HARALD VON BOEHMER

MIDWINTER CONFERENCE

Advances in Immunobiology

CONFERENCE PROGRAM

HARALD VON BOEHMER
MIDWINTER CONFERENCE

Seefeld in Tirol, Austria
January 19 - 23, 2019

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About the MWC

Dear friends and colleagues,

We are pleased to welcome you to the 3rd Harald von Boehmer Midwinter Conference: Advances in Immunobiology (MWC). This conference was initiated in 2014, when Harald von Boehmer and Ludger Klein 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 and other scientific health disciplines 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 to honor his fundamental contributions to the field of immunology, we have renamed the congress the "Harald von Boehmer Midwinter Conference".

The Harald von Boehmer MWC 2019 will feature 26 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". We are very pleased to note that the interest in the MWC continues to grow – the number of participants has reached a new record in 2019!

We sincerely hope you will enjoy your time in Seefeld,

Ludger Klein

Wilfried Ellmeier

Lisa von Boehmer

Conference Speakers

Burkhard Becher
University of Zurich

Julie Magarian Blander
Cornell University

Mark Davis
Stanford University

Andreas Diefenbach
Charité Berlin

Wilfried Ellmeier
Medical University of Vienna

Reinhold Förster
Hannover Medical School

Gillian Griffiths
University of Cambridge

Kris Hogquist
University of Minnesota
Minneapolis

Veit Hornung
Ludwig-Maximilians-Universität
Munich

Matteo Iannacone
San Raffaele Scientific Institute Milan

Stephen Jameson
University of Minnesota
Minneapolis

Axel Kallies
The Walter and Eliza Hall Institute of
Medical Research Melbourne

Wolfgang Kastenmüller
University of Wuerzburg

Adrian Liston
Katholieke Universiteit Leuven

Conference Speakers

Dan Littman
New York University School of Medicine

Michael McHeyzer-Williams
Scripps Research San Diego

Fiona Powrie
Oxford University

Immo Prinz
Hannover Medical School

Klaus Rajewsky
Max Delbrück Center for Molecular Medicine Berlin

Ellen Robey
University of California, Berkeley

Hans-Reimer Rodewald
German Cancer Research Center (DKFZ) Heidelberg

Chiara Romagnani
German Rheumatism Research Centre Berlin (DRFZ) Berlin

Sasha Rudensky
Memorial Sloan Kettering Cancer Center New York

Andreas Schlitzer
University of Bonn

Shannon Turley
Genentech San Francisco

Gabriel Victora
Rockefeller University New York

Saturday, January 19, 2019

KEYNOTE

18.00	Mark Davis	**Tools for T Cells: Specificity and repertoire in infectious disease, autoimmunity and cancer**
19.00	**Welcome Reception**	

Sunday, January 20, 2019

IMMUNE CELL DEVELOPMENT

09.00	Hans-Reimer Rodewald	**Hematopoietic Deconvolution by Fate Mapping and Endogenous Barcoding**
09.30	Kris Hogquist	**Effector Cytokine Production in the Thymus**
10.15	Selected short talk 1	
10.15	Selected short talk 2	
10.30	**Coffee Break**	
11.00	Ellen Robey	**T Cell Fate in the Thymus**
11.30	Burkhard Becher	**The Pathogenic Signature of T Helper Cells in Tissue-Inflammation**
12.00	Selected short talk 3	
12.15	Selected short talk 4	
12.30	**Lunch Break**	

INNATE IMMUNITY

14.00	Shannon Turley	**Stromal Activation in Cancer Immunology and Immunotherapy**
14.30	Andreas Schlitzer	**Understanding Human Innate Immune Memory – From Mechanism to Function**
15.00	Selected short talk 5	
15.15	Selected short talk 6	
15.30	**Coffee Break**	
16.00	Julie Magarian Blander	**Non-canonical Pathway of Cross-presentation**
16.30	Dan Littman	**Role of Serum Amyloid A in Th17 Cell-Mediated Inflammatory Disease**
17.00	Veit Hornung	**Inflammasome Signaling in the Human System**

Monday, January 21, 2019

ILCs and INNATE-LIKE T CELLS

09.00	Andreas Diefenbach	**Innate Lymphoid Cells and Genotoxic Stress**
09.30	Chiara Romagnani	**Activation of Innate Lymphoid Cells**
10.00	Selected short talk 7	
10.15	Selected short talk 8	
10.30	**Coffee Break**	
11.00	Immo Prinz	**Innate and Adaptive Functions of γδ T Cells**
11.30	Selected short talk 9	
11.45	Selected short talk 10	
12.00	**Lunch Break**	

T CELL FUNCTION IN HOMEOSTASIS AND DISEASE I

17.00	Alexander Rudensky	**Cues Promoting Regulatory T Cell Differentiation**
17.30	Selected short talk 11	
17.45	Selected short talk 12	
18.00	**Poster Session – Fingerfood will be served**	

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Tuesday, January 22, 2019

OF B CELLS AND THEIR CROSSTALK TO T CELLS

09.00 Michael McHeyzer-Williams **Regulating B cell Immunity to Non-Self**

09.30 Gabriel Victora **Clonal and Cellular Dynamics in Germinal Centers**

10.00 Selected short talk 13
10.15 Selected short talk 14

10.30 Coffee Break

T CELL FUNCTION IN HOMEOSTASIS AND DISEASE II

11.00 Gillian Griffiths **Fine Tuning the CTL Response: From Genes to Membranes**

11.30 Selected short talk 15
11.45 Selected short talk 16

12.00 Lunch Break

KEYNOTE

17.00 Fiona Powrie **Host Microbe Interactions in the Intestine in Health and Disease**

17.45 Selected short talk 17

18.00 Poster Session – Fingerfood will be served

Poster prizes sponsored by:

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Wednesday, January 23, 2019

IMAGING APPROACHES TO STUDY IMMUNE CELL FUNCTION

09.00	Reinhold Förster	**Lymph node homing of lymph-derived immune cells**
09.30	Wolfgang Kastenmüller	**Concepts of T Cell Activation from a Spatio-temporal Perspective**
10.00	Selected short talk 18	
10.15	Selected short talk 19	
10.30	**Coffee Break**	

T CELL FUNCTION IN HOMEOSTASIS AND DISEASE III

11.00	Matteo Iannacone	**In Vivo Imaging of CD8+ T Cell Responses within the Liver**
11.30	Adrian Liston	**Brain T Cells are essential for Neurological Plasticity**
12.00	Selected short talk 20	
12.15	Wilfried Ellmeier	**HDACs and the Control of CD4+ T Cell-Mediated Immunity**
12.45	**Lunch Break**	
14.00	Klaus Rajewsky	**Therapeutic Gene Editing in Inherited Lympho-proliferative Disease**
14.30	Stephen Jameson	**The Danger Signal Extracellular ATP Promotes CD8+ T Cell Memory**
15.00	Selected short talk 21	
15.15	Selected short talk 22	
15.30	**Coffee Break**	

Wednesday, January 23, 2019

16.00	Selected short talk 23	
16.15	Selected short talk 24	
16.30	Selected short talk 25	
16.45	Selected short talk 26	
17.00	Axel Kallies	**Molecular Regulation of Tissue Regulatory T Cell Differentiation**
19.00	**Farewell Dinner**	

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New insights into Treg biology through deconstructive SCNT

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Using epigenetic reprogramming through Somatic Cell Nuclear Transfer-SCNT, we developed two novel Treg models on pure NOD background, T138 and T143. While both Treg models were derived in the thymus, only T143 displayed characteristics of agonist-selection -aTreg-, such as higher Nur77-levels. T138 showed no signs of agonist-selection, hence we refer to T138 as natural-occurring Treg cell -nTreg-. In addition to agonist-selection, thymic development of aTreg was quite distinct from nTreg. nTreg displayed a stronger interaction between its TCRbeta-chain and MHC-II, causing a bias in T cell development towards CD4, whereas aTreg displayed a CD4low-CD8positive phenotype in the double-positive population. Thus, our TCRbeta-initiated mechanism in Treg development would unify former per se contradicting hypotheses of TCR-dependent and -independent Treg development.
Strikingly, we identified FoxP3- T cells expressing the same TCR as nTreg and aTreg that were heavily biased in their plasticity. Particularly, FoxP3- T138 cells were heavily poised towards differentiation into FoxP3-expressing cells rather than IFN-gamma secreting Th1 cells. We performed ATAC-Seq to identify new transcription factors, that mediated this epigenetically poised state in FoxP3- T138 cells, and to identify accessible chromatin promoters and enhancers. We identified several loci that are more accessible in FoxP3- T138 cells than in FoxP3- WT cells. Motif analysis revealed enrichment of several lineage-determining transcription factor binding sites, such as Atf3. Strikingly, transcriptomic analysis demonstrated that Atf3 is specifically expressed in T138.
Several mechanisms had been shown to mediate immune regulatory function of Treg cells. Strikingly, our transcriptomic analysis identified CTLA4 and LAG3 to be differentially expressed in T138, T143 and the Treg pool in WT mice, suggesting that different Treg subsets might explore different regulatory mechanisms.
Taken together, our data clearly demonstrate the presence of distinct Treg subsets with distinct characteristics, such as development, plasticity and function.

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Aire and Brg1 Define a Fulcrum for Ectopic Gene Expression that Provides Precise Control of Immune Tolerance

Andrew S Koh[*1,4,5], Erik L Miller[4], Jason D Buenrostro[3], David M Moskowitz[1], William J Greenleaf[1,2], Howard Y Chang[1,2], Gerald R Crabtree[*1,4]

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To prevent harm from dangerous self-reactive clones of the T cell repertoire, epithelial cells of the thymic medulla express thousands of genes that are highly restricted to other lineages in specialized organ tissues. How these tissue-restricted genes, within a defined epithelial context, become accessible and activated at levels that prevent physiologic harm is unknown. Here, we identify Brg1 as an essential determinant of this somatic plasticity, promoting chromatin accessibility at tissue-restricted loci to poise transcription and impose central tolerance. We find that Aire, a transcription factor necessary for this ectopic expression, is entirely dispensable for the accessibility at tissue-restricted loci during thymic epithelial differentiation. In fact, we show that Aire harbors an intrinsic repressive function that restricts chromatin accessibility and transcriptional amplitude. Aire imposes this repressive influence within minutes upon recruitment to chromatin and operates over the genome in opposition to the mSWI/SNF chromatin remodeling complex. Human autoimmune mutations that impair Aire's multimerizing and histone-binding activities also inhibit this novel repressive influence indicating dual roles of Aire's functional domains. Together, Brg1 and Aire define a fulcrum in thymic epithelial development that allows the access and activation of tissue-restricted genes at levels that prevent toxicity, yet facilitate immunological tolerance.

