same time, the disruption of their interaction in T cells has been shown to increase anti-tumor responses by promoting T cell accumulation within the tumor and reducing the expression of exhaustion markers. Despite our deep understanding of their biological roles and the growing therapeutic interest, the mechanism of target mRNA recognition and their interdependency remains largely elusive. In this study, we employ an improved crosslinking and immunoprecipitation technology (CLIP) for endogenous Roquin-1 and Regnase-1 in T cells, directly comparing human and mouse species. Creating near-nucleotide resolution maps for the binding of Roquin-1 and Regnase-1 to the transcriptome of T cells, using knockout primary cells, provides insights into the mechanisms of how individual cis-elements in mRNAs employ distinct post-transcriptional machinery and trans-acting factors to regulate gene expression. We can identify specific determinant elements of regulation conserved throughout multiple vertebrates. Interestingly, the presence or absence of Roquin has a profound impact on the binding of Regnase-1 to target mRNA, revealing a dependency of Regnase-1 on Roquin for specific target recognition and in turn, regulation. Delving into evolutionarily conserved binding sites in paralogs and orthologs, we unveiled unrecognized roles within this system in lymphocyte differentiation and stability. Collectively, our findings reveal a cooperative immune regulation mediated by Roquin-1 and Regnase-1, controlling precise immune responses.

Dario Žebčević
Institute of Immunology, Biomedical Center Munich, Germany
Dario.Zebcevic@med.uni-muenchen.de

The role of B cells in central T cell tolerance

Dario Žebčević, João Freitas, Christine Federle, Nadia Khouja, Amina Sayed, Ludger Klein, Iliana Siamishi

Autoreactive CD4 T cells in the thymus are deleted or diverted to become regulatory T cells (Tregs) during tolerance induction. While dendritic cells and medullary thymic epithelial cells are regarded as important antigen presentation cells (APCs) for central tolerance, the significance of thymic B cells in this process is unknown. The thymus has a distinct population of B cells that exhibit powerful APC characteristics such as high levels of CD80 and MHC class II, as well expression of AIRE. To explore the effect of thymic B cells on central T cell tolerance, we compared the TCR repertoires of mature CD4+ thymocytes in WT and B-cell deficient mice. We identified a subset of TCRs whose deletion or diversion is dependent on the presence of thymic B cells. To corroborate the results of our TCR sequencing data, prospective TCRs were rexpressed in the thymi of either B cell-sufficient or B cell-deficient recipient mice via intrathymic injections. This suggests that thymic B cells play an important role in shaping the T-cell repertoire. Curiously, thymic B cells also express a stereotypic pattern of class-switched surface immunoglobulins, raising the possibility that class switching may contribute to their tolerogenic function. However, whether and to which extent class switching in thymic B cells contributes remains to be resolved. To address this, we will compare TCR repertoires of mature CD4+ thymocytes in WT and class-switching deficient (AID-/-) mice.

IN MEMORIAM
OF
HARALD VON BOEHMER

Harald von Boehmer
1942 – 2018

”The curiosity in T cell development is still very much alive even after retirement but I trust that the remaining issues are in good hands of younger scientific colleagues who identify the outstanding questions and think of clever experiments to address them.”

Curriculum Vitae

Harald von Boehmer was born on November 30, 1942 as the youngest of three. His father, Hasso von Boehmer, a lieutenant colonel in the German army, was one of the 20. July Plotters – a group of oppositionists that planned to rid Germany of Hitler. The failed attempt led to Hasso being sentenced to death and executed when Harald was just two years old.

After spending his youth in his grandfather's house and graduating from high school in Freiburg, Harald went on to study medicine in Göttingen, Freiburg and Munich, obtaining his medical doctorate from Ludwig Maximilian University in Munich in 1968. He subsequently received his Ph.D. from Melbourne University, Australia (1974). He joined the Basel Institute for Immunology in 1973 and remained an active member until 1996, when he became director of the Unité INSERM 373 at the René Descartes University in Paris, France. After 3 years in Paris, Harald was recruited to Harvard Medical School and the Faculty of Arts and Sciences of Harvard University, Cambridge where he established the Laboratory for Lymphocyte Biology at the Dana Farber Cancer Institute in Boston which he ran until his retirement in 2013.

In 2024 it is difficult to remember how confused immunologists were in the early 70's about T cells and the conditions that controlled their development and subsequent activation. In the 70's, Harald, together with Jonathan Sprent, showed that stable bone marrow chimerism is the result of donor cell deletion with reactivity against recipient histocompatibility antigens (clonal deletion theory) and that in order to be stimulated by an antigen plus MHC combination, the T cells had to have been exposed, during their development in the thymus, to the MHC allele under study.

Shortly after the thymus result, the T cell receptor was found, and experiments transferring TCR alfa and beta genes from one T cell clone to another allowed Harald, together with Michael Steinmetz, to unequivocally conclude that the MHC-restricted specificity was encoded by a single receptor (Dembic Z. et al, Nature,1986), a finding later-on confirmed by crystallographic studies.

The next question he tackled concerned the issue of immunological tolerance. How is that T cells can respond to many different antigenic peptides, bound by MHC proteins, except peptides that are made by their own host. That is, the immune system, when it is functioning properly, does not attack self. Harald and his colleagues showed that self-reactive cells were destroyed as they developed in the thymus. This was done with a very clever experiment in which mice were produced that expressed T cells with a single T cell receptor, in this case the antigen target of the T cells was a HY-peptide expressed only in male mice. The male specific T cells appeared as expected in female mice, but disappeared during their development in the thymus of male animals. These mice came with an anomaly, the too early expression of the transgenic TCR, which made proper quantitation difficult, but more recently Harald and Taras Kreslavskyi could address this problem and reported deletion of CD4+8+ thymocytes in the absence of TCR editing. This ended a long quest for understanding the deletion of autoaggressive cells at a certain stage of development (NEGATIVE SELECTION).

Curriculum Vitae

The next step was related to POSITIVE SELECTION and the matching of specificity and function. Here, the first realization was that a receptor derived from a CD8+ cell would only be expressed on CD8+ cells in the transgenic mice. The second was that there was in fact POSITIVE SELECTION as mice with inappropriate MHC antigens not restricting the specificity of the cell from which receptor genes were obtained, failed to generate single positive cells and thus development was arrested at the CD4+8+ stage where cells died. This was then named DEATH FROM NEGLECT as opposed to death by NEGATIVE SELECTION which eliminated likewise CD4+8+ cells, at least when the receptor was derived from CD8+ cells. It was then clear that it was the MHC molecules expressed in the thymus and the TCR specificity, which determined positive selection, which also led to the matching of specificity and function, such that CD8+ killer cells were generated from immature cells expressing a class I restricted TCR and as shown later CD4+ helper cells were generated from immature cells expressing a class II restricted TCR and thus in other words helper cells recognized as a rule peptides entering the target cell from the outside whereas killer cells recognized peptides produced in the target cell itself. This relates to the different modes of peptide loading by class I and class II MHC antigens.

In the meantime, the molecular details of this matching process have been worked out mostly by the work of Dietmar Kappes as well as Dan Littman who identified transcription factors guiding this process in dependence of the signaling by the receptor expressed by immature cells. Thus, at present we have a fairly complete picture of positive selection as far as the selectable T cells are concerned while still we know relatively little about the TCR ligands that are responsible for positive selection. Here, one wonders whether thymus-specific proteasome subunits play an essential role. Thus, there are still some secrets in T cell development even after decades of the identification of the TCR.

Later, Harald discovered the preT cell receptor alpha chain (for which he received the Louis Jeantet prize together with Nicole Le Douarin and Gottfried Schatz in Geneva (1990) and an honorary Medical Degree from the University of Technology, Munich (2002)) and studied the role of the receptor in controlling survival and differentiation of developing T cells that have succeeded in productive TCR beta rearrangement. He continued to focus on the generation and function of regulatory T cells that have an essential role in preventing autoimmunity with the goal to exploit these cells in the prevention of and interference with unwanted immune reactions.

Harald summarized the essence of his research work as follows: '**the thymus selects the useful (positive selection), destroys the harmful (negative selection), and ignores the useless (thymocytes fitting neither positive not negative selection die by neglect)**."

Harald von Boehmer retired at the end of 2012 and succumbed to a neurodegenerative disorder in June 2018.

Eulogy

Klaus Rajewsky

Harald von Boehmer was one of my oldest and closest friends and companions who has challenged and provoked me for decades and from whom I continuously learned and profited; and on whom I could always rely. He was one of those rare people of which I knew from the first encounter that here was a connection that would last – even though we had a terrible fight at the time. So we always kept in touch: during his time at the Basel Institute of Immunology; then in Paris at the Hôpital Necker and the beautiful house in Fontainebleau; the ten years in Boston, where we were allowed to live in his home for half a year upon our arrival and subsequently had a friendly neighborly relationship with him and his family, with many joint trips to Crane Beach followed by lobster meals; and finally the time in Seefeld.

Harald was an outstanding scientist who shaped T cell immunology with visionary experiments which today are textbook knowledge, an incorruptible critical mind, and superior intelligence. I know that I am just one of many who sought and received his advice and opinion over decades, in two or three razor-sharp sentences, often highly controversial, sometimes sarcastic or with a crushing verdict, but always helpful and to the point, uncompromising in the search for truth, yet with an open, generous heart. Speaking to the non-scientists in the audience, let me stress that I am saying this in the name of many, many colleagues and scientific friends, a truly global community of eminent researchers and of course his many collaborators and students who all had a special, loving and respectful relationship with Harald and for whom he has been a central, uncompromising scientific, intellectual and human authority.

I am reading from an email I have just received from Fred Alt: "Please give my condolences and best wishes to Harald's family. I have wonderful memories of time spent with Harald (and his family) over the many decades and feel fortunate he moved to Boston so that I got to know him even better. He may be one of the most honest individuals I have ever met. If one got a compliment from him (as you and I both did every now and then) it was worth its weight in gold. I miss all of those wonderful discussions about almost everything the three of us used to have over a bottle of wine or grappa (or both) that usually went well into the night (or morning in some cases)."

Harald's loss is a turning point. For me, it comes at an age when saying farewell becomes harder and at the same time more natural. He was one of my last old, dearest friends. Together with my wife Christine, I would like to express our affection and sympathy to Monica, Lisa, Lotta and Philip and the whole family and convey our admiration for what they did for Harald in these difficult years.

July 2018, Klaus Rajewsky

Translated and slightly modified from German

In Memoriam of Harald von Boehmer (1942–2018)

Hermann Wagner
Technical University Munich, Emeritus of Excellence
European Journal of Immunology, 48: 1269-1270
Published: 6 August 2018

On June 24, 2018, at the age of 75, the highly creative and accomplished immunologist Harald von Boehmer passed away, the consequence of a progressive degenerative disease. He is survived by his wife Monica, and three children, Philip, Lisa and Lotta.

Harald was a larger than life person, both physically and mentally. In addition to his scientific brilliance, he was an accomplished skier and an excellent cellist who adored Pablo Casals and the music of Schumann; if time permitted, Harald and his charming wife Monica played together, he the cello and Monica the magnificent "Bösendorfer Piano Grande". Both of them loved to entertain his many colleagues, always providing outstanding meals, with excellent wine never in short supply. Harald was a skilled conversationalist who enjoyed debating scientific, as well as, countless other topics. He was particularly adept at detecting shallow and superficial ideas, and he could be harsh when dismissing rival ideas. To battle with him was always fun and educational. Possessing a talent for being almost invariably right, few if any friend or foe escaped his criticism. However, Harald was not one to harbor resentment to anyone.

In addition to other institutions, the Deutsche Forschungsgemeinschaft (DFG) relied upon his extensive knowledge. The DFG would often ask him to co-evaluate German research programs. Once Harald was convinced of a program, he would support it even against the will of his co-evaluators. In other words, Harald's decisions were spawned from his quest for truth, his independent spirit, and his commitment to science-based values.

Harald von Boehmer obtained his M.D. from the Ludwig Maximilian University in Munich (1968), and a Ph.D. from Melbourne University, Australia (1974). In Melbourne, he worked as a post-doc with Ken Shortman at the Walter and Eliza Hall Institute (WEHI). From 1973 till 1996, he became a member of the Basel Institute of Immunology, a world- wide renowned "breeding ground" and "talent incubator" for top scientists in immunology. He then moved to Paris to head the Unité INSERM 373 at the René Descartes University (1997-2000). In 1999, he accepted a Professorship for Pathology at Harvard Medical School in Boston (USA), and he became Head of the Laboratory for Lymphocyte Biology. At the end of 2012, Harald's mysterious chronic disease caused him to retire and move to Seefeld in Tirol, Austria. Thereafter, he was a guest-professor at the Institute for Immunology at the LMU in Munich.

Harald von Boehmer was a pioneer in understanding how T cells develop and function in the immune system. Following the old saying "if you want to grow palms, you have to go to places where palms can grow", he moved from Melbourne to the Basel Institute for Immunology. To-

gether with Jonathan Sprent (also from WEHI), he analyzed tolerance to major histocompatibility complex (MHC) antigens in tetraparental bone marrow chimeric mice. Along with Michael Steinmetz, Harald achieved a major break-through in realizing that upon transfer of alpha and beta T cell receptor (TCR) genes (cloned from their H-Y specific T cell clones) a single receptor indeed executed MHC-restricted H-Y specific antigen recognition. This "breakthrough" discovery, however, was subsequently surpassed by the demonstration that clonal deletion of immature CD4+ CD8+ thymocytes is the major mechanism of central tolerance (termed negative selection), while the generation of mature, antigen-reactive T cells requires an interaction of the alpha and beta TCR with MHC antigen (termed positive selection). Today, these experiments are considered "classics" of modern immunology. Working in Paris, Harald von Boehmer's group subsequently reported on the unique role of the pre-TCR in controlling the development of alpha/beta T cells.

Naturally occurring regulatory (suppressor) T cells (Tregs) have an essential role in preventing autoimmunity, such as type 1 diabetes, and it was known that they develop in the thymus. While working at the Harvard Medical School in Boston, Harald's group realized that Tregs are also induced in the course of a peripheral immune response towards an antigen but only if homeopathic antigen doses trigger antigen reactive T cells under non-inflammatory conditions. These results led to the vision that type 1 diabetes can be prevented by Tregs generated via immunization — a new translational aspect in a career that had, until then, focused on basic immunology.

Harald von Boehmer received numerous awards including the Louis Jeantet Prize for Medicine, the Avery Landsteiner Prize (of the German Society for Immunology), the Paul Ehrlich and Ludwig Darmstädter Prize, an honorary Med- ical degree from the Technical University Munich (TUM), and, together with Klaus Rajewsky, the Kurt A. Körber Prize for European Science. He also received the Helmholtz International Fellow Award.

Even though Harald von Boehmer's "classics" (classical experiments) did not enter the Nobel path, his impact on us was immense, both as a scientist and as a person. Harald von Boehmer is no longer with us, however, thanks to his brilliant and titanic work as a scientist, his accomplishments will remain a part of the immunological paradigm — we already miss him a lot.

Harald von Boehmer 1942–2018

Iannis Aifantis & Christine Borowski
Nature Immunology, volume 19 (2018)
Published: 13 August 2018

Today, few topics in immunology receive more attention than efforts to detect, induce or reinvigorate the immune response to tumors. Although many types of immune cells affect and contribute to anti-tumor immune responses, the initial clinical findings that electrified the field focused on the T cell. Cancer biologists who grabbed the nearest immunology textbook in an effort to fully comprehend and build upon those initial clinical successes will have quickly realized that at their core, such approaches rely on understanding how T cells recognize and respond to antigen. What they may not have realized is how important Harald von Boehmer's work was in laying the foundation for this understanding.

Long before translational research was all the rage, Harald charged a segment of his lab with investigating how fundamental immunological principles might influence the onset of autoimmunity and tumor-specific immune responses. Early on he saw that breaking tolerance could result in an attack on healthy tissue or a tumor, and that suppressing the former and inducing the latter would require the study of two sides of a single coin.

Over the course of his scientific career Harald worked in or ran labs in four countries on three continents. After earning his M.D. from the Ludwig Maximilian University in Munich, Harald moved to Melbourne, Australia, where he obtained his Ph.D. under the supervision of Ken Shortman. During these early years he characterized the functions of the various cell types in the mixed-lymphocyte reaction, an assay essential for the understanding of donor–recipient compatibility in transplantation.

Shortly after receiving his Ph.D., Harald was recruited by Niels K. Jerne to the (now-defunct) Basel Institute of Immunology in Switzerland. There he worked closely with superb visiting and resident immunologists. Aided by the recent identification of genes encoding T cell antigen receptors (TCRs) and breakthroughs in transgenic technology, Harald generated mouse models that laid the foundation for understanding of the positive and negative selection of T cells, as well as T cell lineage commitment in the thymus. Through the use of these mice, he delineated the effect of major histocompatibility complex molecules and TCR cognate antigen on various stages of thymocyte development. For example, Harald demonstrated that the interaction between the TCR and peptide–major histocompatibility complex determined whether a thymocyte would differentiate along the CD4+CD8– T cell lineage or the CD4–CD8+ T cell lineage. A few years later, Harald's lab identified a previously unknown TCR, which he called the 'pre-TCR'.