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Ferritin controls the functional integrity and stability of regulatory T cells

Braza F.[1], Amador P.[1], Scicluna B.[3], Ventura P.[1], Cuadrado E.[2], Martins R.[1], Silva J.[1], Weis S.[4,5], Blankenhaus B.[1], Cardoso S.[1], Amsen D.[2], Demengeot J.[1] and Soares M.P.[1]

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Regulatory T cells -Tregs- play vital role in the establishment and maintenance of immune tolerance and homeostasis. Specific genetic programs of Tregs are necessary to enforce their function and maintain both stability and lineage commitment. However, how Treg function and identity are maintained in different tissue environments is still unclear. Here we show that expression of ferritin H chain component of the iron sequestering ferritin complex, is required to enforce Treg stability and functionality. Treg specific deletion of Fth in mice alters Treg suppressive function, compromising immune homeostasis and triggering spontaneous T effector cell activation at steady state, while worsening neuro-inflammation and improving anti-tumor immunity and dampening tumor progression. Supporting the notion that Fth acts as a gatekeeper of Treg identity and maintenance, we found that Fth deletion in Treg resulted in a marked decrease in Treg frequency in spleen, lymph nodes as well as in parenchymal tissues. This was associated with a down-regulation of the expression of Foxp3 and with a concomitant deregulated activation of prototypical gene coding for T helper type 1 and 2 effector cells. Fate mapping revealed that Fth deficient Treg cells lose the expression of Foxp3 over time and transdifferentiate into effector non-Treg cells. Mechanistically this is likely attributed to the i, development of oxidative stress and DNA damage, as reveled by the activation of gene expression profiles characteristic of oxidative stress and DNA damage responses, ii, a metabolic switch towards glycolysis and iii, development of functional exhaustion. Altogether our data revealed a previously unappreciated role of the regulation of iron metabolism by FTH in fine tuning Treg functional integrity.

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Cellular dynamics of intestinal antibody immune responses

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Gut-derived antigens trigger IgA immune responses that are initiated by cognate B cells in the Peyer's patch (PP). These cells colonize the subepithelial domes (SED) of the PP, and subsequently infiltrate into pre-existing germinal centers (GC). Here, we define the pre- GC events and the microanatomical site at which affinity-based B cell selection occurs in PPs. Using whole-organ imaging, we demonstrate that B cell receptor affinity (BCR) of antigen-specific B cells regulates infiltration into GCs, but not the early events in the SED. T follicular helper-like cells reside in the SED and promote its B cell colonization, independently of the magnitude of their BCR affinity. Imaging and immunoglobulin sequencing indicate that selective clonal-expansion ensue during infiltration into GCs. Thus, in contrast to draining lymph nodes and spleen, in PPs, T cells predominantly promote B cell expansion without clonal-selection during pre-GC events. These findings have major implications for oral vaccine design.

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

Elisabetta Petrozziello[1], Jelena Nedjic[1], Sarina Ravens[2], Marc Schmidt-Supprian[3] and Ludger Klein[1]

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Generation of the fittest T cell repertoire is accomplished through thymic positive selection, a process by which cortical thymic epithelium ensures the survival of useful TCR specificities, both in terms of diversity and functionality. While the nature of self-peptides presented to developing T cells remains to be investigated, increasing evidence points at the uniqueness of antigen-processing pathways adopted by cortical epithelial cells.
With our project, we aim at unraveling how a single cortex-specific protease involved in MHCII-antigen processing, namely Cathepsin L, influences the quality of the selected CD4+ T cell compartment. To do so, we take advantage of a conditional knock-out mouse model where the expression of Cathepsin L is ablated in the thymic epithelium, so that antigen-presentation to CD4+ T cells is altered exclusively during positive selection. In this mouse CD4+ T cell development is severely impaired, with regards to both cell numbers and TCR diversity. However, an exhaustive characterization of the polyclonal TCR repertoire is not technically possible, due to its extreme diversity.
To trace the fate of every T cell that survives thymic selection in the absence of Cathepsin L, we generated a new TCR-oligoclonal mouse model, by transgenic insertion of a fixed TCR-β chain and a pre-selected α chain. Surprisingly, CD4+ oligoclonal TCR repertoires are largely overlapping between Cathepsin L-sufficient and deficient animals, with only some TCRs missing from the latter. We are currently re-expressing some putative "Cathepsin L-independent" TCRs in vivo in retrogenic mice, to test if the very same TCR – selected in the presence or absence of Cathepsin L – can be equally functional. These experiments will clarify if the positive-selecting peptides produced by Cathepsin L play a direct role in the generation of a functional CD4+ T cell compartment.

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Class-switch recombination to IgA is under control of Tregs in PPs

Inta Gribonika

Understanding how class-switch recombination [CSR] to IgA occurs in the gut has been insufficiently investigated. Whereas previous studies have suggested Tregs as potentially important for IgA CSR, also Th17 cells have been implicated in gut IgA CSR. In fact, Tregs were exhibiting much plasticity and differentiated into Tfh cells, which were assumed to regulate IgA CSR in the germinal centers [GC]. However, an alternative site for IgA CSR is the subepithelial dome, suggesting that perhaps IgA CSR and Tfh functions in PP GCs are separately regulated. Furthermore, if Treg depletion is undertaken in mice a reduced gut IgA production can be observed, linking IgA CSR to Tregs. We have developed a mouse model to dissect the T cell-subset requirements for IgA CSR in the PPs using CD4 T cells isolated from OVA-specific TCR Tg mice and adoptively transferred into nude mice. This was followed by oral immunizations with OVA with/without cholera toxin [CT] adjuvant. We found that CT adjuvant was not required to mount a strong gut IgA responses, while it was required for an enhanced systemic IgG response to OVA, following i.p immunizations, suggesting differentially regulated CSR to IgA and IgG at local and systemic sites, respectively. However, the OVA-specific gut IgA response required recognition of the microbiota and could be explained on the basis of a rearranged TCR alpha-chain, as TCR Tg mice on a scid background failed to support gut IgA responses. Sorting CD25+ Tregs prior to transfer failed to support gut IgA responses to oral OVA immunizations, indicating that IgA CSR reside with Tregs, while Tfh functions were dependent on CD25- CD44+ CD4 T cells, as these latter cells could elicit a systemic IgG response. Thus, our data support that Tfh and IgA CSR are dissociated functions in the PPs with the former being TCR-dependent while the latter requires Helios+ neuropilin-1+ Foxp3+ nTregs and appears antigen-independent and controlled by TGFβ1.

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Beyond CTLA4 and PD1. Nuclear Receptor NR2F6 as an Alternative Cancer Immune Checkpoint in T Cells

Victoria Klepsch[1], Natascha Hermann-Kleiter[1], Bojana Jakic[1], Zlatko Trajanoski[2], Dominik Wolf[3] and Gottfried Baier[1]

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In contrast to cell surface checkpoints like CTLA-4 and PD-1, additional cancer therapeutic targets are located inside the effector immune cells. Targeting these alternative checkpoints in cancer immunotherapy with the goal to strengthen the patient's immune system are likely to extend the benefits of cancer immunotherapy in the near future. Along this line, we have defined and validated the orphan nuclear receptor NR2F6 [nuclear receptor subfamily 2 group F member 6, also called Ear-2] as an intracellular immune checkpoint in effector T cells by analyzing mouse tumor models in vivo, human T cells ex vivo and human lung cancer samples. Genetic ablation of Nr2f6, particularly in combination with PD-L1 blockade efficiently delayed tumor progression and improved survival in experimental mouse models. In accordance with published data [Hermann-Kleiter 2008, 2010; Kleiter & Klepsch 2016] acute Nr2f6 silencing in both mouse and human T cells induced hyper-responsiveness that established a non-redundant T cell-inhibitory function of NR2F6 by directly repressing transcription of key cytokine genes in T effector cells relevant for tumor cell rejection, such as IL-2, IFNγ and TNFα. In T cell-infiltrating cells of human NSCLC patients, NR2F6 protein expression was found to be upregulated in 54% of the cases [163 of 303 samples] and significantly correlated with PD-1 and CTLA-4 expression. Our data define NR2F6 as an intracellular immune checkpoint that suppresses adaptive anti-cancer immune responses and set the stage for clinical validation of targeting NR2F6 with low molecular weight compounds for improvement of next generation immune-oncological regimens.

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Microbiota-specific CD4 T cells are abundant in steady state and are functionally altered in inflammatory bowel disease

Hegazy AN, West N, Powrie F

The gastrointestinal tract is the largest immune organ and contains a vast amount and diverse populations of commensal bacteria. Immune responses in the gut are tightly regulated to avoid excessive inflammatory responses. However, the specificity of the abundant gut-resident effector and memory CD4 T cells remains unknown. Here, we found an abundance of CD4 T cells reactive to intestinal microbiota in adults who had not been diagnosed with inflammatory bowel disease. The CD4 T cell responses were directed towards both pathogenic and commensal bacteria. More than 80% of the reactive T cells have a memory-phenotype and are detected within circulating and gut-resident memory T cells. The observed reactive T cells were between hundred and thousand cells per million memory CD4 T cells. Microbiota-specific CD4 T cells are functionally heterogeneous in terms of their migratory properties and effector functions. The specific cells expressed CCR4, CCR6, CD161 and produced IFN-g, IL-17A, IL-22 and IL-10. In IBD, we detected a quantitative and qualitative alteration of circulating microbiota-specific CD4 T cells. These results identify CD4 T cells specific to commensals as an integral part of the physiological T cell repertoire and their presence does not indicate disease per se. Thus, these T cell responses might support gut homeostasis and mucosal immunity in steady state.

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The bona fide structure of IgM pentamer and its binding mode with AIM that facilitates the repair of multiple diseases

Toru Miyazaki

Soluble IgM forms a predominantly pentameric complex that also contains a small polypeptide J chain. While the IgM pentamer has various immune functions to defend against foreign pathogens, it also behaves as a carrier of the circulating AIM protein. The binding to IgM pentamer protects AIM from renal excretion, leading to high serum AIM levels. When AIM dissociates from IgM, it becomes functionally active and facilitates the repair of a variety of diseases including acute kidney injury and hepatocellular carcinoma. Since the precise manner of AIM-IgM pentamer binding remains unknown, we examined the structure using a latest single-particle negative-stain electron microscopy. Surprisingly, the IgM-Fc pentamer shapes asymmetric pentagon containing one large gap of 50°, which is markedly different from the textbook prediction of a symmetric pentagon model. A single AIM molecule stably fits into the 50° gap, cross-bridging two IgM-Fc through a disulfide bond and a charge-based interaction. We also found that the association of AIM with IgM pentamer not only inactivates AIM but also progresses inflammatory immune-complex formation in certain autoimmune diseases such as IgA nephropathy. Thus, our current findings of the bona fide structure of the AIM/IgM pentamer complex provides an important insight into the releasing mechanism of AIM from IgM, which could be the basis for the development of new therapeutic strategies against multiple diseases via the increase of active AIM and the disruption of immune-complex formation.

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How central tolerance shapes the polyclonal CD4 T cell repertoire specific for a tissue restricted self antigen

Tobias Haßler, Maria Hinterberger, Christine Federle, Julia Winnewisser, Thamotharampillai Dileepan, Kilian Schober, Dirk Busch, Marc Jenkins and Ludger Klein

The fate of a developing T cell is dependent on the interaction of its T cell receptor and the self-peptide MHC complex on antigen presenting cells. According to the classical affinity model of thymocyte selection, the degree of auto-reactivity determines if potentially harmful T cells are diverted into regulatory T cells or clonally deleted. Nevertheless, how central tolerance induction to a physiological self antigen is set in the polyclonal repertoire is still poorly understood.
We are focusing on the naturally-expressed tissue-restricted antigen proteolipid protein short PLP, the main component of the myelin sheath, as it is one of the putative target antigens in Multiple Sclerosis in humans. Non-tolerant murine strains develop experimental autoimmune encephalomyelitis, a MS-like disease, whereas BL/6 mice are resistant to EAE and lack recall responses upon PLP immunization, due to expression of PLP in thymic epithelial cells.
We previously showed that, upon immunization of PLP deficient BL/6 mice, CD4 T cells react to three immunodominant epitopes within the PLP protein - PLP1, PLP2 and PLP3. To further investigate the mechanisms of central tolerance induction to PLP, we generated a PLP1-MHCII Tetramer and followed the destiny of PLP-specific T cells in the polyclonal CD4 compartment. Through single cell TCR sequencing we were able to identify differences between the T cell repertoires of PLP deficient and PLP wildtype B6/L mice, with regards to abundance, phenotype and TCR usage of endogenous PLP-specific CD4 T cells. We have recently re-expressed some TCRs identified via the PLP-tetramer and single cell sequencing technology in TCR transgenic mice, to validate our findings.

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Origin and differentiation trajectories of fibroblastic reticular cells in the splenic white pulp

HungWei Cheng, Lucas Onder, Mario Novkovic, Charlotte Soneson, Mechthild Lütge, Natalia Pikor, Elke Scandella, Mark Robinson, Jun-ichi Miyazaki, Thomas Ruelicke, Thomas Hehlgans and Burkhard Ludewig

The splenic white pulp is underpinned by poorly characterized stromal cells that demarcate distinct immune cell microenvironments. Here, definition of the embryonic origin and tracing of the differentiation trajectories of fibroblastic reticular cells was enabled by the establishment of FRC-specific fate-mapping in mice. We found that all reticular cell subsets descend from multipotent progenitors that emerge at embryonic day 19.5 from Sca-1+ periarterial progenitors. Commitment of FRC progenitors was concluded during the first week of postnatal life through occupation of niches along developing central arterioles. Single cell transcriptomic analysis facilitated deconvolution of FRC differentiation trajectories and indicated that perivascular reticular cells function both as adult lymphoid organizer cells and mural cell progenitors. Finally, the lymphotoxin-β receptor-independent sustenance of postnatal progenitor stemness unveiled that systemic immune surveillance in the splenic white pulp is governed through subset specification of reticular cells from a multipotent periarterial progenitor cell.

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A novel gut-derived microbiota-specific circulating CD4+/CD8a+ human Treg subset: potential roles in homeostasis and diseases

Emmanuelle Godefroy[1], Harry Sokol[2], Antoine Roquilly[3], Karim Asehnoune[3], Frédéric Altare[1] and Francine Jotereau[1]

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Gut microbiota affects immune homeostasis and susceptibility to diseases. Whether alterations of microbiota-induced Tregs contribute to human immune diseases needs to be further examined. Faecalibacterium prausnitzii, a key member of intestinal microbiota, exhibits anti-inflammatory properties, is decreased in inflammatory bowel diseases (IBD), and can predict relapse in Crohn's disease. Importantly, we have demonstrated that F. prausnitzii induces specific Tr1-like Tregs characterized by the co-expression of CD4 and CD8a (named DP8a). These cells are abundant in the healthy human colon and recirculate in the blood.

Although human colonic DP8a cells seemed homogeneously specific for F. prausnitzii, only a fraction of circulating DP8a cells responded to this bacterium. To specifically track circulating F. prausnitzii-induced Tregs, we investigated the presence of additional markers for both their specificity and suppressive function. We hence unraveled that CCR6 and CXCR6 co-expression characterized F. prausnitzii-specific DP8a cells and that CD39 mediated their regulatory function.

Using these markers, we have observed that circulating CCR6+/CXCR6+ DP8a Tregs are drastically reduced in IBD patients (p=4E-11), as compared with healthy controls or patients with infectious colitis, suggesting this modulation was IBD-specific and valuable regarding early IBD diagnosis. As a second disease model, in severely injured trauma patients, while the frequency of CCR6+/CXCR6+ DP8a Tregs was not altered, interestingly, their CD39 expression was specifically and dramatically heightened, emphasizing an increased regulatory function, which could be linked to the immuno-deficiency status observed in this patient population.

In summary, these two clinical examples illustrate various aspects of DP8a cell biology, whose frequency and/or regulatory function can be altered in pathologic settings. These data suggest this particular subset might play key roles at the diagnostic, prognostic and/or therapeutic levels in defined patient populations.

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Treg identity issues solved by quantitative shotgun proteo-/transcriptomics

D. Amsen

Inflammation is both a requirement and a challenge for regulatory T cell (Treg) function. Inflammatory cues direct Tregs to inflamed sites where they limit tissue destruction and promote repair, but can also coerce some Tregs into assuming functions normally performed by conventional T cells (Tconvs), such as production of pro-inflammatory cytokines. Plasticity is most pronounced in peripherally induced (p)Tregs, while thymus derived (t)Tregs are more stably committed to the Treg lineage. Treg instability is a risk for adoptive therapies using Tregs to mitigate inflammatory conditions and autoimmune disease. It is important, therefore, to understand how Tregs protect their identity from destabilization and to identify markers that allow discrimination between tTregs from more unstable pTregs.
To address these issues, we established a molecular definition of Treg identity using whole cell shotgun proteomics and transcriptomics on human populations of Tregs and Tconvs. We found that proteome and transcriptome compositions markedly differ from one another, but are complementary, underscoring the importance of analysis at both levels. A new marker was discovered that specifically identifies Tregs stemming from the thymus. Furthermore, core and subset-specific Treg signatures revealed adaptations in cytokine-, TCR- and costimulatory receptor signaling pathways. We show that deficiencies in selective pathways allow inflammatory cytokines to mobilize necessary functions in Tregs (such as expression of transcription factors and homing receptors) without compromising Treg identity. Genetic complementation of key signature molecules suffices to cause loss of resistance against destabilization by inflammatory cytokines. We thus identify molecular nodes that determine the unique response characteristics of Tregs to inflammation and provide a means to select stable human Tregs for adoptive cellular therapy.

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B cell zone reticular cell reprogramming governs the germinal center response

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The efficient generation of germinal center (GC) responses requires the directed movement of B cells between distinct microenvironments underpinned by specialized B cell zone reticular cells (BRC). Nevertheless, the cellular identities of BRCs and their functional contributions to the GC response remain unclear. Here, we utilize a functional, genetic targeting strategy to characterize the transcriptional identity and dynamic remodeling of the BRC landscape in the developing GC. Single cell transcriptomic analysis revealed that FDC subsets corresponding to the light and dark zones are pre-determined in the naïve B cell follicle and reprogramed to cater to LZ and DZ B cells in the GC. Furthermore, by genetically uncoupling chemokine gradients in the B cell zone, we reveal that BRC remodeling determines the efficacy of the GC response and is instructed by B cells. Together, our results suggest that spatial stratification and remodeling of specialized BRC subsets determines the efficiency of the GC response.

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Lung-on-a-chip microtechnologies for studies of host-pathogen interactions in Tuberculosis

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A majority of host-M.tuberculosis (Mtb) encounters lead to latent infections in which the bacteria exist in a poorly characterized host-pathogen equilibrium, usually within granulomatous lesions in the lung. Much of the information about the host-pathogen interaction in TB has been obtained from in vitro batch culture infections of macrophages with Mtb. These experiments however capture neither the role of cellular individuality nor the effects of the host environment on the outcomes.
Here we report on experiments that successfully reconstitute the murine alveolar interface entirely from primary cells in a lung-on-a-chip microfluidic device. This builds upon the previously reported human lung-on-a-chip system[1] by incorporating aspects of the innate immune system including alveolar and bone-marrow derived macrophages and monocytes. This system is then inoculated at low multiplicity of infection (MOI) with Mtb, and the progression of the infection is monitored in real-time through a combination of live-cell time-lapse microscopy (7-10 days) followed by confocal microscopy.
This "bottom-up" approach allows for single-cell imaging and temporal tracking of the dynamics of immune cells and their interactions with apoptotic tissue cells and pathogens, and enables us to closely follow the chain of events from initial infection. We are currently investigating unresolved aspects about the innate immune response to the initial infection. We observe an unexpectedly high occurrence of infection of alveolar epithelial cells, which provide a permissive environment for a more rapid initial growth of single bacteria into clumps or long intracellular cords that subsequently prove toxic for uptake by professional phagocytes. Paradoxically, Mtb induced epithelial cell inflammation also appears to 'mask' the bacteria from macrophages. We are currently writing up a manuscript describing these findings. With further development, this is an ideal system to study how tissue-resident memory cells interact and influence the innate immune system. From a TB viewpoint, we will extend the system to study how granulomatous lesions begin to develop, and the roles played by bacterial phenotypic variants and host microenvironments in granuloma formation and in entering and exiting the latent state.

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A novel humanized mouse model to study human antigen-specific cutaneous T cell responses in vivo

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As a barrier organ the skin comprises a complex coordinated system of epithelial tissue cells and immune cells that ensure adequate immune reactions against trauma, toxins and pathogens, while maintaining self-tolerance and preventing allergy and autoimmunity. We hypothesize that interactions between tissue cells (such as keratinocytes) and immune cells are crucial for optimal immunity and the maintenance of homeostasis within the skin. Here we aim to analyze and understand the role of cellular interactions in human skin and manipulate them in settings of tissue damage. Because murine and human skin differ in structure and cellular composition we established a humanized mouse model to investigate human immune mechanisms in human skin in vivo. Importantly, we use a simplified skin tissue generated from fibroblasts and keratinocytes only. This allows us to study the individual contribution of any skin-infiltrating cell to the analyzed immune response in the skin as well as the impact of individual cell-cell interactions, including T cell – APC, T cell – keratinocyte, or T cell – microbe/commensal cross-talk. Using this model we could follow T cell infiltration of the skin, characterize antigen-specific responses to microbial antigen and study the behavior of cutaneous memory T cell populations. Additionally, we started to characterize local T cell – APC interactions and T cell – tissue cell interaction and found that these lead to improved T cell infiltration and immune responses in the skin.
We have generated a powerful novel model with broad applicability in basic and preclinical research, that can be reconstituted with a variety of different cutaneous cell types (such as APCs, T cells, γδTcells) to study their in vivo function.