In 1996, Harald left the Basel Institute of Immunology to join the Institut National de la Santé et de la Recherche Médicale and Institut Necker (Rene Descartes University) in Paris. At the

Institut Necker, Harald found phenomenal immunologists, as well as direct exposure to a hospital with a tradition of the study of immunological conditions, including immunodeficiency and autoimmunity. This environment prompted him to expand the focus of his work to include more translational questions, such as those related to diabetes, T cell anergy and regulatory T cell function. However, during the same period, Harald continued to add substantial basic insights to the understanding of early T cell development; these included the identification of roles for the pre-TCR in thymocyte survival, allelic exclusion and commitment to the αβ or γδ T cell lineage.

Always fascinated by the USA, Harald had many good colleagues and friends there, and on several occasions he considered moving to a US university. In the final days of the 20th century he did, and he remained at Harvard Medical School until his retirement in 2013. Influenced by his new environment in the Smith Building of the Dana Farber Cancer Institute, Harald focused his work even more heavily on human disease. His lab made substantial contributions to the understanding of T cell leukemia, in particular the role of the Notch family of signaling receptors in this malignancy. With colleagues in the lab, Harald also published important insights into the mechanisms through which different T cell populations respond to tumors and destroy pancreatic β-cells. At the same time, he never stopped pursuing knowledge of the basic mechanisms that affect T cell development. While in Boston, his lab described mechanisms that affect the development of regulatory T cells in the thymus and the periphery and continued to publish insights into the structure and function of the pre-TCR.

After closing his Boston lab, Harald returned to his alma mater as a guest professor at the Institute for Immunology of the Ludwig Maximilian University in Munich. In his writings during this period, Harald expressed optimism about the future of immunological research. In one of his final Reviews, he mused that "The curiosity in T cell development is still very much alive even after retirement but I trust that the remaining issues are in good hands of younger scientific colleagues who identify the outstanding questions and think of clever experiments to address them" (von Boehmer, H. Front. Immunol. 5, 424 (2014)).

The immunology community would have enough to thank Harald for if the only thing he left was the enormous body of immunological knowledge he revealed. But he left more than that—he left a global network of trainees, colleagues and friends who benefited from his relentless insistence on rigor, thoroughness, preparedness and creative thinking. As two of Harald's doctoral trainees, we can attest that thanks to his directness, it might not have always felt like we were benefiting while he conveyed his opinion of our work during Monday morning lab meetings. But we can also say with conviction that at the end of the day, it was always obvious that Harald's comments were made with our best interests in mind.

Harald passed away on 24 June 2018 at age of 75. His piercing intelligence, candor and unwavering support will be sorely missed.

SELECTED PUBLICATIONS OF HARALD VON BOEHMER

Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes

Pawel Kisielow*†, Horst Blüthmann‡, Uwe D. Staerz*, Michael Steinmetz‡ & Harald von Boehmer*

* Basel Institute for Immunology, CH-4005 Basel, Switzerland
‡ Central Research Units, F. Hoffmann-La Roche & Co. Ltd, CH-4002 Basel, Switzerland

The mechanism of self-tolerance is studied in T-cell-receptor transgenic mice expressing a receptor in many of their T cells for the male (H-Y) antigen in the context of class I H-2D^b MHC antigens. Autospecific T cells are deleted in male mice. The deletion affects only transgene-expressing cells with a relatively high surface-density of CD8 molecules, including nonmature CD4$^+$CD8$^+$ thymocytes, and is not caused by anti-idiotype cells.

T LYMPHOCYTES recognize antigens on the surface of other cells in the context of molecules encoded by the major histocompatibility complex (MHC)[1] by virtue of the heterodimeric T cell receptor (TRC) which is composed of α and β polypeptide chains[2,3]. In binding to its ligand, the $\alpha\beta$TCR is assisted by CD8 or CD4 accessory molecules[4,5], which presumably interact with nonpolymorphic portions of class I or class II MHC molecules respectively[6–10]. Mature T lymphocytes usually do not respond to self-MHC molecules presenting self-antigens. The question of whether the mechanism of immunological tolerance involved deletion of autospecific lymphocytes has concerned immunologists over decades[11], but no direct evidence for such a mechanism has been obtained, because the great diversity of receptors generated during lymphocyte development had made it impossible to follow individual clones of cells expressing receptors specific for self-antigens.

Recently, two groups of investigators obtained monoclonal antibodies (mAb) against the products of certain Vβ genes that are expressed with unusually high frequency on T cells specific for certain class II MHC-associated alloantigens[12–14]. Using these antibodies, Kappler *et al.* and MacDonald *et al.* were able to show that in mice expressing the relevant class II MHC-associated antigens, cells expressing the particular Vβ gene products were absent from the pool of peripheral T cells and medullary thymocytes[12–14], but were present among cortical $CD4^+8^+$ thymocytes[12,13]. These results can be explained by deletion of autospecific cells, but the alternative possibility that their absence is the result of a change of their phenotype caused by modulation or masking of surface molecules has not been excluded.

The development of transgenic mice offers another approach to analyse the mechanism of self-tolerance. To this end we have constructed transgenic mice expressing in a large fraction of their T cells an $\alpha\beta$TCR specific for a minor histocombatibility antigen (H-Y) present on male, but not female, cells. Fertilized eggs obtained from a cross of C57BL/6J × DBA/2J mice were injected with genomic DNA harbouring the productively rearranged TCR α and β genes isolated from the B6.2.16 cytolytic T-cell clone[15]. This clone is specific for H-Y antigen in the context of class I (H-2D^b) MHC antigen and expresses a TCR β-chain coded in part by the Vβ8.2 gene segment which can be identified by the F23.1 antibody[16].

The transgenic founder mouse 71 contained four copies of the α and two copies of the β transgenes integrated on the same chromosome[17]. It was crossed with C57L mice expressing H-2^b MHC antigens, but lacking the Vβ8 gene family. Here we show that cells with the phenotype of the B6.2.16 clone that responded to H-Y antigen were frequent in female but not in male transgenic offspring, despite the fact that peripheral T cells in animals

† Permanent address: The Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland.

Table 1 Frequency of male (H-Y) antigen-specific precursors of proliferating T cells (PT-P) among $CD4^+8^-$ and $CD4^-8^+$ T cells from normal and $\alpha\beta$TCR transgenic mice

Stimulation: spleen cells (3000R) + IL-2	CD4/CD8 phenotype	C57L female 1/frequency		p*	Donor of responding T cells: $\alpha\beta$TCR transgenic female 1/frequency		p	$\alpha\beta$TCR transgenic male 1/frequency		p
C57BL/6 female	$CD4^-8^+$	>25,000			>25,000			>25,000		
C57BL/6 female	$CD4^-8^+$	2.3	(1.6–3.3)	0.66	1.8	(1.2–2.6)	0.87	2.3	(1.3–4.0)	0.18
+ Con A	$CD4^+8^-$	NT†			6.4	(4.5–9.1)	0.60	NT†		
C57BL/6 male	$CD4^-8^+$	15,985	(5,029–50,802)	0.61	6.6	(4.8–9.0)	0.67	>25,000		
	$CD4^+8^-$	NT†			>25,000			NT†		

Lymph node cells were stained with a mixture of anti CD4-PE and anti-CD8-FITC mabs (see Fig. 1). $CD4^+8^-$ and $CD4^-CD8^+$ T cells were separated on fluorescein activated cell sorter (FACS440, Becton Dickinson). Limiting numbers of $CD4^+8^-$ $CD4^-8^+$ T cells (24 wells per group) were cultured for 8 days together with irradiated (3,000R) spleen cells (5×10^5 cells per cell) and interleukin-w (5% v/v) of partially purified supernatant from Con A-stimulated rat spleen cells[24] without or with Con A (2.5 μg ml^{-1}). Cells were collected after addition of [^{3}H]thymidine for the last 12 h of culture and incorporated radioactivity was measured by liquid scintillation counting. Negative control cultures contained no responder cells. Frequencies were calculated as described elsewhere[25].

* Probability, p, attached to the computed χ^2 (ref. 25). † NT, not tested.

of both sexes expressed both transgenes[17]. T cells in male (but not female) mice had an abnormal CD4/CD8 phenotype: over 90% of T cells in male transgenic mice were $CD4^-8^-$, or expressed only low levels of CD8 molecules, and the numbers of $CD4^+8^-$T cells were very small. The cellular composition of male thymuses revealed that this unusual phenotype of peripheral T cells was the consequence of deletion of auto-specific thymocytes expressing high levels of CD8 molecules, predominantly cortical $CD4^+8^+$ thymocytes. The deletion process spared cells expressing low levels of CD8 molecules, but affected the precursors of single positive $CD4^+8^-$ cells that were not male-specific. This latter observation provides strong evidence that double-positive $CD4^+8^+$ thymocytes contain precursors of single positive $CD4^+8^-$ and $CD4^-8^+$ T cells.

T cells in females

Lymph nodes of female transgenic mice contained normal proportions of $CD4^+8^-$ and $CD4^-8^+$ T ($Thy1^+$) cells which had normal levels of CD4 as well as CD8 accessory molecules (Fig. 1*a*, *b*, *d* and *e*). But these differed in two respects from T cells in normal mice. Firstly, as previously described for β transgenic mice[15], most of them expressed the transgenic β chain on their surface (Fig. 1*b*). Secondly, as shown by limiting dilution analysis of $CD4^-8^+$ T cells, one in six proliferated specifically in response to C57BL/6 male stimulator cells, as compared with one in 16,000 in normal C57L female mice (see Table 1). As only every second plated T cell responded to concanavalin A (Con A), we conclude that at least 30% of $CD4^-8^+$ T cells in transgenic females have a phenotype similar to that of the B6.2.16 clone. $CD4^+8^-$ T cells from transgenic mice did not show any male-specific proliferation, but did respond to Con A (Table 1).

T cells in males

As in transgenic females, lymph nodes of transgenic males contained normal proportions of $Thy1^+$ cells, and most of them expressed the transgenic β chain on their surface (Fig. 1*c*). Northern blot analysis of the α transgene revealed comparable levels of expression in T cells from female and male mice[17]. However, the CD4/CD8 phenotype of T cells in male mice was very different from that of females: 58% of $Thy1^+$ cells were $CD4^-8^-$, 35% were $CD4^-8^+$ but expressed low levels of CD8, and 7% were $CD4^+8^-$ and expressed normal amounts of CD4 (Fig. 1*f*). Limiting dilution analysis showed that one in two $CD4^-8^+$ T cells could be induced to grow by Con A. There was, however, no detectable response to male C57BL/6 stimulator cells (Table 1). Likewise, $CD4^-8^-$ and $CD4^+8^-$ T cells were unresponsive to H-Y antigen (data not shown). These results indicate that male-specific T cells with the phenotype of the B6.2.16 clone are absent from male transgenic mice and that the lack of or low level of CD8 on transgene-expressing cells precluded male-specific responses. This conclusion is supported by the observation that cytolytic activity of the B6.2.16 clone can easily be inhibited by anti-CD8 antibodies (not shown), and by CD8 gene transfection experiments which show that CD8 molecules strongly assist antigen recognition by T cells[4,5]. As shown elsewhere[17], a high proportion of $CD4^-8^-$T cells in male mice expressed both transgenes, but had their endogenous α and β genes in germline configuration. This result, and the fact that only a few T cells expressed normal amounts of CD8 in male transgenic mice, is consistent with the notion that most T cells in male mice and $CD8^+$ T cells in female mice carry transgenic $\alpha\beta$TCR on their surface. But, owing to reduced density of CD8 molecules in male mice, they are not autoreactive.

Thymocytes

The number of thymocytes was drastically lower in male (0.5–1.6×10^7 per thymus) than in female ($1.0–1.6\times10^8$ per thymus) transgenic mice.

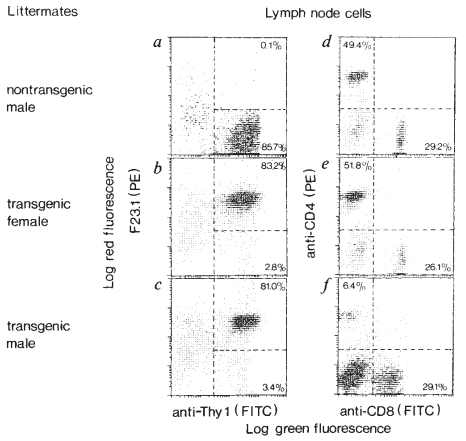


Fig. 1 Comparison of cell surface expression of $F23.1^+$ TCR β chain, CD4 and CD8 molecules on lymph node T ($Thy1^+$) cells from female and male $\alpha\beta$TCR transgenic mice and their nontransgenic male littermate as analysed by two-colour flow cytometry. Lymph node cells were stained with biotinylated F23.1 monoclonal antibody (mAb) followed by a mixture of fluorescein (FITC)-labelled anti-Thyl mab with phycoerythrin-streptavidin (PEA) (*a*, *b* and *c*) or with PE-conjugated anti-CD4 mAb followed by FITC conjugated anti-CD8 mab (*d*, *e* and *f*). In panels *a*, *b* and *c* some $Thy1^-$ cells (B cells) stain nonspecifically with F23.1 mAb due to the binding by Fc receptor. The presented data were obtained with one pair of 7-week-old $\alpha\beta$TCR transgenic female and male littermates. The same results were obtained with 3 other pairs of transgenic mice.

Methods. Single cell suspensions were prepared from lymph nodes (mesenteric, axillary, inguinal) and washed twice in RPM1-1640 and once in PBS with 5% FCS. For staining the following mAbs were used: FITC-conjugated anti-Thyl (ref. 21), biotin-conjugated F23.1 (ref. 16), PE-conjugated anti-CD4 (anti-mouse L3T4, Becton Dickinson) FITC conjugated anti-CD8 (anti-mouse Lyt2, Becton Dickinson). Biotin or FITC conjugation of mAbs was performed by standard procedures. Optimal concentrations of staining reagents were determined in preliminary experiments. All incubations and washings were done at 4 °C. Cells ($0.5–1\times10^6$) were incubated with either biotinylated F23.1 mAb (*a*, *b* and *c*) or anti-CD4-PE mAb (*d*, *e* and *f*). After 20 min, cells were washed twice and incubated again for 20 min with anti-Thyl-FITC mAb plus PEA (Becton Dickinson) (*a*, *b* and *c*) or with anti-CD8-FITC mAb (d, e and *f*). Finally, cells were washed three times in PBS 5% FCS and analysed for two-colour fluorescence on FACScan (Becton Dickinson) flow cytometer with a single Argon laser and logarithmic intensity scales using FACScan research software program (FRSP). Ten thousand viable cells were analysed in each sample. Dead cells were excluded from analysis using a combination of low-angle and sideways light scatter. The results are presented as 'density' plots, generated by analysis of processed data reduced to a 64×64 matrix with 16 levels. Percentages of stained and non-stained cells were calculated using FRSP. Markers were set against the 'density' plots of control samples which involved substitution of diluent alone for either one or both antibodies.

As shown in Fig. 2*a*, double-staining with F23.1 and CD3 antibodies demonstrated that most (>95%) thymocytes from transgenic females and males expressed the β transgene, and that the amount of TCR expression corresponded to the higher values of the normal spectrum of TCR densities observed in C57BL/6 mice.

In the thymus of transgenic females, two populations expressing different levels of TCR could be distinguished (Fig. 2*a*, middle panel). The one with relatively low TCR density included

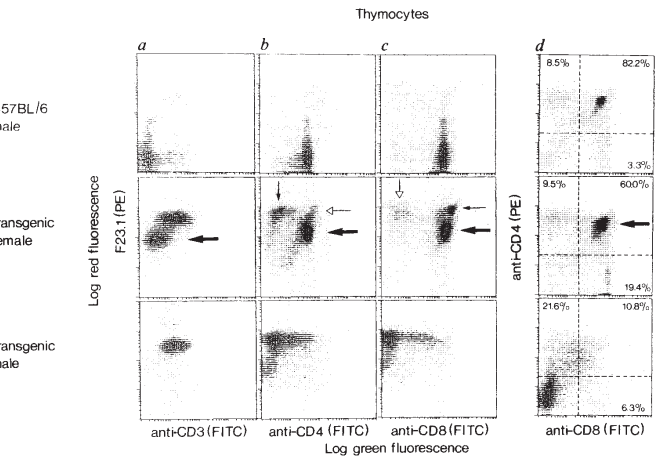


Fig. 2 Expression of F23.1$^+$ TCR β chain, CD3, CD4, and CD8 molecules on thymocyte subpopulations from normal C57BL/6 and from $\alpha\beta$TCR transgenic female and male mice. In panel *a*, cells were incubated consecutively with anti-CD3 mAb, FITC-conjugated goat anti hamster immunoglobulin, mouse immunoglobulin, biotinylated F23.1 mAb and PEA. In panels *b* and *c*, cells were stained bith biotinylated F23.1 mAb, followed by a mixture of anti-CD4-FITC (*b*) or anti-CD8-FITC (*c*) mAbs with PEA. In panel *d*, cells were stained with anti-CD4-PE followed by anti-CD8-FITC mabs. The number of cells per thymus in C57BL/6 male, $\alpha\beta$TCR transgenic female and $\alpha\beta$TCR transgenic male were: 100×10^6, 105×10^6 and 13×10^6 respectively. Thick arrows indicate the population of CD4$^+$8$^+$ thymocytes expressing a lower level of TCR, which is mostly depleted in male thymus. Open-head arrows indicate the population of CD4$^+$8$^-$, and thin arrows of CD4$^-$8$^+$ female thymocytes that express higher levels of TCR. Populations indicated by open-head thin arrows and in the upper left quadrant of middle panels *b* and *c* also contain CD4$^-$CD8$^-$ thymocytes, as indicated by virtual absence of cells in the lower left quadrant of middle panel *c*. Presence of cells in lower left quadrant of middle panel *b* is due to imperfect staining of this particular sample in this experiment. In other experiments, no F23.1$^-$CD4$^-$ cells could be seen under the same conditions.