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Multi-omic single cell RNA sequencing and high-dimensional cytometry of human tissue-resident antigen-presenting cells reveal tissue-specific specialization

Florian Mair, Jami R Erickson, Valentin Voillet, Douglas R Dixon, Raphael Gottardo and Martin Prlic

Studies in the past decade have highlighted the biological significance and the distinct functional properties of immune cell subsets that are resident in non-lymphoid tissues. While tissue-resident memory T cells (TRM) have been well characterized, much less is known about the human myeloid compartment, which includes professional antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages, both of which are critical for shaping the local T cell response. Regulating immune responses may be particularly complex in human mucosal barrier tissues as the immune system has to selectively respond to commensal versus pathogen-derived signals.
A major roadblock in studying the myeloid compartment in human tissues is that APCs are typically rare, hard to cryopreserve and functional analysis is more challenging compared to lymphoid cells. We used a combination of single cell RNA sequencing (sc-RNAseq) and 30-parameter fluorescent flow cytometry to define the APC compartment in fresh healthy and inflamed human mucosal tissue biopsies. Our data suggest that mucosa-resident myeloid cells show tissue-specific specialization and we identified novel myeloid cell clusters specific to the human mucosa with a unique pro-inflammatory signature. Parallel profiling of the adaptive and innate T cell compartment was performed to elucidate the potential impact of these APCs on the local T cell subsets and how these orchestrate mucosal immunity.
Finally, we used multi-omic sc-RNAseq (termed AbSeq) which allows parallel profiling of the transcriptome and surface protein phenotype at the single cell level with the aim of gaining a better understanding of the relationship of APCs in blood and tissue. Parallel analysis of mucosal tumor biopsies will allow to pinpoint myeloid adaptation specific to the tumor microenvironment. Overall, our data provide first insight into the functional heterogeneity of canonical human APC and T cell subsets in mucosal barrier tissues.

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Examining the functional capacity of T cells with a tissue resident phenotype during chronic infection

Jolie Cullen and Dietmar Zehn

During a chronic viral infection, the host fails to clear infectious pathogens. In this situation the immune system must adjust to the dual task of managing the continuous presence of viral antigen-driven inflammation while keeping virus replication at a low level. This is in part achieved through the process of T cell exhaustion, which enables T cells to maintain viral control for long periods of time without causing the level of immunopathology seen in acute infection. Yet, exhausted T cells are heterogeneous and it is so far unclear which cells are capable of such a feat. Interestingly, T cells with a resident phenotype can be found among exhaustion T cell population and they also express an array of inhibitory markers. Yet, they are capable of producing substantial levels of cytotoxic and effector molecules after re-exposure to cognate antigen. Following up in this notion, we have identified and characterised resident CD8+ T cells based on phenotypic markers from previous studies, in large frequencys in salivary glands, liver and kidney. These resident populations are formed early during the infection, are greatly affected by the organ in which they reside and are constantly replenished from the circulating TEX cells. The liver population of resident cells are heterogeneous, CD4-dependent and require antigen for their development. Further, we have shown that the cells recruited for tissue residency in the liver during a chronic infection require high affinity activation, and are dependent on high CXCR6 expression. Thus, resident T cells are formed during a chronic infection, and make up a large frequency of the response during a chronic LCMV infection. On going work is focusing on determining if these cells are aiding the low level of viral control exhibited in chronic infections, or if they contribute negatively to the chronic inflammation. This work has clear implications for future treatment options of chronic infections, as they may represent a population of cells to be targeted for treatment.

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Two distinct Ly49H+ NK cell subsets react to MCMV infection

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Natural killer (NK) cells, as a part of cellular innate immunity, represent a first line of defense against viral infections. The effector functions of NK cells comprise both direct cytotoxicity and the production of inflammatory cytokines, e.g. IFN-gamma and TNF-alpha. So far, it is poorly understood whether one single NK cell can generate both cytotoxic and helper-like NK cells or whether these are two distinct lineages of NK cells. The reactivity of NK cells is controlled by an array of activating and inhibitory receptors. The activating NK cell receptor Ly49H binds to m157, a glycoprotein encoded by murine cytomegalovirus (MCMV), and therefore Ly49H+ NK cells are selectively activated during MCMV infection (Dokun et al. 2001). Thus, monitoring Ly49H+ NK cells during MCMV infection allows to investigate infection-driven NK cell differentiation in vivo.
In order to probe the heterogeneity of the NK cell response to MCMV infection, we mapped the fate of single Ly49H+ NK cells and their progeny in vivo. By monitoring expression of NK cell maturation markers CD62L and CD27, we identified two phenotypically distinct response patterns. We found that these response patterns emerged from two distinct NK cell subsets that differentially express surface markers CD62L, CD69, CD160, CD11b and CD11c as well as cytotoxic effector molecule granzyme B at steady state and differ in the production of TNF-alpha and GM-CSF after stimulation. RNA sequencing of the identified subsets and single cell RNA sequencing of Ly49H+ NK cells suggest that these subsets may constitute distinct NK cell lineages that differ in their relatedness to type I innate lymphoid cells (ILC1s) and play distinct roles in the immune system's response against viral infections.

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Peroxisome proliferator-activated receptor gamma (PPAR-gamma) plays a crucial role in the red pulp macrophage development

Katarzyna Okreglicka, Christoph Schneider, Manfred Kopf

Macrophages derive from hematopoietic cells of the myeloid lineage and play important roles in innate immune response and tissue homeostasis. These phagocytic cells populate every organ and display a unique gene signature, which results from distinct environmental signals, and directs the expression towards effector functions specific for the tissue of residence. We have reported that the induction of nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-gamma) by cytokine GM-CSF is crucial for development of alveolar macrophages (AM) from fetal lung monocytes but dispensable for the development of macrophages in many other tissues.
Performing a more thorough analysis of tissue macrophage subsets in Vav1-Cre-Ppargfl/fl mice, we now found that in addition to AM these mice lack also a subset of VCAM1+ F4/80+ bone marrow macrophages (BMM) and red pulp macrophages (RPM), which are known to be critical for iron homeostasis. Accordingly, Vav1-Cre-Ppargfl/fl mice showed iron accumulation in spleen and bone marrow. Previously, it has been shown that development of RPM and VCAM1+ BMM depends on Spi-C, which gets released from Bach1 repression by heme. We found that the heme induced Spi-C expression occurs independently of PPAR-gamma. Interestingly, GM-CSF is not required for PPAR-gamma-induced RPM development, in contrast to AM.
Taken together, these data suggest that (at least) two transcription factors, PPAR-gamma and Spi-C, induced by distinct environmental triggers, are essential and non-redundant in RPM and VCAM1+BMM development.

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Sensing of phosphorylated isoprenoid metabolites (Phosphoantigens; PAg) by human γδ T cells: Lessons from the alpaca

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All jawed vertebrates possess Ig, αβ-, and γδ–TCR genes. The major subset of human blood γδ T cells uses Vγ9 and Vδ2 TCR genes. These Vγ9Vδ2 T cells kill tumor cells and expand massively in infections like malaria. This response is initiated by TCR-mediated sensing of phosphorylated isoprenoid metabolites (phosphoantigens: PAgs) such as HMBPP (microbial origin) or IPP (host origin) accumulated in stressed or tumor cells. These PAgs bind to an intracellular domain of the B7-like molecule butyrophilin 3A1 (BTN3A1) which induces changes at the cell surface recognized by the Vγ9Vδ2 TCR. These effects are strongly enhanced by cooperation of the PAg-binding BTN3A1 with the PAg-insensitive isoforms BTN3A2 and BTN3A3. Here, we demonstrate trans-molecular interactions of protein domains by co-expressing modified BTN3 isoforms in BTN3-deficient cells. Deletion of the extracellular IgV-like domain of BTN3A1 abolishes cell surface expression and stimulatory potential. Nevertheless, both features are rescued by co-expression with BTN3A2 or BTN3A3 suggesting a cooperation of protein domains in BTN3 heteromers. A BTN3 molecule uniting all features required for efficient PAg sensing is expressed by the camelid species alpaca (Vicugna pacos), which we present as the first non-primate species possessing PAg-sensing cells. Newly developed monoclonal antibodies against alpaca Vδ2 and BTN3 allow the demonstration of expansion of Vδ2-positive T cells upon stimulation with the PAg HMBPP, which is abolished by the BTN3-specific mAb. Murine transductants expressing alpaca Vγ9Vδ2 TCR show a remarkable PAg-induced reactivity to alpaca as well as to human BTN3-expressing cells. Furthermore, analysis of BTN3 chimeras demonstrates that chimeras expressing the human BTN3A1 extracellular domain and the alpaca BTN3 transmembrane/intracellular domain mediate PAg-induced activation of Vγ9Vδ2 TCR transductants even more efficiently than natural human BTN3A heteromers. These findings will be used to identify the minimal structural requirements for efficient PAg-sensing by the γδTCR.

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T cell acute lymphoblastic leukemia as a consequence of thymus autonomy

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Thymus autonomy is the capacity of the thymus to maintain T lymphocyte development and export independently of bone marrow contribution. Prolonging thymus autonomy was shown to be permissive to the development of T cell acute lymphoblastic leukemia (T-ALL), similar to the human disease. Here, performing thymus transplantation experiments in mice we report that thymus autonomy can occur in several experimental conditions, and all are permissive to T-ALL. We show that wild type thymi maintain their function of T lymphocyte production upon transplantation into recipients with several genotypes (and corresponding phenotypic differences), i.e. Rag2-/-gc-/-, gc-/-, Rag2-/-IL-7ra-/-, and IL-7ra-/-. We found that the cellularity of the thymus grafts is influenced exclusively by the genotype of the host, i.e. IL-7ra-/- versus gc-/-. Nonetheless, the difference in cellularity detected in thymus autonomy bore no impact on onset, incidence, immunophenotype or pathology of T-ALL. In all tested conditions, T-ALL reached an incidence of 80%, demonstrating that thymus autonomy bears a high risk of leukemia. Furthermore, we identified a population of thymocytes that persists in the thymus autonomous grafts, presumably maintaining T lymphocyte differentiation. Taken together, our data support that interleukin 7 (IL-7) drives cellular turnover non-cell autonomously, which is required for prevention of T-ALL. We found no influence for T-ALL in the specific combination of the genotypes tested or minor differences in the genetic background of the strains.

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Single-cell fate mapping reveals clonal dynamics of adaptive NK-cell responses

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During infection antigen-specific T-cell populations rapidly expand, contract and then persist at increased frequencies. However, the antigen-driven evolution of the T-cell receptor (TCR) repertoire does not stop at the selection of antigen-specific T-cells per se. It is instead characterized by the further expansion of certain T-cell clones within the antigen-specific T-cell population that harbor TCRs of optimal affinity to the relevant antigen.
Interestingly, an antigen-dependent enrichment of lymphocytes recognizing certain target structures has also been identified for Natural killer (NK) cells in the context of cytomegalovirus (CMV) infection. Whether these "antigen-specific" NK cells respond uniformly to CMV infection or show distinct clonal dynamics similar to those found in T cells remains unknown. Here, we used retrogenic color- barcoding and single-cell adoptive transfer to track clonal immune responses derived from individual Ly49H+ NK cells during murine cytomegalovirus (MCMV) infection. We found that clonal expansion of single NK cells varied dramatically. This variation could not be attributed to the additional presence or absence of inhibitory Ly49 receptors in responding clones. Instead, NK-cell clones showed distinct levels of Ly49H receptor expression that correlated closely with the degree of clonal expansion and persistence during the contraction phase. Thus, akin to adaptive processes shaping an antigen-specific T-cell receptor repertoire, the Ly49H+ NK-cell population adapts to MCMV infection. This is achieved by preferential expansion and maintenance of individual NK-cell clones expressing higher levels of Ly49H.

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Understanding immunity in tumor-targeted therapy of melanoma: development of rationale combination therapy with BRAF inhibitor and DC-based immunotherapy

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Melanoma belongs to the ten most common cancer types both in the US and Europe and its incidence is rapidly increasing. In 60% of melanoma patients mutations occur in the BRAF gene leading to an amino acid substitution of valine to glutamic acid in position 600 (BRAFV600E), which activates the MAPK pathway. This mutation is of clinical interest, because it can be targeted with selective BRAF inhibitors (BRAFi) that have been approved for treatment of melanoma patients. Though BRAFi induces impressive melanoma regression, resistance to BRAFi occurs within the first year of treatment due to different mechanisms. The resistance development is correlated also with alterations in the immune infiltrate of BRAFi-treated tumors. At early time points, the immunosuppressive tumor milieu is reversed as reflected by higher expression of melanoma-associated antigens and infiltration of T cells. In contrast, the resistance phase shows a loss of effector cells and infiltration of myeloid-derived suppressor cells (MDSC). In our project with characterized the immune infiltrate of a transplantable melanoma mouse model, named D4M carrying the BRAFV600E mutation and PTEN loss. In line with earlier observations, we observed enhanced infiltration of activated and more functional T and NK cells and a loss of these cells during resistance phase. The myeloid compartment is affected also as we detected changes in DC, macrophages and MDSC according to the anti-tumorigenic milieu in the BRAFi-sensitive phase and the pro-tumorigenic milieu during resistance. A more thorough analysis of the functional role of tumor-infiltrating DC is required to understand if increased melanoma antigen expression also correlates with tumor-specific T cell responses. Moreover, we have developed DC-vaccines that allow targeting of melanoma antigens to DC in vivo by genetically engineering antibody-antigen fusion proteins. As BRAFi as single therapy does not provide long-term survival, we would like to combine these DC-vaccines with BRAFi to test if we can delay or even prevent the development of resistance of tumor-targeted therapy.

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NK-cell differentiation in the pig is correlated with a down-regulation of NKp46 expression

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The activating receptor NKp46 (CD335) is used as a marker to identify natural Killer (NK) cells across various mammalian species. Nevertheless, in swine three distinct NK-cell subsets can be identified on the basis of NKp46 expression: NKp46-, NKp46+, and NKp46high NK cells. These NK-cell subsets vary in their functional properties and homing preferences to organs. NKp46high NK cells show the highest perforin expression, have an increased capacity to produce IFN-γ and proliferate upon in vitro stimulation. We hypothesize that the NKp46-defined phenotypes correlate with different stages of differentiation. To test this hypothesis, we analyzed expression of transcription factors involved in NK-cell differentiation by multi-color flow cytometry in lymphocytes isolated from blood, spleen, lung and liver. Additionally, the surface molecules CD8α and CD27 were analyzed as they were already shown to correlate with the differentiation of porcine T cells. Increased expression of Eomes could be observed in NKp46high NK cells. In contrast, this NK-cell subset showed much lower levels of T-bet expression compared to the other two NK-cell subsets. The NKp46high phenotype correlated with a CD8αlowCD27+ expression. Both, NKp46- and NKp46+ NK cells, showed an EomesdimT-bethighCD8αhighCD27low phenotype. Eomes and T-bet have been described to regulate different stages of NK-cell maturation in mouse and human. An increase of CD8α and a decrease of CD27 expression in porcine T cells identifies effector memory T cells. Additionally, GATA-3, a transcription factor promoting NK-cell maturation and discussed as a factor required for IFN-γ production in NK cells, was higher expressed in the NKp46high subset compared to the NKp46- and NKp46+ NK cells. These results indicate, that the three porcine NK-cell subsets differ in their differentiation stages and lead to the hypothesis that NKp46high NK cells differentiate into NKp46+/- cells.

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How (CAR-)T-cells Recognize Antigens – a Molecular Imaging Approach

Johannes B. Huppa

T-cells are remarkably sensitive towards antigen; they can detect the presence of even a single antigenic peptide/MHC complex (pMHC) among thousands of non-stimulatory pMHCs on the surface of antigen-presenting cells (APCs). Of note, TCR-pMHC interactions are only of moderate strength when measured in vitro and 2-4 orders of magnitudes weaker than typical antibody-antigen interactions. How can we then explain the phenomenal degree of T-cell antigen sensitivity, a hallmark of adaptive immunity? We think that the specific microenvironment within the immunological synapse, where TCR-pMHC binding takes place, provides at least in part the answer. Binding parameters are severely influenced because receptors and ligands are pre-oriented, to some extent clustered and moreover subjected to cellular forces.
To account for these nonlinear properties of the contacting cells, we have devised a minimally invasive ultrasensitive live-cell imaging approach, in which synaptic TCR-pMHC binding events are directly detected and quantified in situ. While we find antigen recognition to be solely driven by monomeric TCR entities, TCR-pMHC binding is significantly enhanced with both an accelerated association and, due to cellular forces, an accelerated dissociation. Moreover, TCR affinities vary considerably within different synaptic regions and also between different cells.
These observations imply that TCR-pMHC binding and the entire process of antigen recognition are controlled through not well-understood cell-biological parameters, which might also be subject to regulation in T-cell development and differentiation. Identifying these parameters, quantifying their effects on the efficacy of T-cell antigen recognition and understanding their relevance in immunity, autoimmunity and cancer progression stands in the forefront of our research. Last but not least, we wish to apply at least some of the methodological and immunologic lessons learned in the field of cancer immunotherapy, in particular in the design of performance-enhanced chimeric antigen receptor (CAR)- modified T-cell products.

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Abatacept targets Tfh and effector Tregs and disrupts molecular pathways that regulate their proliferation and maintenance

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Interaction of CD28 with CD80/86 expressed by APCs, provides T cells with a second costimulatory signal in addition to TCR engagement and allows them to become fully activated. Therefore, blocking this pathway is an attractive therapeutic target for autoimmune diseases in which dysregulated T cell activation plays a major role in pathogenesis.
Abatacept is a CTLA-4-Ig fusion protein that binds to the co-stimulatory ligands CD80 and CD86 and blocks their interaction with the CD28 and CTLA-4 receptors expressed by T cells, inhibiting T cell activation and function. Longitudinal specimens from the ACCLAIM trial of abatacept in multiple sclerosis were utilized to examine the effects of abatacept treatment on the frequency and transcriptional profile of specific T cell populations in the peripheral blood.
We found that the relative abundance of CD4+ T follicular helper cells and regulatory T cells was selectively decreased in participants following abatacept treatment. Within both cell types, abatacept was associated with changes in gene expression with the altered genes being enriched for functions regarding cell-cycle control and chromatin dynamics. All cellular and molecular changes were reversed following termination of abatacept treatment.
Our findings link the changes in costimulatory signaling to impaired activation, proliferation and decreased abundance. These results identify new transcriptional targets of CD28-mediated costimulatory signaling in human Treg and Tfh cells, further informing on its potential use in diseases associated with dysregulated Tfh activity.

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Nuclear orphan receptor NR2F6 regulates CD8+ T cell effector function and memory formation

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The nuclear orphan receptor NR2F6 is an intracellular immune checkpoint during cancer immune-surveillance. As a transcription factor, NR2F6 directly binds to the Il2 and Ifng minimal gene promoters and antagonizes NFAT/AP-1 binding, thus leading to a decreased production of IFN-gamma and IL-2. How NR2F6 controls CD8+ T cell effector function and memory formation, especially during pathogen encounter, has never been investigated.
Nr2f6-/- mice had an enhanced clearing of bacterial load and a benefit in survival during infection with ovalbumin tagged Listeria monocytogenes (LmOVA) accompanied with an increase in SIINFEKL specific CD8 T cells. In vivo killing assay suggested that Nr2f6-/- CD8+ T cells in OVA immunized OT-I mice do not have an enhanced ability to kill SIINFEKL peptide pulsed target cells per se.
To investigate CD8 T cell intrinsic functions, OT-I wt or Nr2f6-/- naïve cells were adoptively transferred to congenic wt recipients and infected with LmOVA. OT-I Nr2f6-/- cells had an increase memory precursor (KLRG1-CD127+) cell populations, detectable in spleen at day 7 post infection. Moreover, re-stimulation in vitro with SIINFEKL peptide, leads to significantly enhanced IFN-gamma and TNF-alpha production, both by OT-I Nr2f6-/- d7 effector cells and by total Nr2f6-/- CD8 T cells on d70 post infection. Collectively, these data suggest that NR2F6 regulated CD8 T cell effector and memory response in a cell intrinsic manner.

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Dynamic emergence and propagation of cytotoxic T lymphocyte escape mutations during chronic infection

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Cytotoxic T lymphocytes (CTLs) represent key immune effectors of the host response against chronic viruses. Viruses can evade the selective pressure exerted by CTLs through escape mutations in viral T cell epitopes. This mechanism of immune escape is common to both chronic viral infections and malignant tumours. We employ the chronic infection model of lymphocytic choriomeningitis virus (LCMV) to study how CTL escape mutations arise during chronic infection. By using different experimental approaches to modulate CTL selection pressure we identified non-synonymous mutations in viral H2-Db-restricted CTL epitopes. Some of the identified mutations in the immunodominant GP33-41 CTL epitope have been previously verified as escape mutations, yet we observe different kinetics of these variants during chronic infection. These mutations were not found in Rag2-/- mice which are devoid of any CTL selection pressure. By studying the dynamic emergence and propagation of mutations in viral CTL epitopes we expect to gain fundamental insights into the evolutionary dynamics driven by the interplay between pathogens and T cells. The governing principles of which may be applicable to other contexts, such as cancer immune evasion and immunotherapy.

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Regulation of Lymphatic GM-CSF Expression by the E3 Ubiquitin Ligase Cbl-b

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Genome-wide association studies as well as lymphatic expression analyses have linked both Cbl-b and GM-CSF to human multiple sclerosis as well as other autoimmune diseases. Both Cbl-b and GM-CSF have been shown to play a prominent role in the development of murine encephalomyelitis; however, no functional connection between the two has yet been established. In this study, we show that Cblb knockout mice demonstrated significantly exacerbated severity of experimental autoimmune encephalomyelitis (EAE), augmented T cell infiltration into the central nervous system CNS and strongly increased production of GM-CSF in T cells in vitro and in vivo.GM-CSF neutralization demonstrated that the increased susceptibility of Cblb−/− mice to EAE was dependent on GM-CSF. Mechanistically, p50 binding to the GM-CSF promoter and the IL-3/GM-CSF enhancer element "CNSa" was strongly increased in nuclear extracts from Cbl-b-deficient T cells. This study suggests that Cbl-b limits autoimmunity by preventing the pathogenic effects of GM-CSF overproduction in T cells.