Methods. Single thymocyte suspensions were prepared by squeezing the whole thymus through a nylon mesh into medium RPM1-1640 with 5% FCS. After washing, cells were resuspended in PBS with 5% FCS, counted and stained as indicated above with extensive washings between each step (see Fig. 1). For staining with anti-CD3, unconjugated mAb 145.2c11 (ref. 22) was used. To saturate free binding-sites of second-step reagent, cells were incubated with mouse immunoglobulin (Sigma, 1 mg ml^{-1}) for 15 min. FITC-conjugated anti-CD4 (GK1.5, ref. 23) mAb was prepared by standard procedures. Control samples were stained with each reagent alone, or in combinations omitting each single reagent. Ten thousand viable cells were analysed in each sample by FACScan flow cytometry. For details see Fig. 1.

CD4$^+$8$^+$ cells, whereas the other, expressing about tenfold more TCR, contained CD4$^+$8$^-$, CD4$^-$8$^+$ and CD4$^-$8$^-$ cells (Fig. 2*b* and *c*, middle panels).

Double-staining with CD4 and CD8 antibodies revealed significant differences between transgenic and normal C57BL/6 females with regard to the size of CD4$^-$8$^+$, CD4$^+$8$^+$ and CD4$^-$8$^-$ thymocyte subpopulations (Fig. 2*d*, upper and middle panels). The proportion of CD4$^+$8$^-$ thymocytes in transgenic females was normal, but the proportion of CD4$^-$8$^+$ thymocytes was enlarged, resulting in a reversed ratio of CD4$^+$8$^-$ to CD4$^-$8$^+$ cells as compared with normal nontransgenic mice. The proportion of CD4$^-$8$^-$ thymocytes was also noticeably higher in transgenic females than in normal mice. The increase in proportion of CD4$^-$8$^+$ and CD4$^-$8$^-$ cells was matched by a corresponding decrease in the size of the CD4$^+$8$^+$ population.

In contrast to the females, the thymus of transgenic males was severely depleted of CD4$^+$8$^+$ cells with decreased expression TCR, but contained about the same total number of CD4$^-$8$^-$ cells, which constituted the bulk of the population of male thymocytes (Fig. 2*d*, middle and lower panels). Most CD4$^-$8$^+$ cells showed low expression of CD8. Thus, the male thymus was depleted of transgene-expressing cells with relatively high levels of CD4/CD8 accessory molecules and the nonmature CD4$^+$8$^+$ thymocytes expressing decreased amounts of TCR were the main target of depletion.

Because CD4$^+$8$^+$ thymocytes are extremely steroid-sensitive, it was important to find out whether the deletion of these cells in transgenic males was a result of stress rather than of an antigen-specific deletion process. Stress in the male mice could possibly be caused by autoimmunity not detectable by *in vitro* assay. We addressed this question in reconstitution experiments using haemopoietic stem cells from transgenic (F23.1$^+$) and nontransgenic C57L (F23.1$^-$) mice (Fig. 3). T-cell-depleted bone marrow cells (BMC) from transgenic females were transferred either along or together with BMC from normal C57L females into lethally X-irradiated female and male C57L recipients. Five weeks after the transfer of the transgenic BMC, the cellular composition of the thymus in male recipients was very much like that in male transgenic mice (Fig. 3*a*, lower panel). But in the thymus of male recipients which had received a mixture of BMC from transgenic and C57L females, CD4$^+$8$^+$ thymocytes derived from F23.1$^-$ C57L donors developed normally and outgrew the transgenic F23.1$^+$ cells, which were mostly deleted (Fig. 3*b* and *c*, lower panel). On the other hand, in the female recipient, CD4$^+$8$^+$ thymocytes developed from both F23.1$^+$ and F23.1$^-$ donors (Fig. 3*b* and *c*, upper panel). Thus, because the deletion selectively affected transgene-expressing F23.1$^+$ CD4$^+$8$^+$ thymocytes in the male recipients, this experiment indicates that the deletion is a result of the interaction of autospecific thymocytes with radioresistant male cells in the thymus, and not of stress and steroid release. If the latter possibility were true, the F23.1$^-$ CD4$^+$8$^+$ thymocytes derived from C57L donors of BMC should also have been affected.

Discussion

Our study with $\alpha\beta$TCR transgenic mice provides new observations relevant to the understanding of the mechanism of self-tolerance and relevant to the clarification of the function of cortical CD4$^+$8$^+$ thymocytes in T-cell development. The drastically decreased number of thymocytes in male but not female mice is direct evidence for a deletion of autospecific cells at the level of CD4$^+$8$^+$ nonmature thymocytes. Furthermore, our results indicate that CD4 and CD8 accessory molecules are involved in the deletion process of autospecific cells.

Two questions relating to the function of double-positive

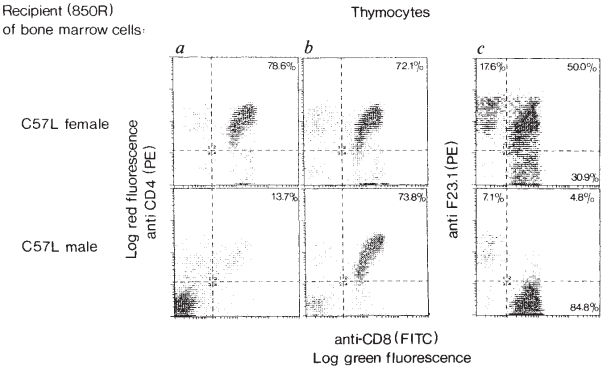


Fig. 3 Surface phenotype of thymocytes from C57L male and female irradiation chimeras reconstituted with bone marrow of $\alpha\beta$TCR transgenic female, either alone (*a*) or with normal bone marrow from C57L female (*b*, *c*). Thymocytes were stained with anti-CD4-PE (*a*, *b*) or biotinylated F23.1 (*c*) mAbs, followed by anti-CD8-FITC mAb (*a*, *b*) or a mixture of CD8-FITC mAb with PEA (*c*). In (*a*), 80% of thymocytes in the female recipient and 67% in the male recipient were stained with F23.1 mAb (data not shown).

Methods. Bone-marrow cells from transgenic or normal C57L dnor were treated with cytotoxic anti Thyl mAb (T24, ref. 21) plus rabbit complement (Cedar Lane, Ontario, Canada) for 45 min at 37 °C. After washing, 5×10^6 viable cells from the transgenic donor were injected intravenously (i.v.) into lethally irradiated (850R) eight-week-old C57L females and males, either alone or together with 0.5×10^6 viable bone marrow cells from normal female C57L. Five weeks later the mice were killed, their thymuses removed and single-cell suspensions prepared, counted and stained with anti-CD4, -CD8 and -F23.1 mAb, and analysed as described in Figs 1 and 2.

$CD4^+8^+$ thymocytes are why so many of these cells should die within the thymus[18] and whether or not they contain precursors of single positive $CD4^+8^-$ and $CD4^-8^+$ cells[19]. Our results show that the death of at least some cortical thymocytes can result from antigen-specific elimination of autoreactive cells. The deletion of nonfunctional, antigen-specific $CD4^+8^+$ thymocytes would make sense if $CD4^+8^+$ thymocytes contained precursors of functional $CD4^+8^-$ and $CD4^-8^+$ cells. Consistent with this view is our observation that $CD4^+8^-$ cells were severely depleted in male transgenic mice, despite the fact that such cells from transgenic female mice cannot be activated by male cells. An analogous finding has been reported by MacDonald *et al.*[14], who observed that $CD4^+8^-$ and $CD4^-8^+$ T cells staining with V_{β^6} antibodies were reduced to the same extent in animals positive for the *Mls*^{a}-allele of the minor lymphocyte stimulating locus (*Mls*), even though $CD4^-8^+$ from *Mls*^{a}-negative animals lacked specificity for *Mls*^{a}.

We thus favour the view that at least some double-positive $CD4^+8^+$ thymocytes act as precursors for functional single positive cells[20], even though further investigation is needed. Although we have shown that the deletion predominantly affects $CD4^+8^+$ thymocytes, we could argue that it might occur independently of accessory molecules at any stage of T-cell development. But this view is not compatible with our observation that the deletion process spares T cells that lack accessory molecules, or even T cells having low expression of CD8. Thus our experiments provide the first direct evidence that these molecules play a crucial role in the induction of tolerance. Taken together, the three observations made in male transgenic mice, namely the drastically reduced number of $CD4^+8^+$ thymocytes, the reduction of $CD4^+8^-$ cells and the occurrence of transgene-expressing cells with virtually no CD8, argue that the deletion of autospecific cells is dependent on CD4 and CD8 accessory molecules.

The results of our experiments with transgenic mice differ in at least two important aspects from others recently reported[12–14] for normal mice. Firstly, in the experiments of Kappler *et al.*[12,13], the depletion of autospecific T cells did not appear to affect $CD4^+8^+$ thymocytes. One possible reason for the difference is that different antigens are under investigation: we are looking at an antigen in the context of class I MHC antigens found throughout the cortex whereas Kappler *et al.*[12,13] are looking at an entity[21] related to class II MHC antigens which are usually not detected in the outer cortex. Thus in the latter case $CD4^+8^+$ cells can meet antigen only when reaching the cortico-medullary junction. Consequently only a minor subset of $CD4^+8^+$ cells would be deleted in the experiments of Kappler *et al.*, and this would be difficult to detect. Another possible reason for the different findings is the fact that in our transgenic mice the expression of TCR proteins is skewed towards higher levels of the range observed in $CD4^+8^+$ from normal mice. This phenomenon, as well as the increased proportion of $CD4^-8^+$ thymocytes in transgenic females, could reflect a positive selection of thymocytes by H-2^b antigens, or alternatively may be a direct consequence of expression of transgenes. We could argue therefore that the deletion of $CD4^+8^+$ thymocytes was easily detected because the majority of $CD4^+8^+$ thymocytes in transgenic mice represent a minor and more mature population of $CD4^+8^+$ thymocytes that may escape detection in normal mice, especially when representing only a fraction of cells expressing a certain idiotype. Whatever the reason for the apparent discrepancy in the results, our data indicate that the nonmature $CD4^+8^+$ populaton can be a target of deletion, whereas it is not clear whether $CD4^+8^-$ or $CD4^-8^+$ cells are susceptible to the same deletion process.

The second difference between our results and those of Kappler *et al.*[12,13] and MacDonald *et al.*[14] is that these authors did not report the presence of cells with few or no accessory molecules, spared by the deletion. Again in this case, such cells would constitute a very minor population in their experimental system, because the pool of T cells in normal mice can be easily replenished by T cells expressing different TRCs, which is not the case in transgenic mice.

As we observed normal numbers of T cells in the periphery, but not in the thymus, of transgenic males, we propose that the number of peripheral T cells can be adjusted independently of the export of newly formed cells from the thymus. This would allow the accumulation of cells with rare phenotypes in the periphery of male mice, as shown here and in the accompanying paper[17]. The fact that transgene-expressing cells with few or no accessory molecules accumulate in male mice, tends to rule out a role of anti-idiotypic cells in the deletion process; such a mechanism should eliminate transgene-expressing cells, rather than cells expressing high levels of accessory molecules.

We would like to thank Katrin Hafen, Abigail Garner and Verena Stauffer for technical assistance, Drs Werner Haas and Hung Sia Teh for critical reading and S. Kirschbaum for typing the manuscript. The Basel Institute for Immunology was founded and is supported by F. Hoffman-LaRoche & Co. Ltd, Basel, Switzerland.

Received 7 April; accepted 6 May 1988.

1. Schwartz, R. M. *A. Rev. Immun.* **3**, 237–261 (1985).
2. Dembić, Z. *et al. Nature* **320**, 232–238 (1986).

3. Saito, T., Weiss, A., Miller, J., Norcross, M. A. & Germain, R. N. *Nature* **325,** 125–130 (1987).
4. Dembić, Z. *et al. Nature* **326,** 510–511 (1987).
5. Gabert, J. *et al. Cell* **50,** 545–554 (1987).
6. Swain, S. L. Proc. natn. Acad. Sci. U.S.A. **78,** 7101–7105 (1981).
7. Marrack, P. *et al. J. exp. Med.* **158,** 1077–1091 (1983).
8. Biddison, W. E., Rao, P. E., Talle, M. A., Goldstein, G. & Shaw, S. *J. exp. Med.* **156,** 1065–1076 (1982).
9. Gay, D. *et al. Nature* **328,** 626–629 (1987).
10. Doyle, C. & Strominger, J. L. *Nature* **330,** 256–259 (1987).
11. Burnet, F. M. *The Clonal Selection Theory of Acquired Immunity* (Cambridge University Press, 1959).
12. Kappler, J. W., Roehm, N. & Marrack, P. *Cell* **49,** 273–280 (1987).
13. Kappler, J. W., Staerz, U. D., White, J. & Marrack, P. C. *Nature* **332,** 35–40 (1988).
14. MacDonald, H. R. *et al. Nature* **332,** 40–45 (1988).
15. Uematsu, Y. *et al. Cell* (in the press).
16. Staerz, U., Rammensee, H., Benedetto, J. & Bevan, M. *J. Immun.* **134,** 3994–4000 (1985).
17. Blüthmann, M. *et al. Nature,* submitted.
18. McPhee, D., Pye, J. & Shortman, K. Thymus **1,** 151–162 (1979).
19. Shortmann, K. & Scollay, R. in *Recognition and Regulation in Cell-mediated Immunity* (eds Watson, J. D. & Marbrook, J.) 31–60 (Dekker; New York and Basel, 1985).
20. von Boehmer, H. *Immunol. Today* **Vol?** 333–336 (1986).
21. Dennert, G., Hyman, R., Lesley, J. & Trowbridge, I. S. *Cell Immun.* **53,** 350–364 (1980).
22. Leo, O., Foo, M., Sachs, D. M., Samelson, L. E. & Bluestone, J. A. *Proc. natn. Acad. Sci. U.S.A.* **84,** 1374–1378 (1987).
23. Dialynas, D. P. *et al. Immunol. Rev.* **74,** 29–56 (1983).
24. Schreier, M. H., Iscove, N. N., Tees, R., Aarden, L. & von Boehmer, H. *Immunol. Rev.* **51,** 315–336 (1980).
25. Fazekas de St. Groth, S. *J. immunol. Meth.* **49,** R11–R23 (1982).
26. Marrack, P. & Kappler, J. *Nature* **332,** 840 (1988).

Thymic major histocompatibility complex antigens and the $\alpha\beta$ T-cell receptor determine the CD4/CD8 phenotype of T cells

Hung Sia Teh*‡, Pawel Kisielow*‡, Bernadette Scott*, Hiroyuki Kishi*, Yasushi Uematsu*, Horst Blüthmann† & Harald von Boehmer*

* Basel Institute for Immunology, 487 Grenzacherstrasse, CH-4058 Basel, Switzerland
† Central Research Units, F. Hoffmann-LaRoche, CH-4002 Basel, Switzerland

T-cell receptors and T-cell subsets were analysed in T-cell receptor transgenic mice expressing α and β T-cell receptor genes isolated from a male-specific, H-2D^b-restricted CD4$^-$8$^+$ T-cell clone. The results indicate that the specific interaction of the T-cell receptor on immature thymocytes with thymic major histocompatibility complex antigens determines the differentiation of CD4$^+$8$^+$ thymocytes into either CD4$^+$8$^-$ or CD4$^-$8$^+$ mature T cells.

THYMUS-derived lymphocytes (T cells) recognize antigen on the surface of antigen-presenting cells in the context of class I or class II major histocompatibility complex (MHC) molecules using the heterodimeric $\alpha\beta$ T-cell receptor (TCR)[1,2]. CD4 and CD8 molecules, expressed on the surface of T cells, bind to nonpolymorphic portions of class II and class I MHC molecules, respectively, and enhance the binding of the TCR to its ligand[3,4]. This binding of CD4 and CD8 molecules to MHC antigens may, in addition, contribute to signals leading to T-cell activation.

It is thought that the selection of the antigen-specific T-cell repertoire involves the negative selection (suppression or deletion) of autospecific T cells[5–8]. Some authors have also proposed that T cells are positively selected by thymic MHC antigens such that T cells, emerging from the thymus, bind foreign antigens predominantly in the context of self-MHC molecules[9–12]. To examine both aspects of T-cell repertoire selection we constructed TCR transgenic mice which expressed, on a large fraction of their T cells, a receptor which binds to H-Y antigen in the context of class I H-2D^b molecules. We used monoclonal antibodies that identify the transgenic receptor expressed in these mice to analyse negative selection in male $\alpha\beta$ transgenic H-2^b mice, which express the H-Y antigen as well as H-2D^b molecules. In addition, the analysis of female $\alpha\beta$ transgenic mice which express different thymic MHC antigens should reveal the possible impact of MHC molecules on the selection of T cells in the absence of nominal (H-Y) antigen.

In a previous report we have described our experiments on the mechanism of self-tolerance: from the comparison of $\alpha\beta$ transgenic male and female H-2^b mice we concluded that autospecific T cells were deleted in male mice. It was shown that this deletion involved predominantly immature CD4$^+$8$^+$ thymocytes, which contain the precursors of mature, single positive, CD4$^+$8$^-$ and CD4$^-$8$^+$ T cells[8,13].