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Mesenchymal cell fate decisions during lymph node and peyer`s patch organogenesis

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The development and function of secondary lymphoid organs depends on the differentiation of distinct subpopulations of fibroblastic reticular cells from embryonic mesenchymal organizer cells. However, the delineation of fibroblastic cell fate decisions during the formation of lymphoid niches and specific molecular requirements within lymphoid tissue organizer cells remained elusive. Here, we apply fibroblastic reticular cell specific fate mapping and conditional ablation of lymphorganogenic molecules to identify fibroblastic lineage decisions during lymphoid organ formation. We found that fibroblastic reticular cells in lymph nodes arise from unique peri-venous organizer cells in a lymphotoxin-beta-receptor dependent manner, while fibroblastic reticular cells in peyer`s patches originate from perivascular and subepithelial niches in the embryonic intestine. Molecular dissection of fibroblastic cell lineages in both organs revealed profound differences in their differentiation trajectories and unravel novel cues for organ-specific lymphoid niche formation. Our findings broaden the current knowledge about mesenchymal signals which are necessary to build lymphoid tissues during development and under pathological conditions.

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Superior capacity of fetal monocyte compared to primitive macrophage precursors in development and function of tissue resident macrophages

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Tissue-resident macrophages have a vital tissue-specific function in homeostasis and first-line defense against invaders. They develop during embryogenesis independent from bone marrow HSC. Fate-mapping studies revealed at least two distinct waves of erythroid myeloid progenitors (EMP) arising in the yolk sac (YS) at E7.5 and E8.5. Each has been proposed to give rise to tissue-resident macrophages with the latter going through a monocyte intermediate. However, the relative potential of these precursors in determining the development and functional capacity of tissue-resident macrophages remains unclear. Here, we studied AM development after adoptive transfer of EMP, primitive macrophage and monocyte precursors from YS, fetal liver and lung into neonatal Csf2ra-/- mice, which lack endogenous AM. Compared to fetal monocytes, E10.5 EMP and primitive MΦ showed a strikingly impaired c-Myb-dependent potential to develop into AM associated with a poor glycolytic capacity. Mice containing primitive MΦ-derived AM failed to efficiently clear alveolar proteinosis and protect from mortality following influenza virus infection. Together, our results provide an explanation, why primitive macrophage precursors are replaced by monocytes in fetal tissues with advancing embryogenesis.

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Evolutionary trajectory of the thymopoietic niche

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The thymus is a lymphoid organ unique to vertebrates. During evolution, the niche supporting T cell development has undergone dramatic changes. In mammals, the transcription factor Foxn1 is essential for the differentiation of thymic epithelial cells, and thus, efficient T cell development.
We hypothesize that during evolution the elaboration of a supporting microenvironment predates innovations in the haematopietic system. In order to test this idea, we subjected the evolutionary trajectory of the thymic microenvironment to direct experimental analysis, and asked whether the primordial, non-vertebrate version of the Foxn1 transcription factor might be pre-adapted to support some form of thymopoiesis. To this end, we complemented Foxn1 deficiency in mice using the transcription factor Foxn4 derived from the cephalochordate amphioxus (Branchiostoma lanceolatum, B.l.). The expression of B.l. Foxn4 supported the development of a microenvironment conducive to T cell development until the DP stage, but failed to support later stages of T cell development. Transcriptome analysis showed that B.l. Foxn4 drives the expression of only a subset of those genes that are known as targets of the mammalian Foxn1 transcription factor. This result suggested the possibility of a stepwise expansion of Foxn1´s targets, and hence, the functional complexity of the niche fostering T cell development. In support of this conclusion, we show that the replacement of mouse Foxn1 by Foxn4 and Foxn1 versions derived from shark (Callorhinchus milii) and lamprey (Lampetra planeri) results in generation of CD4 and CD8 single-positive T cells, albeit with different efficiency. We propose that the evolutionary reconstruction will allow us to assess the contribution of different gene expression profiles to thymus and T cell development.

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BCAP promotes Lupus-like disease and TLR-induced IFNa production in pDC

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Systemic lupus erythematosus (SLE) severity is correlated with elevated serum levels of type I interferons (IFN), specifically IFNa. pDC are important in the pathogenesis of SLE due to their ability to produce large amounts of IFNa in response to endocytosed nucleic acids. TLR7 and TLR9-induced IRF7 translocation to the nucleus and subsequent IFNa production by pDC is dependent on phosphatidylinositol-3 kinase (PI3K), but how PI3K is activated and regulates this process remains undefined. We showed that the cytosolic signaling adapter B cell adaptor for PI3K (BCAP) links TLRs to PI3K activation in macrophages, thus we asked whether BCAP plays a role in pDC IFNa production and SLE pathogenesis. Here, we show BCAP promoted many aspects of TLR7-driven lupus-like disease including interferon-stimulated gene expression in the blood. Consistent with these findings, BCAP promoted TLR7 and TLR9-induced IFNa production in pDC, and BCAP-/- mice produced significantly less serum IFNa after injection of TLR9 agonist than WT mice, consistent with a pDC IFNa defect. RNA or CpG DNA-induced IFNa production in pDC requires two signaling pathways. In the endosome, RNA or DNA is recognized by TLR7 or TLR9, respectively, leading to MyD88 activation. RNA or DNA signaling from the cell surface activates a TLR7/9-independent cascade involving DOCK2-mediated activation of Rac1. These two pathways are both required for and converge upon the phosphorylation and activation of IKKa leading to the phosphorylation and nuclear translocation of the transcription factor IRF7 and IFNa gene transcription. We show BCAP and PI3K activation were required for CpG DNA-induced early actin remodeling, a readout of Rac1 activation, and IKKa phosphorylation in pDC. We found BCAP associated with DOCK2 through its N-terminal domain and was required for early TLR-induced Rac1 activation in pDC. Overall, we reveal a novel role for BCAP in regulating SLE pathogenesis and TLR-induced IFNa production in pDC by regulating DOCK2-mediated Rac1 activation.

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Deltex1 Regulates Foxp3 and Regulatory T cell Stability by Antagonizing HIF-1α

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Regulatory T cell (Treg) is one of the major components in immune tolerance machinery by suppressing inflammatory T cell activation. Foxp3 is the master transcription factor essential for the functional activities of Tregs, but the maintenance of Foxp3 protein stability in vivo remains incompletely understood. Here we identified E3 ligase deltex1 (DTX1) determining Foxp3 protein stability and Treg suppressive function in vivo. Dtx1-/- Tregs are as effective as WT Tregs in the inhibition of CD4+CD25- effector T cells activation in vitro. However, the ability of DTX1-knockout Tregs to inhibit effector T cells-induced colitis is greatly impaired in vivo. The selective deficiency of DTX1 within Tregs is sufficient to dramatically inhibit tumor growth. We find that decrease in Foxp3 expression and small increases in the expression of IFN-γ, IL-17, TNF-α and IL-4 in the Dtx1-/- tTreg cells recovered from Rag1 -/ - mice . We further find that DTX1 interacts with HIF-1α and promotes HIF-1α degradation. DTX1 increases Foxp3 stability through interfering with HIF-1α mediated Foxp3 downregulation. Knockout of HIF-1α rescues the Foxp3 instability and the defective suppressive ability in Dtx1-/- Treg in vivo. Our results reveal another level of control on Treg stability in vivo by sustaining the expression of Foxp3 protein stability in Tregs by DTX1.

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The gain-of-function STAT5B N642H mutation as a driver of mature T-cell leukemia/lymphoma

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Mature T-cell leukemias/lymphomas (MaTCL) are a group of rare hematological malignancies of mostly incurable prospects due to limited efficient therapies and faithful pre-clinical models. It is increasingly evident that hyperactive JAK/STAT signalling plays a role in many of these diseases. Notably, a hotspot gain-of-function (GOF) mutation in STAT5B, N642H, has been found in over 100 patients with T-cell neoplasia, and is associated with more aggressive disease, therapy resistance and worse prognosis. To investigate the role of STAT5B N642H in MaTCL, we generated transgenic mice harboring moderate expression of human STAT5B N642H in the hematopoietic compartment. These mice rapidly develop aggressive mature CD8+ T-cell disease, suggesting that CD8+ T-cells are particularly susceptible to transformation by this mutation. Examination of these mice revealed prominent lethal infiltration of neoplastic T-cells into peripheral organs including lung, skin, brain and liver. Interestingly, in addition to CD8+ T-cells, infiltration of CD4+ as well as γδ T-cells was also observed in various organs of the STAT5B N642H mice, suggestive of STAT5B N642H-driven transformation of other T-cell lineages. This would be more consistent with human patients carrying this mutation, which suffer predominantly from MaTCL of CD4+ as well as aggressive γδ T-cell subtypes. Indeed, isolation and transplantation of different mature T-cell subsets (CD8+, CD4+ or γδ T-cells) from donor STAT5B N642H transgenic mice into immunocompetent recipients resulted in the onset of subtype-specific T-cell neoplasia, demonstrating the transforming capacity of STAT5B N642H in multiple T-cell lineages. Overall, these data highlight the aggressive nature of the STAT5B N642H driver mutation in MaTCL. Furthermore, investigating and modelling the transformation of various T-cell lineages by STAT5B N642H will allow us to develop novel pre-clinical models more closely recapitulating human MaTCLs, which are urgently needed to assist with testing new treatment strategies.

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Short term cold acclimation enhances human Treg induction

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Foxp3+regulatory T cells (Tregs) function as critical immune regulators, thereby controlling tissue-specific differentiation, homeostasis and local inflammation. We recently found that murine Tregs represent key components of the molecular interface connecting adipose tissue (AT) function with environmental cold or low-dose beta3-adrenergic stimulation. Specifically, by loss- and gain-of-function experiments, including Treg depletion and transfers in vivo, we identified a T cell-specific Stat6/Pten signaling axis that links cold exposure or beta3-adrenergic stimuli with Treg activity and AT function. However, the translational relevance of these findings for human Treg induction in response to beta3-adrenergic stimulation or cold remains currently unknown.

Here, we show that beta3-adrenergic stimulation using Mirabegron (Mira) induces human Tregs in a preclinical setting of humanized NSG mice (human CD3+CD4+CD-127lowCD25high Foxp3+Tregs [% of CD4+T cells]: control: 2.0±0.5 vs. +Mira: 4.9±1.0; p=0.0319) accompanied by increased Treg induction potential from naïve CD4+T cells in vitro. Moreover, human CD4+T cell analyses of subcutaneous AT biopsies after an acute cold stimulus of 2 hours to healthy subjects provide first evidence for a trend towards an increase in local Tregs (CD3+CD4+CD127lowCD25highFoxp3+Tregs [% of CD3+CD4+]; t0=4.36±0.5% vs. t2=5.19±1.7%). Of note, short-term human cold acclimation in vivo also enhanced human Treg induction potential from naïve CD4+T cells in peripheral blood (females; CD3+CD4+CD127lowCD25highFoxp3+Tregs [% of CD25highCD127low]; t0=39.0±3.9% vs. t2=47.5±4.0%; p=0.0002). As observed in the murine system upon beta3-adrenergic stimulation, in vivo cold exposure triggered an increase in BORCS6 expression in human CD4+T cells. These findings support the concept that cold exposure or beta3-adrenergic stimulation can exert pro-tolerogenic functions on human CD4+T cells. Further mechanistic analyses are required to dissect molecular underpinnings of human Foxp3+Treg induction in response to cold or beta3-adrenergic stimulation in health and metabolic disease.

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Targeting miRNA-mediated immune activation in islet autoimmunity

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Islet autoimmunity, the presymptomatic phase of type 1 diabetes (T1D), is characterized by aberrations in T-cell activation versus T-cell tolerance. However, the molecular underpinnings remain poorly understood. MiRNAs regulate multiple targets and thereby alter complex cellular states. Here, using CD4+T cells from children at an early stage of islet autoimmunity we find miRNA181a and miRNA142-3p to be increased, thereby interfering with Foxp3+regulatory T cell (Treg) induction. Mechanistically we show that a miRNA181a-mediated increase in signal strength of stimulation and costimulation links Nuclear factor of activated T cells 5 (NFAT5) with impaired Treg induction and autoimmune activation. Enhancing miRNA181a activity increases NFAT5 expression while inhibiting Treg induction in vitro. Accordingly, Treg induction is improved using T cells from NFAT5ko animals (CD4+CD25+Foxp3+Tregs [% of CD4+T cells]: WT: 17.1±2.8 vs. NFAT5ko: 30.3±1.2, P<0.01) while altering miRNA181a activity does not affect Treg induction in NFAT5ko T cells. Furthermore, we demonstrate that a miR142-3p-mediated decrease in Tet2 expression links epigenetic remodeling and impairments in Treg induction and stability with islet autoimmunity. Inhibiting miRNA142-3p increases in vitro Treg induction while increasing Tet2 expression and Treg stability in restimulation experiments (control inhibitor vs. miR142-3p inhibitor: 88.3 ± 0.9% vs. 92 ± 1.2% of CD4+; P = 0.0440). In line with a role of Tet2 in DNA demethylation, higher abundance of miRNA142-3p during ongoing islet autoimmunity resulted in reduced expression of Tet2 and increased methylation of the Treg-specific demethylated region (TSDR) in the Foxp3 locus of Tregs. Of note, blocking either signaling pathway with a NFAT5 inhibitor or a miRNA142-3p antagomir can increase Treg induction in murine and humanized in vivo models. Additionally, inhibiting miRNA142-3p in vivo increases Treg stability. These findings therefore suggest that future targeting of miRNA181a/NFAT5 and miRNA142-3p/Tet2 signaling can help to interfere with ongoing T1D islet autoimmunity.

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Modulation of allergy and vaccine responses through maternal helminth infection

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Chronic infection with the parasitic helminth Schistosoma mansoni is characterized by a modified Th2 response coupled to immunosuppression. This protects the host against overwhelming inflammatory responses against the parasite, but has spillover effects to bystander antigens, such as allergens. There is recent evidence that schistosomiasis during pregnancy similarly influences offspring allergic responses, as well as to vaccines. We have shown that OVA-induced allergic airway inflammation (AAI) in adult murine offspring from schistosome-infected mothers is strongly modified by the phase of maternal infection, and suppressed when pregnancy was initiated during late chronic stages. Further, this was associated with changes at the fetomaternal interface, specifically in terms of an infection-phase-specific shifts in placental transcriptional profile as well altered cytokine production to schistosome antigens. We have since further investigated the potential effect of this maternal infection within the immune cell compartments of these offspring, and in our in vivo model explored the responses to early sensitization stages of antigens as well as vaccines in these offspring. In line with epidemiological trends, we have found differential T and B cell responses to vaccination of offspring exposed to transmaternal schistosomiasis, with these changes relying heavily on vaccine vector and the mode of antigen delivery. Through altered vaccine strategies, we have been able to further modify these antigen-specific responses, and our continued work investigates the mechanisms underlying this. Our studies will help to understand the effects of the maternal immune status during pregnancy on immune predisposition in later life, and mechanisms for fine tuning immune responses.

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Erythro-myeloid progenitors emerge from Toll-like receptor 2+ c-kit+ cells at E7.5

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Hematopoiesis in mammalian embryos proceeds through three successive waves of hematopoietic progenitors. Specifically, primitive erythroid progenitors emerging at E7.5 in the yolk sac (YS) are followed by a second "transient definitive" wave of mostly erythro-myeloid progenitors (EMPs) at E8.5. Finally, progenitors of definitive hematopoietic stem cells emerge from the hemogenic endothelium of dorsal aorta at E10.5. Since the emergence of hematopoietic progenitors is spatially and temporally overlapping and phenotypic markers are often shared, the specifics regarding their origin, development, lineage restriction and mutual relationships have not been fully determined. The identification of wave-specific markers would aid to resolve these uncertainties. Here, we show that Toll-like receptors (TLRs) are expressed during early embryogenesis. We provide phenotypic and functional evidence that the expression of TLR2 on E7.5 YS derived c-kit+ cells, accompanied by the high expression of Tie2, CD31, CD41 and Runx1, marks the emergence of EMPs. Additionally TLR2 engagement leads to the enhanced production of myeloid cells from E8.5 TLR2+ c-kit+ cells in a Myd88 dependent manner. Using in vivo fate mapping, we show that at E7.75-E8.5 Tlr2 locus is already active in emerging EMPs, which contribute to tissue resident macrophages in the brain as well as granulocytes, monocytes and macrophages in the fetal liver. Together, these data demonstrate that the activation of Tlr2 locus at ~E7.5 tracks the earliest events in the process of EMP specification.

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Quantifying the Effect of TCR Avidity on the Development of Immunological Memory starting out from single naïve T cells

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During a CD8+ T-cell response the magnitude of immunological memory is determined by the precursor frequency of T cells for a given epitope, as well as the processes of recruitment, clonal expansion and memory differentiation. A key factor that influences these processes is the binding avidity of a given T-cell receptor (TCR) to its cognate peptide MHC (pMHC) antigen. In how far TCR avidity determines the memory potential of an individual CD8+ T cells is incompletely understood.
Utilizing the adoptive transfer of single OTI T cells and Listeria monocytogenes strains expressing either the native SIINFEKL antigen or altered peptide ligands, showing decreased affinity to the OTI TCR, we studied how the memory development of single T cells is scaled by TCR avidity. We found that TCR-pMHC avidity is a critical determinant during the primary response and a strong predictor of response size, upon transfer of physiologically low numbers of OTI T cells. Lower affinity stimulation resulted in smaller progenies containing higher frequencies of central memory precursor (TCMp) cells. However, absolute numbers of TCMps were substantially under these conditions. Furthermore, we could show that differences in the absolute numbers of TCMps at the peak of the primary response, were directly mirrored in the absolute response size during heterologous secondary immunization. Importantly, we found that decreases in TCR avidity led to reduced recruitment of naive T cells into the primary response. These recruitment differences amplify avidity dependent expansion differences and therefore the size of the memory T-cell pool.

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Lineage-specific microRNA: mRNA regulatory networks in the differentiation of effector T cell subsets

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The (patho)physiological functions of T cells in infection, cancer and autoimmunity are often associated with the production of the potent pro-inflammatory cytokines IFN-γ and IL-17. MicroRNAs (miRNAs) have emerged as key post-transcriptional regulators of IFN-γ and IL-17 expression, but a global understanding of the underlying miRNA: mRNA networks is still lacking. Here we aimed to provide an unbiased and holistic analysis of miRNA regulators of effector T cell differentiation. We employed a double reporter IL-17-GFP: IFNg-YFP mouse (naïve or challenged) to isolate pure IL-17+ or IFN-γ+ T cells from the main T-cell lineages: CD4+, CD8+ and gamma-delta T cells. The various effector T cell subsets were analyzed by next generation sequencing (NGS) of both miRNA and mRNA repertoires, and the results were integrated using bioinformatics tools taking into account differential expression and potential interactions at the sequence level. Top candidates were validated experimentally via gain-of-function or loss-of-function approaches. These allowed us to identify novel miRNA determinants of effector T cell differentiation that were strikingly different across the various T cell subsets. Namely, the miRNAs differentially expressed between IL-17+ and IFNg+ subsets of CD4+ versus gamma-delta T cells were poorly overlapping, with none of the top differentials being shared between the two T-cell lineages. Moreover, IFNg expression was regulated by different miRNAs in each of the T cell lineages, as highlighted by the novel inhibitory roles disclosed for miR-181a and miR-451a in CD8+ T cells; and miR-326 and miR-450b in gamma-delta T cells. Each of these miRNAs was integrated in specific networks with putative candidate mRNA targets (including cytokine genes and their master transcription factors) that were validated independently and are here presented. Our study thus provides unique insight into the distinct miRNA: mRNA networks that control IFN-γ and IL-17 expression in the main T-cell lineages generating pro-inflammatory effector T cells in vivo.

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Interleukin-17 determines brain-derived neurotrophic factor levels and short-term learning

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The conventional notion of "immune privilege" of the brain has been revised to accommodate its infiltration, at steady state, by immune cells that participate in normal neurophysiology. Surprisingly, such neuroimmune functions have been linked to typically "pro-inflammatory" cytokines like IL-4 or IFN-gamma, shown to control behavioural and social cognition. Here we identify a novel neuroimmune role for IL-17 produced by a foetal-derived mouse gamma-delta (gd) T cell subset that populates the meninges at birth and remains throughout life. Strikingly, the establishment of this meningeal IL-17+ gd T cell population was independent of microbiota or inflammatory signals such as IL-1 or IL-23. When tested in classical spatial learning paradigms, mice lacking gd T cells or IL-17 displayed deficient short-term working memory, while retaining long-term reference memory. Mechanistically, we show that the plasticity of glutamatergic synapses is reduced in the absence of IL-17, which results in impaired long-term potentiation in the hippocampus. Conversely, IL-17 enhanced glial cell production of brain derived neurotrophic factor (BDNF), whose exogenous provision rescued the phenotype of IL-17-deficient animals, including upon intra-cranial injection in vivo. By identifying a novel meningeal-resident, non-inflammatory IL-17+ gd T cell population, our work provides fundamental clues on cognitive dissociation of working versus reference memory in the hippocampus, while providing exciting new avenues to explore in Neuroimmunology.