There is less compelling evidence for the positive selection of T cells by thymic MHC antigens in the absence of nominal (H-Y) antigen: there have been reports of T cells recognizing foreign antigens predominantly in the context of those MHC molecules which they encountered during their maturation in the thymus[9–12]. It was also reported that animals that received large doses of class II MHC-antigen-specific antibodies were devoid of CD4$^+$8$^-$ T cells[14]. This could mean that antibodies can interfere with the positive selection of CD4$^+$8$^-$ T cells by thymic class II MHC antigens. These experiments do not, however, address the question of whether the $\alpha\beta$ TCR is involved in the selection process. On the basis of these and other experiments[15] one of us proposed that the interaction of the TCR on immature thymocytes with thymic MHC antigens will rescue immature T cells from programmed cell death and determine their further differentiation into mature CD4$^+$8$^-$ and CD4$^-$8$^+$ T cells. In the absence of nominal antigen, the interaction of the TCR with class II or class I thymic MHC antigens will direct the differentiation of immature T cells into CD4$^+$8$^-$ and CD4$^-$8$^+$ mature T cells, respectively[16,17]. This model predicts that in $\alpha\beta$ transgenic H-2^b mice the transgenic $\alpha\beta$ TCR should be expressed only on CD4$^-$8$^+$ and not CD4$^+$8$^-$ T cells because it was originally expressed on a class I-restricted CD4$^-$8$^+$ T cell which presumably was selected by class I MHC antigens in the thymus of C57B1/6 mice. Here we describe several observations, made in female $\alpha\beta$ transgenic mice, that are consistent with this model.

Firstly, the proportion of CD4$^-$8$^+$ thymocytes was elevated in $\alpha\beta$ transgenic H-2^b but not H-2^k or H-2^d mice. Secondly, using monoclonal antibodies specific for the transgenic receptor,

‡ Permanent addresses: Department of Microbiology, University of British Columbia, Vancouver, Canada V6T 1W5 (H.S.T.) and Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland (P.K.).

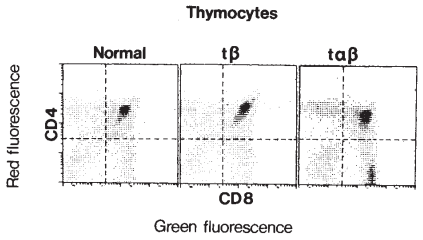


Fig. 1 The proportion of CD4/CD8 thymocyte subsets in female normal, β transgenic and $\alpha\beta$ transgenic mice. Thymocytes were stained by a mixture of phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibodies.

Methods. Transgenic β and $\alpha\beta$ mice of the H-2^b haplotype were produced as previously described[18,13]. PE-conjugated anti-CD4 (anti-mouse L3T4, ref. 20) and FITC-conjugated anti-CD8 (anti mouse Lyt-2) were purchased from Becton Dickinson and used at a final dilution of 1 in 50. The staining of thymocytes from female C57B1/6 (normal), transgenic β and $\alpha\beta$ mice were performed as previously described[8]. Two-colour fluorescence was analysed using a FACScan (Becton Dickinson) flow cytometer with a single Argon laser. Ten thousand viable cells were analysed in each sample. Dead cells were excluded from analysis using a combination of low-angle and sideways-light scatter. The results are presented as density plots, generated by analysis of processed data reduced to a 64×64 matrix with 16 levels. Where applicable, percentages were calculated using FACScan research software programs. The markers were set against negative controls which involved cells that were incubated in diluent alone and analysed in the same manner as the double-stained cells.

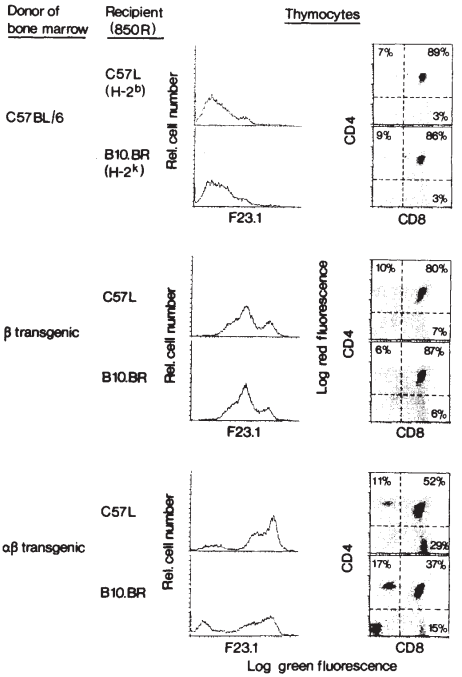


Fig. 2 The expression of transgenic β chain, CD4 and CD8 molecules on thymocytes obtained from various mice.

Methods. Female C57L and B10.BR recipient mice were lethally X-irradiated (850 rads) and reconstituted by intravenous injection of 5×10^6 anti-thy-1 treated bone marrow cells from C57Bl/6, β transgenic or $\alpha\beta$ transgenic donors. Both non-transgenic and transgenic donors of marrow cells were of the H-2^b haplotype. After six weeks the thymocytes from the recipients of marrow cells were removed, single-cell suspensions prepared and cells counted and analysed by single and double colour staining. Double staining of thymocytes with anti-CD4 and anti-CD8 antibodies was performed as described in Fig. 1. For single staining with the F23.1 antibody[21], which detects the expression of the transgenic β-chain, the thymocytes were first incubated with 10 μg ml^{-1} of F23.1 monoclonal antibody for 20 mins on ice, washed twice and then incubated with FITC-labelled sheep $(Fab')_2$ fragment anti-mouse immunoglobulin (Silenus Laboratories) at a 1 in 100 dilution for 20 mins on ice. The cells were washed three times and analysed using the FACScan flow cytometer. Five thousand viable cells were analysed in each sample.

we found that in H-2^b mice only $CD4^-8^+$ T cells expressed high levels of both the α and β transgenic TCR chains. In contrast, $CD4^+8^-$ T cells expressed high levels of the transgenic β chain only, which was usually paired with endogenous α chains. The new specificity of these receptors allowed the selection of $CD4^+8^-$ T cells by thymic class II MHC antigens. Thirdly, in $\alpha\beta$ transgenic H-2^d mice, obtained by back-crossing $\alpha\beta$ transgenic mice to DBA/2 mice, both $CD4^+8^-$ and $CD4^-8^+$ T cells expressed receptors composed of the transgenic β-chain and endogenous α-chains. The specificity of these receptors allowed the selection of $CD4^+8^-$ and $CD4^-8^+$ T cells by thymic class II and class I H-2^d MHC antigens, respectively.

T-cell subsets in transgenic mice

The proportions of thymocyte subsets classified by which of the CD4 and CD8 antigens they bear were compared in normal H-2^b and H-2^b mice that expressed either the β or $\alpha\beta$ transgenes. No significant difference was observed between C57L and β transgenic mice. In $\alpha\beta$ transgenic mice, however, the proportion of single positive $CD4^-8^+$ T cells was significantly elevated[8,13] (Fig. 1). These results indicate that the specificity of the transgenic TCR, which was originally expressed by a $CD4^-8^+$ T cell, influences the composition of thymocyte subsets. To determine whether the elevated proportion of $CD4^-8^+$ thymocytes depended on the interaction of the transgenic receptor with polymorphic domains of thymic MHC antigens, we analysed the composition of T-cell subsets in thymuses of different MHC haplotypes which had been repopulated by haemopoietic stem cells from $\alpha\beta$ transgenic, β transgenic or normal mice. In initial experiments, C57L and B10.BR recipient mice were lethally X-irradiated and repopulated with stem cells from the T-cell-depleted bone marrow of the three different donor mice. When transgenic donor cells were used, most thymocytes expressed the β transgene as detected by staining with the F23.1 monoclonal antibody showing that the repopulation was by donor cells (Fig. 2). The colonization of the H-2^b, but not the H-2^k, thymus by $\alpha\beta$ transgenic cells resulted in an elevated proportion of $CD4^-8^+$ cells compared with $CD4^+8^-$ cells. This observation was extended in a large series of repopulation experiments involving recipients expressing H-2^b MHC antigens (C57L, C57B1/6, B10.HTG) and recipients lacking H-2^b MHC antigens (B10.BR, B10.D2, DBA/2). We consistently found that only those thymuses that expressed H-2^b MHC antigens and were repopulated by $\alpha\beta$ transgenic stem cells, had a higher proportion of $CD4^-8^+$ thymocytes. We also observed that the

thymuses of MHC-mismatched ($H\text{-}2^k$ or $H\text{-}2^d$), but not partly mismatched $(C57Bl/6 \times DBA/2)F_1$ recipients, contained fewer thymocytes (10–20%) than thymuses of $H\text{-}2^b$ animals. Furthermore, in completely allogeneic thymuses, $\alpha\beta$ transgenic stem cells yielded only one-third to one-half of the progeny of normal stem cells. Despite differences in absolute cell numbers, which may depend in part on a reaction of MHC-mismatched recipient cells towards donor cells, mice expressing $H\text{-}2^b$ antigens in their thymus always had an elevated proportion of $CD4^-8^+$ thymocytes, and they were the only ones to do so.

We adopted an alternative approach to document the influence of thymic MHC and the specificity of the TCR on the development of thymocytes and back-crossed $\alpha\beta$ transgenic animals to DBA/2 mice (see below). In this case the thymuses of $\alpha\beta$ transgenic $H\text{-}2^d/H\text{-}2^d$ homozygous but not $\alpha\beta$ transgenic $H\text{-}2^d/H\text{-}2^b$ heterozygous mice contained normal numbers of thymocytes as well as a normal ratio of $CD4^+8^-$ to $CD4^-8^+$ thymocytes (3:1 to 10:1). Taken together, the results indicate that the specificity of the TCR, as well as thymic MHC antigens determine the subset composition of thymocytes.

T-cell receptors in transgenic $H\text{-}2^b$ mice

If an interaction of the transgenic, heterodimeric TCR with thymic $H\text{-}2^b$ MHC antigens was responsible for the elevated proportion of $CD4^-8^+$ thymocytes, most of these cells would presumably express both transgenic TCR chains. In contrast, one would expect that the interaction of TCRs with thymic class II MHC antigens, required for the selection of $CD4^+8^-$ cells, would depend on the expression of endogenous TCR chains generating TCRs with new specificities. We have previously shown that in our transgenic mice the β transgene prevents the rearrangement of endogenous V_β genes[18]. Thus, new specificities can result only from the rearrangement and expression of endogenous V_α genes, which was observed in our $\alpha\beta$ transgenic mice[13]. To investigate the expression of α-TCR genes on various T-cell subsets we prepared a monoclonal antibody that detects the transgenic α-chain. For this purpose we immunized BALB/B mice with the B6.2.16 clone from which the α and β transgenes were isolated. A B-cell hybridoma, referred to as T3.70, was obtained by fusing the immune spleen cells with the myeloma cell line AG8.653. This hybridoma produced antibodies which

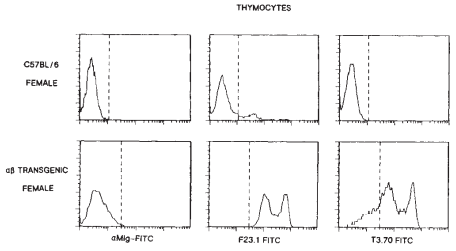


Fig. 3 Staining of thymocytes from female C57Bl/6 and $\alpha\beta$ transgenic $H\text{-}2^b$ mice by F23.1 and T3.70.
Methods. Thymocytes from a female $\alpha\beta$ transgenic mouse were incubated with phosphate-buffered saline, F23.1 or T3.70 followed by incubation with FITC-labelled second antibody as described in Fig. 2. The stained cells were then analysed using the FACScan flow cytometer. The markers were set against thymocytes that were incubated with the FITC-labelled second antibody alone. Five thousand cells from each sample were analysed. In this experiment the percentage of thymocytes stained specifically by the F23.1 and the T3.70 monoclonal antibodies were 99.6% and 76.1%, respectively. The percentage of C57Bl/6 thymocytes stained by F23.1 and T3.70 were 10.2% and 0.0%, respectively. αMIg is anti-mouse immunoglobulin.

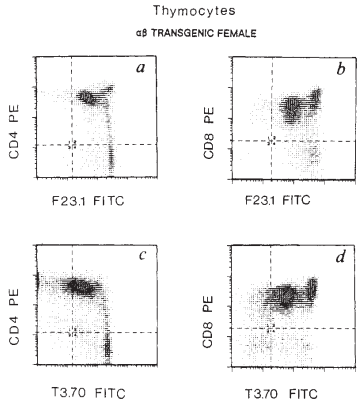


Fig. 4 Staining of thymocytes from female $\alpha\beta$ transgenic $H\text{-}2^b$ mice by F23.1, T3.70, CD4 and CD8 antibodies. Thymocytes (yield 1×10^8) were doubled stained for F23.1 and CD4 or CD8 (*a* and *b*) and for T3.70 and CD4 or CD8 (*c* and *d*).
Methods. Thymocytes were double stained with F23.1 and anti-CD4 or anti-CD8 monoclonal antibodies by incubating the cells first with unconjugated F23.1 followed by FITC-labelled sheep $(Fab')_2$ fragment anti-mouse immunoglobulin. To saturate free mouse immunoglobulin binding sites for the FITC-labelled antibody the cells were incubated with whole mouse serum (2% v/v) for 20 mins after the FITC step. The cells were then stained directly with PE-labelled anti-L3T4 or with biotin-labelled anti-Lyt-2 followed by streptavidin phycoerythrin (both from Becton Dickinson). A similar procedure was used to stain thymocytes with T3.70 and anti-CD4 or anti-CD8. Non-specific binding of PE to the splenic T cells was minimized by washing the cells four times after incubation with the biotinylated antibody. Two-colour fluorescence was analysed as described in Fig. 1. Proportions of single positive cells expressing high levels of the F23.1 or T3.70 idiotypes were: $CD4^+$, $F23.1^+ > 95\%$; $CD8^+$, $F23.1^+ > 95\%$; $CD4^+$, $T3.70^+ < 5\%$; $CD8^+$, $T3.70^+ > 90\%$.

stained the B6.2.16 T-cell clone but not the 93.2.20 T-cell clone (derived from a β transgenic mouse), T cells from normal C57Bl/6 mice or T cells from α transgenic mice; it also precipitates a disulphide-linked heterodimer with a relative molecular mass of 90,000 (in preparation). Thus the T3.70 antibody seems to be specific for an idiotypic determinant that is dependent on the co-expression of both the α and β transgenic TCR chains.

Single staining of thymocytes from female $\alpha\beta$ transgenic $H\text{-}2^b$ mice shows that they express low and high levels of the idiotypes recognized by either the F23.1 or T3.70 antibodies (referred to as F23.1 and T3.70 idiotypes). We already know that low receptor levels are found on $CD4^+8^+$ thymocytes. It is also clear from Fig. 3 that some thymocytes do not bear the T3.70 idiotype but are F23.1 positive, and therefore do not express the transgenic α-chain. Because α- and β-chains are present in equimolar concentrations on T cells this is consistent with our previous observation that some endogenous α genes are being expressed by T cells from $\alpha\beta$ transgenic $H\text{-}2^b$ mice. In further experiments the differential expression of the transgenic α-chain on thymocyte subsets was analysed by double staining with one of the F23.1 or T3.70 antibodies and one of the CD4 or CD8 antibodies. From the data in Fig. 4 it is apparent the majority of $CD4^+8^+$ cells express low levels of both the T3.70 and F23.1 idiotype and therefore low levels of both α and β transgenic TCR chains (Fig. 4*a*–*d*). $CD4^+8^-$ thymocytes express high levels of the

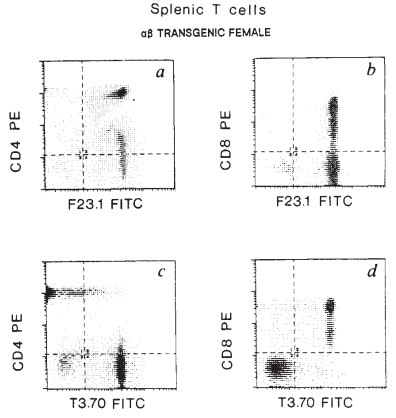


Fig. 5 Staining of peripheral, nylon-wool-purified splenic T cells from female $\alpha\beta$ transgenic H-2^b mice by F23.1, T3.70, CD4 and CD8. Nylon wool-nonadherent spleen cells from a female $\alpha\beta$ transgenic mouse were double stained with F23.1 and CD4 or CD8 (*a* and *b*) and with T3.70 and CD4 or CD8 (*c* and *d*).
Methods. Spleen cells from a female $\alpha\beta$ transgenic mouse were enriched for T cells by passing them over a nylon wool column as described[22]. The nylon wool nonadherent cells were 88.3% Thy-1^+ and 9.4% immunoglobulin-positive by single colour FACScan analyses. They were stained and analysed as described in Fig. 4. Proportions of single positive cells expressing high levels of the F23.1 or T3.70 idiotypes were: $CD4^+$, $F23.1^+ > 95\%$; $CD8^+$, $F23.1^+ > 95\%$; $CD4^+$, $T3.70^+ < 1\%$; $CD8^+$, $T3.70^+ > 90\%$.

transgenic β- but not α-chain (Fig. 4*a* and *c*). In contrast, $CD4^-8^+$ cells express high levels of both transgenic chains (Fig. 4*c* and *d*). These results indicate that the selection of $CD4^+8^-$ but not $CD4^-8^+$ cells from immature precursors requires the expression of endogenous α-chains.

The same conclusion is reached from the analysis of splenic T cells from female $\alpha\beta$ transgenic H-2^b mice (Fig. 5): again $CD4^+8^-$ T cells express high levels of the transgenic β-chain but low levels of the transgenic α-chains (Fig. 5*a* and *c*) whereas the vast majority of $CD4^-8^+$ cells clearly express high levels of both transgenic α- and β-chains (Fig. 5*b* and *d*).

T-cell receptors in transgenic H-2^d mice

Transgenic H-2^d mice were obtained by crossing $\alpha\beta$ transgenic mice with DBA/2 mice and selecting offspring that expressed α and β transgenes and were homozygous at the MHC. As the B6.2.16 clone was obtained from H-2^b mice, we expected that the transgenic TCR will not be selected in H-2^d mice because the B6.2.16 clone is not restricted by H-2^d MHC molecules. Therefore, in H-2^d mice the selection of both the $CD4^+8^-$ as well as the $CD4^-8^+$ subset should depend on the expression of endogenous α genes. The results in Figs 6 and 7 confirm this: we observe that thymocytes from five independent $\alpha\beta$ transgenic H-2^d mice (but not H-$2^d \times$ H-2^b heterozygous mice) contain more $CD4^+8^-$ than $CD4^-8^+$ single positive thymocytes (ratio 4:1, Fig. 6*a* and *b*) compared to thymocytes from $\alpha\beta$ transgenic H-2^b mice (Figs 1 and 2). Most of the $CD4^+8^+$ thymocytes in H-2^d mice express low levels of both transgenic chains similar to those observed in H-2^b mice suggesting that MHC antigens do not influence the selection of these immature cells. However, the H-2^d mice differ from the H-2^b mice in the levels of α-chain expression by single positive $CD4^+8^-$ and $CD4^-8^+$ T cells: both subsets lack high levels of the transgenic α-chain (Fig. 6*c* and *d*). This is also apparent on lymph node T cells where T cells display wide variation in the level of the T3.70 idiotype, with most cells expressing levels that are much lower (Fig. 7) than those observed on $CD4^-8^+$ T cells from transgenic H-2^b mice (Fig. 5). These data indicate that the selection of all single positive T cells in H-2^d mice requires the expression of endogenous α-chains, and that at least some of the $CD4^+8^-$ T cells in these mice can express relatively high levels of the transgenic α chain (Fig. 7*c*). This is unlike the situation in H-2^b mice because the expression of high levels of transgenic α- and β-chains in H-2^d mice does not lead to differentiation of immature $CD4^+8^+$ T cells into mature $CD4^-8^+$ T cells.

Discussion

The data reported here provide evidence that the class I MHC-restricted $\alpha\beta$ heterodimeric TCRs and thymic H-2^b MHC antigens are involved in the selection of $CD4^-8^+$ T cells. This selection occurs in the absence of the nominal (H-Y) antigen in $\alpha\beta$ transgenic female mice. The contribution of thymic MHC antigens to this selection process is evident from the fact that an elevated proportion of $CD4^-8^+$ thymocytes is observed in H-2^b, but not in H-2^k or H-2^d, thymuses repopulated by the progeny of $\alpha\beta$ transgenic stem cells. An elevated proportion of $CD4^-8^+$ cells is observed with $\alpha\beta$ transgenic stem cells but not with stem cells from β transgenic or normal C57L mice demonstrating that the $\alpha\beta$ transgenic TCR influences the selection process. $CD4^-8^+$, but not $CD4^+8^-$, cells in H-2^b mice express

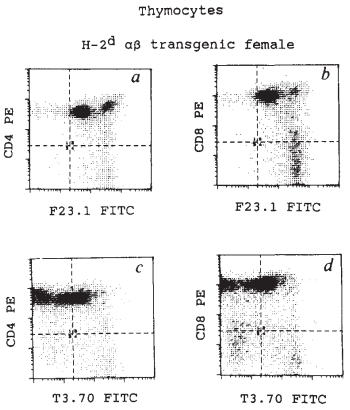


Fig. 6 Staining of thymocytes from female $\alpha\beta$ transgenic H-2^d mice by F23.1, T3.70, CD4 and CD8 antibodies. Thymocytes (yield 7×10^7) were double stained with F23.1 and CD4 or CD8 (*a* and *b*) and with T3.70 and CD4 or CD8 (*c* and *d*).
Methods. Female $\alpha\beta$ transgenic H-2^d mice were produced by backcrossing the $\alpha\beta$ transgenic founder C57B1/6 $\times$ DBA/2. (H-$2^{b/d}$)F_1 hybrid mouse with DBA/2 (H-$2^{d/d}$) mice[13]. The H-2 haplotype of the backcrosses was determined by subjecting peripheral blood lymphocytes to killing by specific antisera against K^b or K^d plus complement. Five independent $\alpha\beta$ transgenic H-2^d/H-2^d mice were analysed with similar results as shown here. Thymocytes were double stained with the indicated antibodies and analysed as described in Fig. 4. Proportions of single positive cells expressing high levels of F23.1 or T3.70 idiotypes were: $CD4^+$, $F23.1^+ > 95\%$; $CD8^+$, $F23.1^+ > 95\%$; $CD4^+$, $T3.70^+ < 5\%$; $CD8^+$, $T3.70^+ < 5\%$.

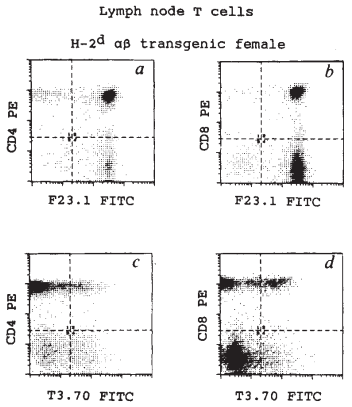


Fig. 7 Staining of lymph node cells (yield 2×10^7) from female $\alpha\beta$ transgenic H-2^d mice by F23.1, T3.70, CD4 and CD8 antibodies. **Methods.** Female $\alpha\beta$ transgenic H-$2^{d/d}$ mice were produced as described in Fig. 6. Lymph node cells were enriched for T cells by passing lymph node cells over a nylon wool column as described[22]. This preparation of nylon wool non-adherent cells contained 98.9% Thy-1^+ cells and 0.9% immunoglobulin-positive cells. The immunoglobulin-negative were double stained with F23.1 and CD4 or CD8 (*a* and *b*) and with T3.70 and CD4 or CD8 (*c* and *d*) as described in Fig. 4. Proportions of single positive cells expressing high levels of the F23.1 or T3.70 idiotypes were: $CD4^+$, $F23.1^+ > 95\%$; $CD8^+$, $T3.70^+ > 95\%$; $CD4^+$, $T3.70^+ < 10\%$; $CD8^+$, $T3.70^+ < 10\%$.

high levels of both α and β transgenic TCR chains whereas in $\alpha\beta$ transgenic H-2^d mice both subsets express lower levels of the α transgenic TCR chain confirming the importance of the $\alpha\beta$ TCR. These results are consistent with the hypothesis that the interaction of class I MHC antigens in the thymus with the $\alpha\beta$ heterodimeric T-cell receptor determines the CD4/CD8 phenotype of mature T cells in the absence of nominal antigen.

It is possible that CD4 antigens and the $\alpha\beta$ transgenic receptor are incompatible on the surface of the same cell or that CD4 molecules change the idiotype recognized by the T3.70 antibody. This does not seem likely as the majority of $CD4^+8^+$ T cells express similar levels of the determinants recognized by the F23.1 and T3.70 antibodies. In addition, this reasoning does not explain the fact that a few $CD4^+8^-$ cells in $\alpha\beta$ transgenic H-2^d mice express high levels of the T3.70 determinant although, in the same mice, most $CD4^-8^+$ cells express low levels of idiotypes recognized by the T3.70 antibody. It is also possible that the expression of the T3.70 idiotype requires CD8 molecules on the cell surface, but this is not consistent with our observation that $CD8^-$ T cells and hybridomas express the T3.70 idiotype (unpublished results) and, in general, class I-restricted T cells can express a class I-MHC-antigen restricted $\alpha\beta$ heterodimeric receptor in the absence of CD8 molecules[15]. It is possible that some interaction of the $\alpha\beta$ heterodimer with the CD8 molecule on immature $CD4^+8^+$ T cells is essential for the generation of $CD4^-8^+$ thymocytes although this would not explain the elevated proportion of $CD4^-8^+$ T cells, which express both α and β transgenic TCR chains, in H-2^b but not H-2^k or H-2^d thymuses. We therefore propose that thymic MHC antigens play an important role in the interaction of the $\alpha\beta$ heterodimer with the CD8 molecule, possibly by cross-linking the two molecules[16], which may lead to the generation of $CD4^-8^+$ T cells[16,17].

To investigate this further, we will test whether the MHC antigens needed for obtaining a high proportion of $CD4^-8^+$ cells are in fact the restricting class I H-$2D^b$ MHC antigens. It will also be important to determine whether these antigens select $CD4^-8^+$ T cells expressing high levels of both α and β transgenic TCR chains. At present, we cannot rule out the possibility that some suppression mechanism interferes with the development of $CD4^+8^-$ cells which express high levels of α and β transgenic TCR chains. We hope to investigate this possibility in $\alpha\beta$ transgenic mice which have been back-crossed to mice with severe combined immune deficiency; such mice should only express the α and β transgenes as these mice are defective in the rearrangement of endogenous TCR genes[19] and are therefore expected to be devoid of any endogenous effector T-cell population including suppressor cells.

The experiments reported here also support our earlier conclusion[8] that $CD4^+8^+$ cells contain the precursors of single positive cells. $CD4^+8^-$ T cells lack high levels of the transgenic α-chain, indicating that these T cells are not male-specific, but their numbers were significantly reduced in male $\alpha\beta$ transgenic mice[8]. The best explanation for this observation is that most of their precursors are deleted in male mice. This implies that the precursors of the $CD4^+8^-$ T cells initially express the male-specific, transgenic receptor and, later, rearrange endogenous α loci leading to the expression of new receptors selectable by class II MHC antigens. Therefore, both positive and negative selection can occur at the same stage of T-cell development, that is, negative selection by nominal self-antigen need not occur after positive selection by thymic MHC antigens. These conclusions would imply that the signals leading to positive and negative selection are different.

We thank Katrin Hafen, Beatrice Schmutz and Verena Stauffer for technical assistance and Abby Garner, Uwe Staerz and Felipe Fegueroa for providing some of the antibodies. The manuscript was typed by Shirley Kirschbaum. The Basel Institute for Immunology is supported by Hoffmann-LaRoche Co. Ltd.

Received 2 June; accepted 9 August 1988.

1. Dembic, Z. *et al. Nature* **320**, 232-238 (1986).
2. Saito, T., Weiss, A., Miller, J., Nacross, M. A. & Germain, R. N. *Nature* **325**, 125-130 (1987).
3. Gabert, J. *et al. Cell* **50**, 545-554 (1987).
4. Gay, D. *et al. Nature* **328**, 626-629 (1987).
5. Kappler, J. W., Roehm, N. & Marrack, P. *Cell* **49**, 273-280 (1987).
6. Kappler, J. W., Staerz, U. D., White, J. & Marrack, P. *Nature* **332**, 35-40 (1988).
7. MacDonald, H. R. *et al. Nature* **332**, 40-45 (1988).
8. Kisielow, P., Blüthmann, H., Staerz, U. D., Steinmetz, M. & von Boehmer, H. *Nature* **333**, 742-746 (1988).
9. Bevan, M. J. *Nature* **269**, 417 (1977).
10. Zinkernagel, R. M. *et al. J. exp. Med.* **147**, 882-896 (1978).
11. von Boehmer, H., Haas, W. & Jerne, N. K. *Proc. nat. Acad. Sci. U.S.A.* **75**, 2439-2442 (1978).
12. Kappler, J. & Marrack, P. *J. exp. Med.* **148**, 1510 (1978).
13. Blüthmann, H. *et al. Nature* **334**, 156-159 (1988).
14. Kruisbeck, A. *et al. J. exp. Med.* **161**, 1029-1047 (1985).
15. Dembić, Z. *et al. Nature* **326**, 510-512 (1987).
16. von Boehmer, H. *Immun. Today* **7**, 333-336 (1986).
17. von Boehmer, H. *A. Rev. Immunol.* **6**, 309-326 (1988).
18. Uematsu, Y. *et al. Cell* **52**, 831-841 (1988).
19. Schuler, M. *et al. Cell* **46**, 963-972 (1986).
20. Dialynas, D. P. *et al. Immunol. Rev.* **74**, 29-55 (1983).
21. Staerz, U., Rammensee, H., Benedetta, J. & Bevan, M. J. *J. Immun.* **134**, 3994-4000 (1985).
22. Julius, M. H., Simpson, E. & Herzenberg, L. A. *Eur. J. Immun.* **3**, 645-649 (1973).

RESEARCH ARTICLE

Analysis and Expression of a Cloned Pre–T Cell Receptor Gene

Claude Saint-Ruf,* Katharina Ungewiss,* Marcus Groettrup, Ludovica Bruno, Hans Joerg Fehling, Harald von Boehmer

The T cell antigen receptor (TCR) β chain regulates early T cell development in the absence of the TCRα chain. The developmentally controlled gene described here encodes the pre-TCRα (pTα) chain, which covalently associates with TCRβ and with the CD3 proteins forms a pre-TCR complex that transduces signals in immature thymocytes. Unlike the λ5 pre–B cell receptor protein, the pTα chain is a type I transmembrane protein whose cytoplasmic tail contains two potential phosphorylation sites and a Src homology 3 (SH3)–domain binding sequence. Pre-TCRα transfection experiments indicated that surface expression of the pre-TCR is controlled by additional developmentally regulated proteins. Identification of the pTα gene represents an essential step in the structure-function analysis of the pre-TCR complex.

T cell development takes place in discrete steps during which the TCR genes are rearranged and expressed in temporal order. During development of TCRαβ-expressing cells the TCRβ gene is rearranged and expressed before the TCRα gene (*1*, *2*). Without TCR rearrangement the development of T cells is arrested at an early stage (*3*–*5*). By introducing TCRβ transgenes into mice that are defective for rearrangement of antigen receptor genes, it was shown that TCRβ proteins, in the absence of TCRα chains, are sufficient to promote early T cell development (*6*–*8*). Although such mice are still rearrangement-defective, their immature thymocytes (which express neither the CD4 nor CD8 proteins) begin to express CD4 and CD8 coreceptors, transcripts of the TCRα locus become detectable (*7*), and the number of thymocytes increases (*6*–*8*). Introduction of TCRβ transgenes into normal mice suppresses rearrangement of endogenous TCRβ genes (*9*, *10*). The TCRβ transgene is expressed on the cell surface in the absence of TCRα proteins in both normal (*11*) as well as in rearrangement-defective mice (*7*, *8*, *12*) in an 80-kD disulfied-linked complex and as a glycosyl-phosphatidylinositol (GPI)–linked 40-kD monomer.

The presence of the TCRβ chain in the 80-kD complex suggested that either the complex was a homodimer or that an unknown TCR chain was involved that may affect T cell maturation. A glycosylated chain of 33 kD (gp33) is paired with TCRβ proteins in a TCRβ-transfected immature T cell line (SCB.29) from severe combined immunodeficient (SCID) mice (*12*), but could not be identified in normal thymocytes (*12*, *13*). The gp33-TCRβ complex of SCB.29 cells is associated with CD3 proteins (*8*, *12*) and cross-linking of TCR chains initiates Ca^{2+} mobilization. This suggested that this TCRβ complex could be responsible for the developmental progression observed in TCRβ transgenic, rearrangement-deficient mice, whereas the TCRβ GPI-linked monomer could represent a transgenic artifact (*14*, *15*). We have now cloned the gene encoding gp33 and examined its structure and expression. Because of its properties, the gp33 protein was named the pre-TCRα (pTα) chain.

Pre–T cell receptor α (pTα) expression in immature T cells. The pTα chain can be identified by two-dimensional (diagonal) gel electrophoresis, in which the disulfide-linked pTα protein under reducing conditions migrates away from the diagonal just underneath the TCRβ protein (*12*) (Figs. 1 and 2). The analytical method was scaled up to obtain sufficient amounts of pTα protein for microsequencing. In a first attempt a 20-amino-acid-long NH_2-terminal sequence was obtained; a peptide of the 18 NH_2-terminal residues was synthesized and injected into rabbits to obtain a pTα-specific antiserum. The antiserum was tested for binding to the pTα protein. To this end lysates from the TCRα-negative SCB.29 cell line as well as the TCRαβ-expressing B6.2.16BW hybridoma (*12*) were precipitated with the monoclonal antibody (mAb) F23.1 to $V_\beta 8$ proteins (*16*). Precipitates

C. Saint-Ruf is at the Unité INSERM 373, Institut Necker, 75730 Paris, Cedex 15, France. K. Ungewiss, M. Groettrup, L. Bruno, H. J. Fehling, and H. von Boehmer are at the Basel Institute for Immunology, CH-4005 Basel, Switzerland.

*The first two authors contributed equally to this work.

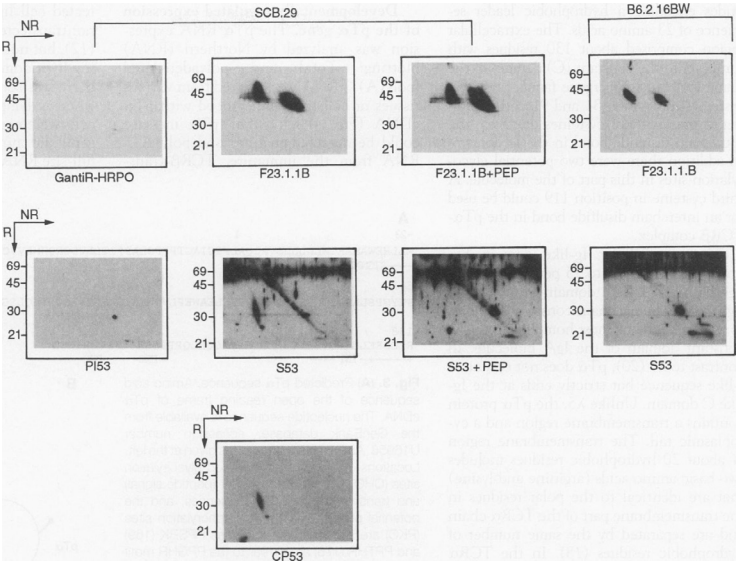


Fig. 1. Identification of the pTα chain by antibodies. Immunoprecipitations of TCRβ from either SCB.29 or B6.2.16BW cells were immunoblotted with control reagents or antibodies to TCRβ or pTα, with or without peptide (PEP) from the NH_2-terminus of pTα (*38*). SCB.29, cell line from SCID mouse transfected with TCRβ; B6.2.16BW, TCRαβ-expressing T cell hybridoma; GantiR-HRPO, goat antibodies to rabbit immunoglobulin conjugated to horseradish peroxidase; F23.1, mAb to TCRβ ($V_β8$); PI53, pre-immune serum from rabbit 53; S53, immune serum from rabbit 53 injected with NH_2-terminal peptide of pTα; CP53, affinity column–purified S53. Molecular sizes are given in kilodaltons.

were separated on diagonal gels and the proteins analyzed in Western immunoblots by staining with either mAb F23.1 or serum from immunized rabbit 53 (Fig. 1). The TCRβ mAb stained proteins off and on the diagonal, corresponding to disulfide-linked and -unlinked TCRβ proteins, respectively, in the lysates of both SCB.29 and B6.2.16.BW cells (Fig. 1, top) whereas the control GantiR-HRPO [goat anti-rabbit immunoglobulin (Ig) conjugated to horseradish peroxidase] produced no signal. The pre-immune serum from rabbit 53 (PI53) likewise produced no signal (except for some weak reactivity with the 30-kD marker on the diagonal) whereas the immune serum (S53) stained in SCB.29 lysates a protein off the diagonal beneath the TCRβ protein that was not stained in B6.2.16BW lysates (Fig. 1, middle). The staining of the pTα protein could be completely inhibited by the specific peptide, whereas the extensive background staining could not. Affinity purification on a peptide column finally yielded a reagent of exquisite specificity for the pTα protein that became visible as a streak of differentially glycosylated proteins as well as a nonglycosylated dot (Fig. 1, bottom). These results established that the protein sequence was obtained from the pTα protein and that the antiserum could be used to analyze expression of the pTα-TCRβ complex in various cells.

Because the pTα-TCRβ complex in thymocytes was not previously identified, we used the above approach to examine this complex in thymocytes from TCRα-deficient ($TCRα^{-/-}$) mice (Fig. 2). We could detect the pTα-TCRβ complex by precipitation with the mAb H57 to all TCRβ proteins (*17*) from lysates of $TCRα^{-/-}$ thymocytes. Thus the pTα-TCRβ complex does not represent a peculiarity of a single cell line, but is also formed and glycosylated in immature thymocytes.

Cloning of the pTα chain complementary DNA (cDNA). To obtain a pTα chain cDNA sequence we prepared a cDNA library from the SCB.29 cell line in lambda ZAP II (Stratagene). A lysate of the library was prepared and amplified by PCR (polymerase chain reaction) with degenerate or nondegenerate oligonucleotides, corresponding to the most 3′ part of the pTα NH_2-terminal protein sequence and an oligonucleotide complementary to the lambda ZAP vector-sequence at the 5′ end of the cDNA inserts. Specific PCR products were identified by Southern (DNA) hybridization with another set of degenerate oligonucleotides corresponding to the pTα sequence between the PCR primers. Hybridizing PCR fragments were subcloned and sequenced. In this way a fragment of approximately 180 bp was obtained that encoded part of the 5′ untranslated region, the leader sequence, and the sequence corresponding to the first five amino acids of the pTα protein. This fragment was then used as a probe for conventional screening of the SCB.29 cDNA library. Several independent lambda clones were isolated that contained the full-length coding sequence of pTα. The sequence revealed an open reading frame of 618 nucle-

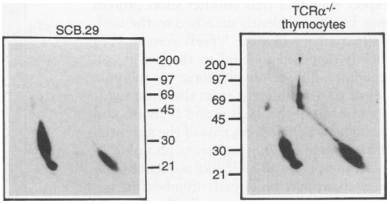


Fig. 2. Identification of the pTα chain in $TCRα^{-/-}$ thymocytes (4×10^9 cells) and SCB.29 (2×10^7 cells). Two-dimensional SDS-PAGE of immunoprecipitates with H57 (*17*). The pTα chain is revealed by CP53 serum. The spot on the diagonal may represent nondisulfide-linked pTα in the precipitate. Staining with mAb F23.1 was similar to that in Fig. 1. Methods were as in Fig. 1. Molecular sizes are indicated in kilodaltons.

otides encoding a hydrophobic leader sequence of 23 amino acids. The extracellular region comprised about 130 residues with similarity to the constant (C) domain of the immunoglobulin supergene family with two cysteines in position 31 and 91 corresponding to the invariant cysteines that form the intrachain disulfide bond in the Ig domain. In addition there were two potential glycosylation sites in this part of the molecule. A third cysteine in position 119 could be used for an interchain disulfide bond in the pTα-TCRβ complex.

The extracellular Ig-like domain has only some weak (20 to 25 percent) homology with the Ig-like domain of TCRα, Ig light and Ig heavy chain constant domains, as well as λ5, being most homologous to the constant domain of the IgA molecule. In contrast to λ5 (*20*), pTα does not contain a J-like sequence but strictly ends at the Ig-like C domain. Unlike λ5, the pTα protein contains a transmembrane region and a cytoplasmic tail. The transmembrane region of about 20 hydrophobic residues includes two basic amino acids (arginine and lysine) that are identical to the polar residues in the transmembrane part of the TCRα chain and are separated by the same number of hydrophobic residues (*18*). In the TCRα chain, these polar residues are essential for the assembly and transport of the TCRαβ-CD3 complex (*18*). The cytoplasmic region of about 31 residues is rich in proline residues and could constitute a SH3-domain binding region. In addition, there are PPSRK and PPTHR sequences similar to the PPGHR motif present in the cytoplasmic tail of CD2, known to be involved in CD2-dependent T cell activation (*19*) and containing two potential phosphorylation sites for protein kinase C (Fig. 3).

The sequence analysis indicates that the pTα protein is well suited for pairing with the TCRβ chain. Even though there is only moderate identity to any particular member of the Ig supergene family, this protein bears the hallmarks of this family. The cysteine in position 119 as well as the transmembrane part seem designed to form a complex with TCRβ and CD3 proteins. The pTα-TCRβ heterodimer has an asymmetrical shape because the TCRβ has two and the pTα only one extracellular Ig-like domain. One might expect therefore that another short protein may be noncovalently attached to the molecule, perhaps like the VpreB domain (*20*) that is supposed to bind to the pre–B cell receptor. The relatively long cytoplasmic tail of pTα is different from the short cytoplasmic tail of the TCR α and β chains and the Ig heavy chains. Because of the potential SH3-domain binding sequence, phosphorylation sites, and the CD2 like sequences, the pTα chain may be directly involved in signal transduction by the pre-TCR.

Developmentally regulated expression of the pTα gene. The pTα RNA expression was analyzed by Northern (RNA) blotting: Total and polyadenylated [poly(A)$^+$] RNA was isolated from various tissues or cell lines and probed with pTα cDNA (Fig. 4, left). The pTα message could be detected in total and poly(A)$^+$ RNA from the immature, TCRβ-transfected cell line SCB.29 as well as in the nontransfected parental line SCI.ET.27F (*12*), but not at all in the TCRαβ-expressing hybridoma B6.2.16.BW (*12*). In mice, there was some expression in the thymus, whereas lymph node (Fig. 4) and spleen cells were negative. Expression of the pTα gene did not require rearrangement, as full-size RNA was abundant in the thymus

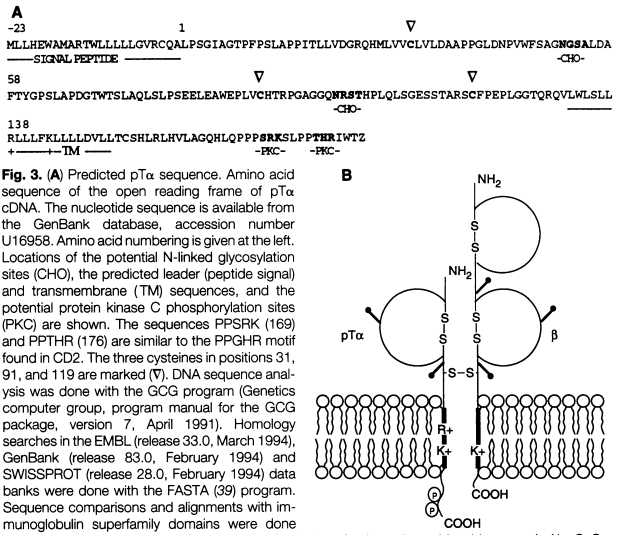


Fig. 3. (**A**) Predicted pTα sequence. Amino acid sequence of the open reading frame of pTα cDNA. The nucleotide sequence is available from the GenBank database, accession number U16958. Amino acid numbering is given at the left. Locations of the potential N-linked glycosylation sites (CHO), the predicted leader (peptide signal) and transmembrane (TM) sequences, and the potential protein kinase C phosphorylation sites (PKC) are shown. The sequences PPSRK (169) and PPTHR (176) are similar to the PPGHR motif found in CD2. The three cysteines in positions 31, 91, and 119 are marked (∇). DNA sequence analysis was done with the GCG program (Genetics computer group, program manual for the GCG package, version 7, April 1991). Homology searches in the EMBL (release 33.0, March 1994), GenBank (release 83.0, February 1994) and SWISSPROT (release 28.0, February 1994) data banks were done with the FASTA (*39*) program. Sequence comparisons and alignments with immunoglobulin superfamily domains were done with the PILEUP (*40*) program. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B**) The pTα-TCRβ complex. ▬, Corresponds to the N-linked glycosylation sites. P, represents protein kinase C phosphorylation sites in the pTα cytoplasmic tail. Two basic amino acids, arginine (R$^+$) and lysine (K$^+$), are located in the pTα transmembrane region.

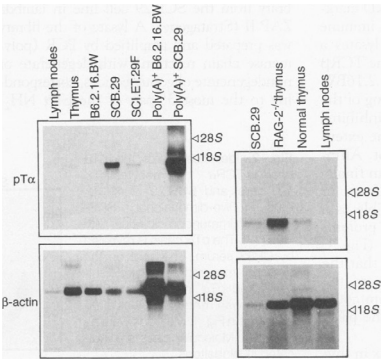


Fig. 4. pTα mRNA expression in various cells and tissues. Total or poly(A)$^+$ RNA was isolated from cell lines and tissues and the RNA was separated on agarose gels. After transfer, the RNA was probed with a labeled cDNA specific for pTα; after the original probe was stripped from the filter, it was again hybridized with a labeled β-actin probe (*41*).

of RAG-2$^{-/-}$ (recombination activating gene 2) rearrangement-defective mice (*21*) and it was expressed higher in RAG-2$^{-/-}$ thymocytes than in thymocytes from normal mice (Fig. 4). The latter result suggested that the pTα gene was predominantly expressed in the more immature thymocytes.

Expression in early thymocytes was confirmed by PCR analysis with oligonucleotides specific for the pTα gene (Fig. 5). The developmentally regulated pTα message was expressed in different amounts at successive stages of thymic development. There was high expression in CD44$^-$CD25$^+$ and CD44$^-$CD25$^-$ double negative (CD4$^-$CD8$^-$) cells but there was weaker expression in earlier and later stages of development. Small single positive (expressing only CD4 or CD8) thymocytes had no detectable pTα RNA. The difference in the intensity of the pTα RNA bands between RAG-2$^{-/-}$ and normal thymocytes in Fig. 4 is explained by the fact that most cells in the normal thymus are small CD4$^+$CD8$^+$ cells, whereas in the RAG-2$^{-/-}$ thymus most cells are CD44$^-$CD25$^+$ and because CD44$^-$ CD25$^+$ cells express more pTα RNA than small CD4$^+$CD8$^+$ thymocytes. Thus pTα is expressed in the thymus before TCRβ and TCRα genes, but there may be some overlap in the expression of pTα and TCRα genes. Coexpression of pTα-TCRβ and TCRα-TCRβ heterodimers on the cell surface of TCRα-transfected SCB.29 cells has been observed (*22*). Therefore this coexpression is theoretically possible in immature thymocytes.

The transfection of pTα in a TCRα-deficient, mature T cell line. The above results show that a glycosylated pTα-TCRβ complex is transported to the cell surface in immature T cells. Because TCRβ has two and pTα only one extracellular Ig-like domain, one would expect that the pTα-TCRβ complex has an additional, noncovalently associated Ig-like domain, much like the VpreB domain in the pre–B cell receptor (*23*). One might further expect that without such an additional protein the pTα-TCRβ complex may not be transported to the cell surface.

To test whether or not the pTα-TCRβ complex was sufficient for cell surface expression, we transfected a pTα construct into the 58α$^-$β.1 mature T cell line (*8*), which expresses a TCRβ gene and CD3 proteins that are essential for surface expression of the αβ TCR, but lacks TCRα chains. When these cells were supertransfected with the pTα construct, intracellular pTα-TCRβ dimers were observed, but in contrast to dimers in the immature T cell line SCB.29, the disulfide-linked pTα proteins in the mature T cell line were poorly or not at all glycosylated, indicating that most of the heterodimer did not leave the endoplasmic reticulum (Fig. 6). Also, surface expression of TCRβ in the pTα transfectants was not increased, whereas TCRαβ surface expression was observed after TCRα transfection. This result was consistent with the hypothesis that another developmentally regulated protein (VpreT) may be required for proper assembly, glycosylation, and transport to the cell surface of the pTα-TCRβ complex. The result is of course equally consistent with the hypothesis that mature T cells may contain proteins that prevent pTα-TCRβ complexes from leaving the endoplasmic reticulum and that the pTα-TCRβ complex can only be transported to the cell surface in the absence of these retention molecules.

Regulation of T cell development by the pre-TCR complex. In conjunction with experiments in TCRβ transgenic, rearrangement-defective SCID mice (*6–8*) and analogous experiments in TCRβ transgenic RAG$^{-/-}$ mice (*21*, *24*), our results are consistent with the hypothesis that the pre-TCR complex is sufficient to promote T cell development in the absence of other TCR chains encoded by rearranging genes. Although the experiments in the TCRβ transgenic, rearrangement-deficient mice have indicated that TCRβ proteins are sufficient to induce T cell maturation, studies in TCRβ$^{-/-}$ mice suggest that a TCRβ protein is not essential for the expression of CD4 and CD8 molecules in development because CD4$^+$CD8$^+$ cells are present in TCRβ$^{-/-}$ mice (*24*). The possibility was considered that CD4$^+$CD8$^+$ thymocytes in TCRβ$^{-/-}$ mice were of the γδ lineage (because they were absent in TCRβ$^{-/-}$ TCRγ$^{-/-}$ mice) and that the TCRβ protein was essential for expression of CD4 or CD8 coreceptors in the αβ lineage (*24*). There are, however, no experiments supporting this view. In contrast, reconstitution of thymuses from SCID (*25*, *26*) or RAG$^{-/-}$ mice (*22*) with either pro-T cells or γδT cells have shown that thymocytes devoid of TCR proteins can express CD4 and CD8 molecules as long as other TCR-bearing cells are present. Thus the pre-TCR complex may not directly regulate CD4 and CD8 expression through intracellular signaling.

Intracellular signaling by the pre-TCR complex may, however, be essential to obtain large numbers of immature thymocytes. This can be deduced from experiments that showed that the proportion of productive

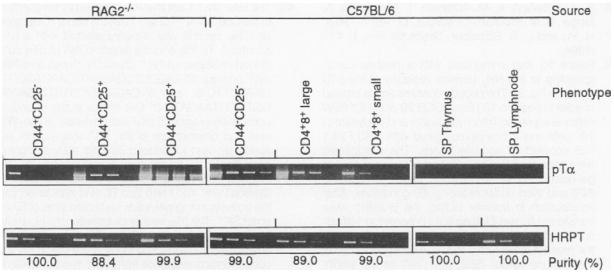


Fig. 5. Semiquantitative analysis of pTα expression on lymphocyte subsets. Thymocytes from RAG-2$^{-/-}$ and normal C57BL/6 mice were separated by fluorescence-activated cell sorting (FACS) or complement-dependent killing. Subsets of T cells were subjected to polymer chain reaction (PCR) as described in (*42*). Samples were stained with ethidium bromide. SP, single positive (CD4 or CD8) T cells; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

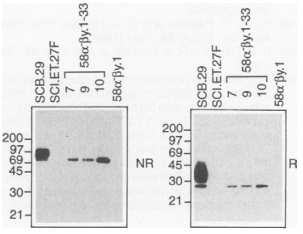


Fig. 6. Transfection of a pTα construct into a mature, TCRα-deficient T cell line. Immunoprecipitations of TCRβ from SCB.29, SCI.ET.27F (the nontransfected SCID cell line), the pTα transfectants of 58α$^-$β.1 (a TCRα-mature T cell line) (*43*) named 58α$^-$β.1-33 and represented by clones 7, 9, and 10, and 58α$^-$β.1 cells were separated under nonreducing (NR) and reducing (R) conditions and immunoblotted with the CP33 pTα antiserum.

rearrangements in CD44$^-$CD25$^-$ (*28*) and total thymocytes (*29*) from TCR$\alpha^{-/-}$ mice were much higher than expected if cells of this phenotype could similarly expand, irrespective of whether or not they carried productive TCRβ genes. Thus the pre-TCR complex may regulate survival and expansion of immature T cells rather than regulating directly CD4 and CD8 gene expression in the lineage of $\alpha\beta$ T cells.

The role of the pre-TCR complex in T cell development is supported by other studies. In SCID (*30*) as well as in TCRβ- or RAG-deficient (*31*, *32*) mice, the effects of productive TCRβ transgenes on thymocyte development can be mimicked by treating thymocytes of these mice with antibodies to CD3ε. These results support the hypothesis that the TCRβ protein exerts its function through a pre-TCR complex that associates with CD3 proteins. It is not clear from these studies whether signaling through CD3 is all that is required or whether the cytoplasmic part of the pTα chain plays an essential role. A potential role of the cytoplasmic tail of pTα needs to be analyzed under more physiological conditions.

A role of p56^{lck} in the signaling by the pre-TCR is implicated by studies in mice that are Lck-defective (*33*) or carry a dominant negative Lck mutation (*34*). The Lck dominant negative mutation results in a developmental arrest that resembles that of rearrangement-deficient mice. On the other hand the introduction of an active *lck* gene in rearrangement-deficient mice (*35*) or in normal mice (*36*) causes the same effects as a TCRβ transgene. Thus p56^{lck} may be part of the signaling cascade of the pre-TCR complex. Because the cytoplasmic tail of the pTα chain contains a proline-rich, potential SH3-binding domain (*37*), p56^{lck} may be recruited by the pTα protein into the pre-TCR complex.

The cloning and expression analysis of the pTα gene has established that the pre-TCR is expressed in immature thymocytes and opens the way to determine the precise structure and function of the pre-TCR complex in T cell development.

REFERENCES AND NOTES

1. D. H. Raulet, R. D. Garman, H. Saito, S. Tonegawa, *Nature* **314**, 101 (1985).
2. R. H. Snodgrass, Z. Dembic, M. Steinmetz, H. von Boehmer, *ibid.* **315**, 232 (1985).
3. M. Bosma and A. M. Carrol, *Annu. Rev. Immunol.* **9**, 323 (1991).
4. Y. Shinkai, *Cell* **68**, 855 (1992).
5. P. Mombaerts *et al.*, *ibid.*, p. 869.
6. H. von Boehmer, *Annu. Rev. Immunol.* **8**, 531 (1990).
7. H. Kishi *et al.*, *EMBO J.* **10**, 93 (1991).
8. M. Groettrup, A. Baron, G. Griffiths, R. Palacios, H. von Boehmer, *ibid.* **11**, 2735 (1992).
9. Y. Uematsu *et al.*, *Cell* **52**, 831 (1988).
10. R. G. Fenton, P. Marrack, J. W. Kappler, O. Kanagawa, J. G. Seidman, *Science* **241**, 1089 (1988).
11. H. von Boehmer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9729 (1988).
12. M. Groettrup *et al.*, *Cell* **75**, 283 (1993).
13. M. Groettrup and H. von Boehmer, *Eur. J. Immunol.* **23**, 1393 (1993).
14. H. von Boehmer, *Cell* **76**, 219 (1994).
15. M. Groettrup and H. von Boehmer, *Immunol. Today* **14**, 6101 (1993).
16. U. D. Staerz, H.-G. Rammensee, J. D. Benedetto, M. J. Bevan, *J. Immunol.* **134**, 3994 (1985).
17. R. T. Kubo, W. Born, J. Kappler, P. Marrack, M. J. Pigeon, *ibid.* **142**, 2736 (1989).
18. P. Cosson, S. P. Lankford, J. S. Bonifacio, R. D. Klausner, *Nature* **351**, 414 (1991).
19. H. C. Chang, *J. Exp. Med.* **172**, 351 (1990).
20. H. Karasuyama, A. Kudo, F. Melchers, *ibid.*, p. 969.
21. Y. Shinkai *et al.*, *Science* **259**, 822 (1993).
22. M. Groettrup, thesis, University of Basel, Switzerland (1993).
23. A. Kudo and F. Melchers, *EMBO J.* **6**, 2267 (1987).
24. P. Mombaerts *et al.*, *Nature* **360**, 225 (1992).
25. E. W. Shores, S. O. Sharrow, A. Singer, *Eur. J. Immunol.* **21**, 973 (1991).
26. F. Lynch and E. M. Shevach, *Int. Immunol.* **5**, 991 (1993).
27. H. R. Rodewald, unpublished data.
28. E. C. Dudley, H. T. Petrie, L. M. Shah, M. J. Owen, A. C. Hayday, *Immunity* **1**, 83 (1994).
29. C. A. Mallick, E. C. Dudley, J. L. Viney, M. J. Owen, A. C. Hayday, *Cell* **73**, 513 (1993).
30. C. N. Levelt, A. Ehrfeld, K. Eichmann, *J. Exp. Med.* **177**, 707 (1993).
31. C. N. Levelt, P. Mombaerts, A. Iglesias, S. Tonegawa, K. Eichmann, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11401 (1993).
32. H. Jacobs *et al.*, *Eur. J. Immunol.* **24**, 934 (1994).
33. T. J. Molina *et al.*, *Nature* **357**, 161 (1992).
34. S. D. Levin, S. J. Anderson, K. A. Forbush, R. M. Perlmutter, *EMBO J.* **12**, 1671 (1993).
35. P. Mombaerts, S. J. Anderson, R. M. Perlmutter, T. W. Mak, S. Tonegawa, *Immunity* **1**, 261 (1994).
36. S. J. Anderson, K. M. Abraham, T. Nakayama, A. Singer, R. M. Perlmutter, *EMBO J.* **11**, 4877 (1992).
37. H. Yu and L. S. Schreiber, *Structural Biol.* **1**, 417 (1994).
38. Rabbit 53 was immunized with a peptide corresponding to the NH_2-terminal residues of the pTα shown in Fig. 3. (The peptide contains an Ala instead of a Ser in position 12.) Either SCB.29 or B6.2.16BW cells were lysed in Triton lysis buffer and the lysate of 10^7 cells was immunoprecipitated with mAb F23.1 (*16*) coupled to agarose beads. The precipitates were separated by SDS–10 percent polyacrylamide gel electrophoresis (PAGE), first under nonreducing (NR) and then under reducing (R) conditions. After equilibration in transfer buffer, the proteins were transferred by wet blotting onto polyvinyliden difluoride membranes. Filters were incubated with either the preimmune serum (PI53, at a dilution of 1:100), the immune serum (S53, at 1:100) or the affinity column–purified (CP53, at 1:100) in phosphate-buffered saline containing 0.4 percent Tween and 2 percent fetal calf serum. For peptide competition, peptide (35 μg/ml) was first incubated with S53 serum or mAb F23.1 for 1 hour. For detection of TCRβ protein a F23.1-biotin conjugate was used. After the first-stage reagents were removed, GantiR-HRPO at 1:20,000 or streptavidine-HRPO (1:5000) was added for visualization of the rabbit and F23.1 antibodies, respectively.
39. W. M. R. Pearson and D. J. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444 (1988).
40. D. F. Feng and R. S. Doolittle, *J. Mol. Evol.* **35**, 359 (1987).
41. Total RNA was prepared from cell suspensions (thymocytes from C57BL/6 mice and RAG-2$^{-/-}$ mice, and lymph node cells from C57BL/6 mice) and from cell lines (SCB.29, SCI.ET.27F and B6.216.BW cells) with RNAzol B (TM Cinna Scientific, Friedswood, TX). The mRNA was prepared from total RNA with the use of the Oligotex TM mRNA Kit (QIAGEN). Both total RNA and mRNA were separated on 1.2 percent agarose–formaldehyde gels and transferred to GeneScreen membranes according to the manufacturer's recommendations (Du Pont). Filters were first hybridized at 65°C for 8 hours and hybridized with the same solution [1 mM EDTA, 0.5 M NaH_2PO_4, 7 percent SDS, and salmon sperm DNA (100 μg/ml)] containing the specific probe at 65°C overnight. To remove any unspecifically bound probe, the membranes were washed first with 1 mM EDTA, 40 mM NaH_2PO_4, 1 percent SDS at 65°C. Filters were hybridized first with a cDNA probe corresponding to the entire coding sequence of pTα and subsequently, after dishybridization, with a probe specific for β-actin message. Washed membranes were exposed to Kodak X-OMAT AR film at −70°C for 1 to 5 days.
42. CD44$^+$CD25$^-$, CD44$^+$CD25$^+$, and CD44$^-$CD25$^+$ double-negative (CD4$^-$CD8$^-$) thymocytes were obtained from RAG-2$^{-/-}$ mice. CD44$^-$CD25$^-$ thymocytes were obtained from C57BL/6 mice by complement-dependent killing of thymocytes with antibodies to CD4 and CD8. The thymocytes were then stained with antibodies specific for CD4, CD8, CD44, CD25, and CD3 surface markers to obtain the desired populations by cell sorting. The purity of the subsets was deduced from reanalysis of sorted cells. In order to quantitate pTα RNA, cells (5×10^4) were directly sorted in 500 μl of RNAzol (TM Cinna Scientific), and total RNA was extracted according to the manufacturer's protocol. The cDNA was prepared with random hexamer primers and reverse-transcribed with Superscript kit (Gibco BRL). Dilutions (1:3) of cDNA in water were then used in PCR amplification reactions. Primers used were oligonucleotides recognizing sequences in the 5′ and 3′ regions of the pTα (5′-CTGCAACTGGGTCAT-GCTTC-3′ and 5′ TCAGACGGGTGGGTAAGATC-3′) and HRPT (5′-CACAGGACTAGAACACCTGC-3′ and 5′-GCTGGTGAAAAGGACCTCT-3′) genes. Amplification was done for 35 cycles at an annealing temperature of 55°C with a thermal cycling machine (Perkin-Elmer Cetus). A 15-μl portion of each amplified product was separated through a 1.2% agarose gel by electrophoresis and stained with ethidium bromide.
43. The 58$\alpha^-\beta$y.1 T cell line was obtained by transfection of the C58 TCRα^-TCRβ^- T cell line with a TCRβ gene (*8*). This cell line was supertransfected with a pTα construct. To this end, full-length cDNA of pTα subcloned in Bluescript SK$^+$ (clone D2.1) was amplified with primers 5′-TAGCCTCGAGCTGCAACTGGGT-CATGCTTC-3′ and 5′-CAGAGAATTCTCAGACG-GGTGGGTAAGATC-3′ that anneal in the 5′ and 3′ untranslated region of pTα just upstream of the ATG start and downstream of the TGA stop codon, respectively, and introduced specific artificial cloning sites: Xho I in the former case, Eco RI in the latter. The resulting PCR fragment of about 660 bp was purified, digested with Xho I and Eco RI, and subcloned into the corresponding polylinker restriction sites of Bluescript SK$^+$. The pTα sequence between the Nco I site at the second in-frame ATG and the one Bgl II site downstream of the stop codons was replaced by the corresponding sequence from D2.1 in order to eliminate potential mutations introduced during the PCR amplification. The resulting plasmid was digested with Xho I and Not I to release the cDNA fragment encoding pTα, which was then inserted in the corresponding sites of expression vector BCMGSHyg (*20*). The construct was transfected into 58$\alpha^-\beta$y.1 cells by electroporation.
44. We thank D. Avila, for microsequencing of the pTα protein; J. Kaufman, M. Reth, and J. Cébrian for help with the analysis of the pTα sequences; C. Benoist, D. Mathis, and M. Wiles for advice on pTα cloning; J. Garcia-Sanz for advice on the RNA analysis; S. Goude, V. Stauffer, and C. Geiger for secretarial help; and K. Hafen and C. Laplace for expert technical assistance. The Basel Institute for Immunology is supported by Hoffmann–La Roche, Basel. Supported in part by the Institut National de la Santé and Recherche Medicale, Paris, and by the Faculté Necker Enfants Malades, Descartes Université, Paris. H.v.B. is affiliated with the Faculté Necker, Paris, and also supported by the Human Frontier Science Project Organization (HFSPO).

9 September 1994; accepted 18 October 1994

15.
16.
17.

18.
19.
20.
21.
22.
23.
24.
25.
26.
27.
28.

29.

ACP
gen
L. C

Crucial role of the pre-T-cell receptor α gene in development of αβ but not γδ T cells

Hans Jörg Fehling*, Anna Krotkova*, Claude Saint-Ruf† & Harald von Boehmer*

* Basel Institute for Immunology, CH-4005 Basel, Switzerland
† Institut Necker, Unité INSERM 373, 156 rue de Vaugirard, 75730 Paris Cedex 15, France

In T-cell precursors, the T-cell-receptor β chain is expressed before the T-cell-receptor α chain[1,2] and is sufficient to advance T-cell development in the absence of T-cell receptor α chains[3–7]. In immature T cells, the T-cell-receptor β protein can form disulphide-linked heterodimers with the pre-T-cell-receptor α chain[8,9] and associate with signal-transducing CD3 molecules[5]. The recently cloned pre-T-cell-receptor α gene encodes a transmembrane protein that is expressed in immature but not mature T cells[9,10]. Here we show that αβ, but not γδ, cell development is severely hampered in pre-T-cell-receptor α-gene-deficient mice, which establishes a crucial role for the pre-T-cell receptor in early thymocyte development.

Intrathymic T-cell development proceeds from $CD4^-8^-$ precursors through $CD4^+8^+$ intermediates into $CD4^+8^-$ and $CD4^-8^+$ mature thymocytes[3,11]. In rearrangement-deficient mice, thymocyte development is arrested at the $CD4^-8^-3^{low}25^+$ stage[6,7,12]. Productive T-cell-receptor (TCR)-β transgenes can partly relieve the developmental block, allowing the accumulation of immature $CD4^+8^+$ thymocytes but not mature $CD4^+8^-$ and $CD4^-8^+$ T cells[3–7] that require positive selection by TCRαβ (ref. 13). In T-cell precursors the TCR-β chain forms disulphide-linked heterodimers with the pre-TCRα (pTα) chain, and can associate with signal-transducing CD3 molecules[5,8,9].

Here we report on the role of the TCRβ–pTα heterodimer in development, based on experiments with pTα-deficient mice. These animals were generated by gene targeting in embryonic stem (ES) cells using a deletion-type targeting vector. On homologous recombination, this construct eliminated exons 3 and 4 of the pTα gene encoding the connecting peptide, which contains the cysteine required for heterodimer formation, the transmembrane region, the cytoplasmic tail and most of the 3′ untranslated region (Fig. 1). Homologous recombination in ES cells and the absence of the deleted gene segment in $pT\alpha^{-/-}$ mice was verified by Southern blotting with appropriate probes (Fig. 1, and results not shown). Offspring from intercrosses of $pT\alpha^{+/-}$

TABLE 1 Absolute number of thymocytes with different phenotypes

Phenotypes	$pT\alpha^+$ ($\times 10^{-6}$)	$pT\alpha^{-/-}$ ($\times 10^{-6}$)
$CD4^-8^-3^{low}25^+$	25.6	54.0
	22.0	40.4
$CD4^-8^-\delta^+$	12.4	55.1
	14.6	32.0
$CD4^+8^+\delta^+$	10.0	6.5
	14.5	11.0
$CD4^+8^+$	34.54	1.19
	34.62	1.13
$CD4^+8^+TCR\beta^{int.}$	14.50	0.24
	13.75	0.23
$CD4^+8^-TCR\beta^{high}$	3.51	0.13
	3.05	0.13
$CD4^-8^+TCR\beta^{high}$	0.81	0.09
	1.39	0.13

Numbers were obtained from two different mice of each genotype from a 5-day-old litter.

TABLE 2 Subsets among $CD4^-8^-3^{low}$ thymocytes

Phenotypes	$pT\alpha^+$ (%)	$pT\alpha^{-/-}$ (%)
$CD44^+25^-$	12.1	11.0
$CD44^+25^+$	2.20	2.30
$CD44^-25^+$	40.7	86.6
$CD44^-25^-$	45.0	0.10

Percentages were calculated from the same litter as described in Fig. 2 and Table 1.

mice were killed, thymus and bone marrow removed, and single cell suspensions prepared and analysed by cytofluorometry. There were no significant differences between numbers of marrow cells from age-matched mice and thymocytes from $pT\alpha^{+/+}$ and $pT\alpha^{+/-}$ age-matched mice, whereas the number of thymocytes in pTα-deficient animals was reduced to less than 10%.

Figure 2 shows subsets from thymus of $pT\alpha^+$ and $pT\alpha^{-/-}$ mice only, because there was no difference in subsets of bone marrow and because lymphoid organs from $pT\alpha^{+/-}$ and $pT\alpha^{+/+}$ mice did not differ. Both $pT\alpha^+$ and $pT\alpha^{-/-}$ mice contain $CD4^-8^-$, $CD4^+8^+$ and single-positive $CD4^+8^-$ and $CD4^-8^+$ thymocytes, but cells with CD4 and CD8 co-receptors are proportionally under-represented in $pT\alpha^{-/-}$ mice whereas the proportion of $CD4^-8^-25^+$ cells is drastically increased (Fig. 2). Both types of mice contain CD4 and CD8 co-receptor expressing cells with low, intermediate and high levels of TCR-β chain on the cell surface. The fraction of $CD4^-8^-$ thymocytes with TCRδ chains on the cell surface was more prominent in $pT\alpha^{-/-}$ mice, and both TCRβ and TCRδ chains were stoichiometrically associated with CD3 molecules. The data also show that the TCRβ chains were associated with TCRα chains, as revealed by double-staining with TCRβ and TCR Vα2, 3.2, 8 and 11 antibodies (Fig. 2, lower right). From triple stainings with various antibodies listed in Fig. 2 and thymocyte numbers, we calculated the absolute number of cells belonging to various thymocyte subsets as shown in Table 1; the data show that the pre-TCR is not required to generate normal numbers of $CD4^-8^-3^{low}25^+$ precursors of αβ T cells. In fact, their number is increased in $pT\alpha^{-/-}$ mice, probably because of the developmental arrest in these mice (see below). There is likewise no decrease in the number of $CD4^-8^-$ or CD4 and CD8 co-receptor expressing γδ T cells (Table 1). Table 2 shows that among $CD4^-8^-3^{low}$ cells, $pT\alpha^{-/-}$ mice have normal or increased numbers of $CD44^+25^-$, $CD44^+25^+$ and $CD44^-25^+$ cells, but no $CD44^-25^-$ cells, which represent 45% in $pT\alpha^+$ mice. As most thymocyte expansion occurs in this subset[11], it is not surprising that the absolute number of $CD4^+8^+$ cells as well as mature TCR $\alpha\beta^{high}$ $CD4^+8^-$ and $CD4^-8^+$ cells is much lower in $pT\alpha^{-/-}$ than in $pT\alpha^+$ mice. Also, the proportion of TCR-αβ-positive cells among $CD4^+8^+$ cells is lower in $pT\alpha^{-/-}$ than in $pT\alpha^+$ mice. Lymph-node cells from 28-day-old $pT\alpha^{-/-}$ mice contain mature single positive CD4 and CD8 cells representing about 5% of numbers found in $pT\alpha^+$ littermates (results not shown).

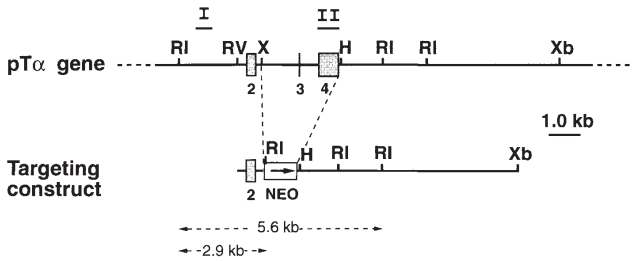


FIG. 1 Disruption of the pTα gene by homologous recombination: partial organization of the pTα locus[22] and structure of the targeting vector. The pTα gene was cloned from a 129/Ola-derived genomic library (kind gift of A. Berns, Amsterdam). The targeting vector was constructed by replacing approximately 2.6 kilobases (kb) of the pTα sequence between the unique *Xho*I site in intron 2 and a *Bgl*II site ~60 base pairs (bp) upstream of the AATAAATAAA polyadenylation site with a 1.2-kb *Xho*I–*Bam*HI fragment of pMC1neopA (Stratagene) carrying the neomycin resistance gene (*neo*). The isogenic targeting construct was electroporated into E14.1 embryonic stem cells as described[18]. ES colonies surviving G418 selection were analysed by the polymerase chain reaction (PCR) in pools of 12 using primers specific for the *neo* cassette within the *tk* promotor (ATTCGCCAATGACAAGACGCTGC) and for the pTα gene just upstream of the *Eco*RV site (GTTGGATGTTATTGGTTACTACTCCTGA), respectively. Colonies within positive pools were re-screened individually by PCR and eventually by Southern analysis using *Eco*RI-digested DNA and probe I (a 470-bp PCR fragment specific for pTα sequences outside the targeting construct, ~1.3 kb upstream of exon 2 (primers: TAGGTTTGAACTCAGAT; TGATTTCTCTCTGTAGC)). Out of ~1,600 colonies screened, 3 had undergone homologous recombination, and one of these clones (pT355) gave rise to chimaeric mice. Chimaeric males were backcrossed with $(C57BL/6 \times DBA/2)F_1$ females and heterozygous offspring carrying a mutant pTα allele were intercrossed to obtain mice deficient in pTα. The absence of the deleted pTα sequences in homozygous knockout mice was confirmed by Southern blotting of *Eco*RI-digested genomic tail DNA and hybridization with a 310-bp *Apa*I/*Bsp*EI complementary DNA fragment spanning the region of exon 4 that encodes the transmembrane portion, the cytoplasmic tail and 120 nucleotides of 3′ untranslated sequence. Abbreviations for restriction sites: RI, *Eco*RI; RV, *Eco*RV; X, *Xho*I; H, *Hin*dIII; Xb, *Xba*I.

The above data indicate that pTα has no role in the development of most γδ T cells for one of three reasons: (1) because it is not expressed in the γδ lineage; (2) it cannot pair with the γ chain; or (3) because there is no need for a putative TCRγ–pTα heterodimer in γδ T-cell development. Although this issue requires further investigation, we have been unable to detect RNA for pTα in thymocytes of TCRδ surface-positive thymocytes[10]. The increase in the number of γδ cells could depend on the availability of space, and/or the possibility that ongoing γδ rearrangement is not terminated by the pre-TCR. The fact that a few TCRαβ-positive CD4/8-co-receptor-expressing thymocytes can be generated in the absence of the pre-TCR is consistent with an earlier finding of TCRα rearrangements in $TCR\beta^{-/-}$ mice deficient in pre-T-cell-receptors[6]. The authors argued that $CD4^+,8^+$ cells in $TCR\beta^{-/-}$ mice (which are often claimed erroneously not to contain $CD4^+8^+$ cells) could be of the δ lineage because they were absent in $TCR\beta^{-/-} \times TCR\delta^{-/-}$ mice. We find that in $pT\alpha^{-/-}$ mice $CD4^-8^-3^{low}25^+$ precursors of αβ T cells can differentiate, albeit inefficiently, into $CD4^+8^+$ cells with TCRαβ on the surface. We therefore propose that the pre-TCR is sufficient and necessary for the generation of $CD4^-8^+$ precursors when no other TCR-expressing cells are present, and that in the presence of TCR-positive cells, the pre-TCR is required for the transition of $CD4^+8^+25^+$ αβ T-cell precursors, through rapidly dividing $CD4^-8^-25^-$ cells into TCRαβ-expressing $CD4^+8^+$ thymocytes.

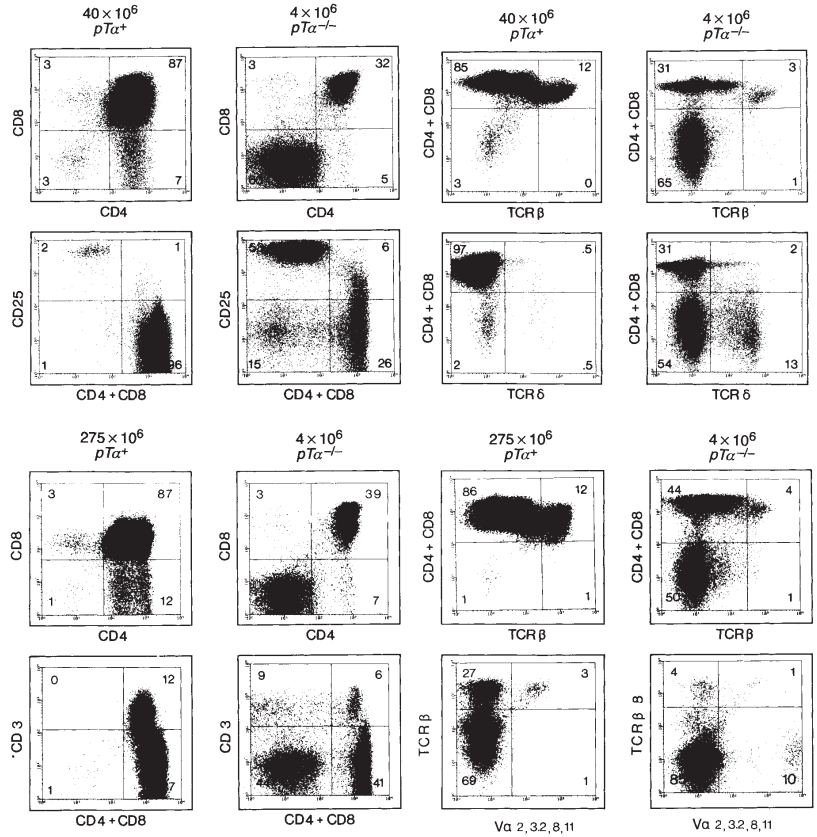


FIG. 2 Thymocyte subsets from $pT\alpha^+$ and $pT\alpha^{-/-}$ mice. Staining of thymocytes from 5-day-old (top two rows) and 28-day-old (bottom two rows) litters with CD4, CD8, TCRβ, TCRδ and TCR-V_α antibodies. All stainings were three-colour stainings with the following antibody-conjugates: anti-CD3–biotin and anti-CD3–FITC conjugates (500 A2 (ref. 19)), anti-TCRβ–biotin and anti-TCRβ–FITC (H57 (ref. 20)), anti-CD4–phycoerythrin (H129.19 Gibco), anti-CD8–biotin and anti-CD8–phycoerythrin (53-6.7 Pharmingen, San Diego), Anti-CD25–biotin (Pharmingen, San Diego), anti-CD44–phycoerythrin (ATCC collection KM81), anti-HSA–biotin (M1169 (ref. 21)), and anti-TCRδ–FITC (G3, Pharmingen, San Diego). A cocktail of TCR-V_α antibodies, namely anti-V_α2 (B20.1),anti-V_α3.2 (RR3.16), anti-V_α8 (KT50) and anti-V_α11 (RR8.1) was obtained from D. Mathis, Strasbourg. The biotin conjugates were revealed by streptavidin–Tricolor, (Caltag, San Francisco). For each of the three colour stainings ~4×10^5 cells were incubated with various reagents. In each case, 10^5 events were acquired for fluorescence-activated cell sorting by FACScan. The bright staining in the thymus (lower right) is due to thymic B cells that stain brightly with sheep anti-mouse Ig–FITC reagent, which was used to reveal the V_α antibodies.

This view is consistent with data showing that TCR-positive thymocytes can induce the development of $CD4^+8^+$ cells when injected into rearrangement-deficient mice[14,15] and explains the absence of $CD4^+8^+$ thymocytes in rearrangement-deficient[6,7], TCR-negative[6] as well as CD3-negative mice (B. Malissen, personal communication). Differentiation through the $CD4^-8^-25^-$ subset requires cell-autonomous signals delivered by the pre-TCR, and for that reason in normal mice almost all $CD4^+8^+$ T cells contain productive TCR-β genes[16], whereas in the absence of the pre-TCR, only ~2% of TCRαβ-positive $CD4^+8^+$ precursors are generated by some aberrant differentiation. Nevertheless, positive selection will operate on these cells and generate mature αβ T cells whose number is regulated by homeostasis independent of the pre-TCR[17]. □

Received 12 May; accepted 8 June 1995.

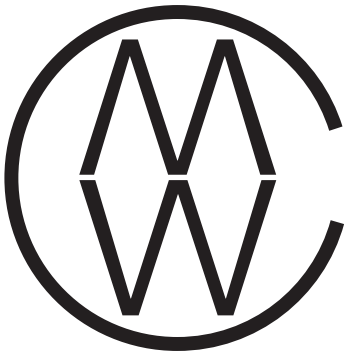
1. Snodgrass, H. R., Dembic, Z., Steinmetz, M. & von Boehmer, H. *Nature* **315,** 232–233 (1985).
2. Raulet, D. H., Garman, R. D., Saito, H. & Tonegawa, S. *Nature* **314,** 103–107 (1985).
3. von Boehmer, H. *A. Rev. Immun.* **8,** 531–556 (1990).
4. Kishi, H. *et al. Embo J.* **10,** 93–100 (1991).
5. Groettrup, M., Baron, A., Griffiths, G., Palacios, R. & von Boehmer, H. *Embo J.* **11,** 2735–2745 (1992).
6. Mombaerts, P. *et al. Nature* **360,** 225–231 (1992).
7. Shinkai, Y. *et al. Science* **259,** 822–825 (1993).
8. Groettrup, M. *et al. Cell* **75,** 283–294 (1993).
9. Saint-Ruf, C. *et al. Science* **266,** 1208–1212 (1994).
10. Bruno, L. E. A., Rocha, B., Rolink, T., von Boehmer, H. & Rodewald, H. R. *Eur. J. Immun.* (in the press).
11. Kisielow, P. & von Boehmer, H. *Adv. Immun.* **58,** 87–209 (1995).
12. Rothenberg, E. V. *Adv. Immun.* **51,** 85–214 (1992).
13. Scott, B., Blüthmann, H., Teh, H. S. & von Boehmer, H. *Nature* **338,** 591–593 (1989).
14. Shores, E. W., Sharrow, S. O., Uppenkamp, I. & Singer, A. *Eur. J. Immun.* **20,** 69–77 (1990).
15. Lynch, F. & Shevach, E. M. *Int. Immun.* **5,** 991–995 (1993).
16. Dudley, E. C., Petrie, H. T., Shah, L. M., Owen, M. J. & Hayday, A. C. *Immunity* **1,** 83–93 (1994).
17. von Boehmer, H. & Hafen, K. *J. exp. Med.* **177,** 891–896 (1993).
18. Kuhn, R., Rajewsky, K. & Muller, W. *Science* **254,** 707–710 (1991).
19. Allison, J. P. in *UCLA Symposia on Molecular and Cellular Biology, New Series* (eds Kappler, J. & Davies, M.) (Lissabon, New York, 1987).
20. Kubo, R. T., Born, W., Kappler, J. W., Marrack, P. & Pigeon, M. *J. Immun.* **142,** 2736–2742 (1989).
21. Springer, T., Galfre, G., Secher, D. S. & Milstein, C. *Eur. J. Immun.* **8,** 539–551 (1978).
22. Fehling, H. J., Laplace, C., Mattei, M.-G., Saint-Ruf, C. & von Boehmer, H. *Immunogenetics* (in the press).

ACKNOWLEDGEMENTS. We thank U. Müller for injection and implantation of blastocysts; C. Laplace and K. Hafen for technical assistance and C. Geiger for typing; D. Mathis and C. Benoist (Strasbourg, France) for the gift of TCR-V_α antibodies, and K. Karjaleinen (Basel, Switzerland) for critical reading of the manuscript. The Basel Institute for Immunology is supported by F. Hoffmann–La Roche Ltd, Basel. The work was also supported by INSERM through the Unité INSERM 373. H.V.B. is supported by the Human Frontier Science Program Organization.

Speaker Schedule

(alphabetical order)

Burkhard Becher	Monday, 22 January	11:30
Thomas Boehm	Saturday, 21 January	18.15
Chunaram Choudhary	Sunday, 22 January	14.00
Adrian Erlebacher	Tuesday, 23 January	11.00
Donna Farber	Tuesday, 23 January	17.00
Ananda Goldrath	Monday, 22 January	17.00
Muzlifah Haniffa	Wednesday, 24 January	09.00
Matteo Iannacone	Wednesday, 24 January	14.00
Nicole Joller	Wednesday, 24 January	14.30
Susan Kaech	Wednesday, 24 January	11.00
Axel Kallies	Wednesday, 24 January	11.30
Taras Kreslavskyi	Monday, 22 January	09.00
Maria Mittelbrunn	Tuesday, 23 January	17.30
Marion Pepper	Wednesday, 24 January	16.00
Caetano Reis e Sousa	Tuesday, 23 January	09.00
Ellen Robey	Sunday, 21 January	09.00
Chiara Romagnani	Monday, 22 January	09.30
Romain Roncagalli	Sunday, 21 January	16.00
Alexander Scheffold	Wednesday, 24 January	09.30
Michael Sixt	Tuesday, 23 January	09.30
Georg Stary	Wednesday, 24 January	10.00
Martin Turner	Sunday, 21 January	14.30
Gabriel Victora	Sunday, 21 January	11.15
Carola Vinuesa	Sunday, 21 January	10.45



Imprint

Verein zur Förderung der Immunbiologie e.V.
Association for the Advancement of Immunobiology (AAIB)
c/o Dr. L. Klein
Petra-Kelly-Strasse 26
80797 München