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Selection for gain-of-function Notch1 ΔPEST mutations in T-ALL

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Acute lymphoblastic T cell leukemia (T-ALL) is a highly aggressive pediatric cancer. Despite intense research and the identification of typical mutations associated with the disease, the mechanisms underlying malignant T cell progenitor transformation and leukemia progression are not yet fully understood. Our laboratory has identified a mouse model for spontaneous development of T-ALL due to disrupted cell competition and deregulated thymocyte development in the absence of de novo progenitor supply from the bone marrow. T-ALL in this novel murine model resembles the human disease in key aspects from pathology to genomic lesions. These include characteristic frameshift mutations within the last exon of Notch1, causing PEST domain deletions (ΔPEST) due to the presence of STOP codons in the alternative reading frames of Notch1. To investigate the role of such ΔPEST truncations for T-ALL development, we introduced into mice a modified Notch1 allele, devoid of alternative STOP codons. Prior to mutation, this modified allele is fully functional, but signaling incompetent when frameshift mutated. In our disease model, heterozygous and wild type thymi developed T-ALL with equal kinetics and incidence, but Notch1 mutations were selectively found only on the wild type allele. Interestingly, even thymi homozygous for the modified Notch1 allele gave rise to T-ALL with the same kinetic and incidence and even displayed a similar Notch1 mutation frequency. However, in this situation, all mutations found in the PEST domain of the modified Notch1 allele were direct STOP codons. Such direct ΔPEST mutations are also known from human patients, albeit at much lower frequencies compared to frameshifts. We conclude that T-ALL transformation or progression is highly dependent on functional Notch1 signaling and therefore strongly selective for functionally active ΔPEST Notch1 mutations.

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Circulating monocytes and lymphocytes in first-degree relatives of type 2 diabetes patients

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Aim: Development of type 2 diabetes (T2DM) is associated with disturbances of immune status, that may be reflected by alterations of the profile of circulating immune cells. In order to study whether there exists genetic predisposition to these alterations we investigated relative content of circulating monocyte and lymphocyte subpopulations at fasting condition and upon stimulation by short-term hyperinsulinemia in non-diabetic first degree relatives (FDR) of T2DM patients and in control subjects.

Materials and methods: 19 non-diabetic male FDR and 19 control subjects without family history of diabetes matched for age and BMI underwent 2hours' hyperinsulinemic-euglycemic clamp. Blood samples taken before and at the end of the clamp were used for flow cytometry analysis of lymphocyte and monocyte populations and for cytokine measurement.

Results: At fasting conditions FDR showed higher CD4/ CD8 ratio of peripheral lymphocytes, higher percentage of Th17 lymphocytes and lower content of intermediate monocytes when compared to controls. The CD4/CD8 ratio correlated to fat mass, insulin and HOMA-IR in the entire group of subjects. Hyperinsulinemia decreased a relative content of peripheral CD4+ and increased a relative content of CD8+ T lymphocytes, thus decreasing the CD4/CD8 ratio by 18-22% in both groups of subjects, the magnitude of decrease not being different in the two groups. In FDR but not in controls , the hyperinsulinemia-induced decrease of CD4+ T lymphocyte content was partially due to the decrease of TH2 and TH17 lymphocytes subpopulations. In control subjects but not in FDR, the number of intermediate monocytes has declined in response to hyperinsulinemia.

Conclusion: The alterations in the balance in CD4/CD8 lymphocytes ratio and in the content of TH17 cells and intermediate monocytes at fasting condition and divergent response of TH17 cells and intermediate monocytes under stimulation by insulin may be features of genetic predisposition to T2DM.

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The transcription factor Duxbl mediates elimination of pre-T cells that fail β-selection

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T-cell development is critically dependent upon successful rearrangement of antigen-receptor chains. At the β-selection checkpoint only cells with a functional rearrangement continue in development. However, how non-selected T cells proceed in their dead-end fate is not clear. We identified low CD27 expression at the double-negative (DN) 3 stage to mark pre-T cells that have failed to rearrange their β-chain and that will therefore undergo apoptosis. Expression profiling and single-cell transcriptome clustering identified a developmental trajectory through β-selection and revealed specific expression of the transcription factor Duxbl at a stage of high recombination activity prior to β-selection, whereas its expression was not detected in cells that have passed this checkpoint. Conditional transgenic expression of Duxbl resulted in a developmental block at the DN3-to-DN4 transition due to reduced proliferation and enhanced apoptosis. RNA silencing of Duxbl, on the other hand, led to a decrease in apoptosis at the DN3 stage. Thus, Duxbl seems to mediate apoptosis induction in cells at the β-selection checkpoint. Transcriptome analysis linked Duxbl to elevated expression of the apoptosis-inducing Oas/RNaseL pathway. RNaseL deficiency as well as sustained Bcl2 expression led to a partial rescue of cells in Duxbl transgenic mice, thereby suggesting that the induction of apoptosis by Duxbl is at least partly mediated through the Oas/RNaseL system and is sensitive to Bcl2 expression. These findings identify Duxbl as a regulator of β-selection by mediating the elimination of cells with a non-functional rearrangement.

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Analysis of EV-decorated CD8+ T cells during viral infection

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The exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane is a classical feature of apoptosis but also common on extracellular vesicles (EVs). EVs have been shown to play important roles in cellular communication and are involved in the regulation of CD8+ T cell responses during infection and cancer. However, the investigation of EV-attached CD8+ T cells in complex and dynamic in vivo settings has not been reported yet. Here, we have generated a fusion protein of the PS-binding protein Mfge8 and the reporter molecule eGFP (MFGe8-eGFP) to analyze naturally occurring EV-T cells-interactions in situ. PS+ cells were detected by intravenous injection of Mfge8-eGFP followed by imaging flow cytometry. In order to reliably discriminate apoptotic from live EV-decorated cells, we developed a deep learning algorithm. By using this novel approach, only low frequencies of EV-bound T cells were detected in the absence of infection. In contrast, upon LCMV-infection we found an approximately 10-fold increase in the frequency of EV-decorated CD8+ T cells. EV-binding was mostly confined to activated effector CD8+ T cells, but almost absent on naïve CD8+ T cells. Furthermore, bound EVs were enriched for proteins normally found on antigen presenting cells (APCs), suggesting an APC-origin. Our data demonstrate the propensity of activated CD8+ T cells to bind EVs. Moreover, we here introduce a new method to study EVs and their CD8+ T cell targets in vivo, which will give new insights into the complex immune modulating role of EVs in infection and antitumor immunity.

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STAT5-driven T cell neoplasia: a closer look at thymic T cell development

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The recurrent activating point mutation in human STAT5B, N642H, has been found in more than 100 patients with aggressive mature or immature T cell neoplasias, and is linked to poor survival and increased risk of relapse. Accordingly, transgenic mice expressing hyperactive STAT5BN642H or STAT5AS710F under the vav promoter develop lymphoid neoplasia with massive expansion of mature CD8+ T cells in the periphery, leading to organ infiltration and organ failure. The diseased cytotoxic lymphocytes are fully functional and hypersensitive to cytokines, and the mice succumb to pulmonary infiltration and obstruction as the main cause of death. To understand lineage commitment of early T cell precursors (ETP) harboring STAT5 mutations, we analyzed thymi from diseased STAT5BN642H and STAT5AS710F mice and controls. We observed highly increased numbers of CD8+ single positive (SP) T cells and less CD4+ CD8+ double positive (DP) cells compared to wildtype (WT) controls, underlining the strong bias towards commitment to the cytotoxic T cell lineage in both models.
Under physiologic conditions, murine CD4- CD8- double negative (DN) cells undergo maturation from stage DN1 to DN4, defined by expression patterns of CD25 and CD44, as a prerequisite to successfully pass T cell receptor selection. Here, we show that this development is perturbed in STAT5BN642H mice, as CD25 expression is downregulated. This trend was not observed in STAT5AS710F mice, which display less aggressive tumor progression. Further, well defined medullary and cortical structures in the thymus are disrupted in STAT5BN642H but not STAT5AS710F mice.
Interestingly, in a Rag2-/- background, STAT5 hyperactive mice overcome arrest in DN stage and develop lethal thymic lymphomas. Diseased mice bear high numbers of thymic DP as well as CD8+ CD3- TCRβ- T cells, which are absent in Rag2-/- mice. Here, RNA-seq analysis of these populations is used to understand STAT5-driven transition from DN to DP thymocytes. Our results indicate that STAT5 hyperactivation 1) disrupts thymic T cell development in a mature T cell lymphoma model and 2) drives thymic lymphomagenesis of an immature T cell immunophenotype in Rag2-/- mice.

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T cell-expressed microRNAs critically regulate germinal center Tfh and B cell function and maintenance

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Cell fate decisions of T helper cells are particularly sensitive to precise dosing of regulatory factors, and are therefore subject to the fine-tuning activity of microRNAs (miRNAs) (1). We have previously shown that global miRNA expression in T cells was absolutely required for the generation of T follicular helper (Tfh) cells2. This phenotype was in striking contrast to Th1, Th2, and Th17 cells, which still develop in the absence of mature miRNAs (1). The miR-17~92 cluster promoted robust Tfh and T follicular regulatory (Tfr) cell differentiation and restrained subset-inappropriate gene expression in Tfh cells (2). Following up on these results, we recently showed that miR-17~92 is also an important regulator of Th17 cells and the conversion of Treg into Tfr cells (3,4). In contrast to miR-17~92, conditional deletion of miR-146a in T cells exhibited an opposing effect on Tfh cell differentiation (unpublished), thus providing another example of how Tfh cells are tightly regulated by miRNAs (5,6). Since it was not possible before to study the role of global T cell-expressed miRNAs in the maintenance of already established Tfh cells and ongoing germinal center (GC) responses, we have now developed a system that allows for the temporally guided depletion of mature miRNAs specifically in CD4+ T cells (unpublished). We found that T cell-intrinsic miRNA expression during ongoing LCMV infection was critical for the maintenance of Tfh cell identity by regulating the size of the antigen-specific T helper cell compartment as well as the phenotype of Tfh cells and their relationship to Th1 cells. In addition, CD4+ T cell-specific depletion of miRNAs resulted in dramatically impaired GC B cell responses. In conclusion, our results highlight miRNAs as important regulators of Tfh and Tfr cells, thus providing novel insights into the molecular events that govern T cell-B cell interactions and T helper cell plasticity.

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2 Baumjohann et al., Nat Immunol 2013
3 Montoya et al., J Immunol 2017
4 Essig et al., Immunity 2017
5 Maul & Baumjohann, Trends Immunol 2016
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Mapping memory and effector fate decisions of CD8 T cells

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During an adaptive immune response CD8+ T cells have to differentiate into effector and memory subsets to efficiently eradicate the pathogen and to provide long lasting protection against reinfection with the same pathogen. It was shown that in response to antigen challenge an individual naïve T cell can generate progeny encompassing all major effector and memory CD8+ T cell subsets (Stemberger et al., Immunity 2007). In addition to that single T cell-derived population expansion and differentiation show strong variability in vivo with many small single cell-derived progenies consisting of only some 100 to 1000 mainly memory-differentiated daughter cells and just a few single cell-derived progenies that consist of up to 100 000 daughter cells and are mainly effector-differentiated (Buchholz et al., Science 2013, Gerlach et al., Science 2013). To elucidate the origin of variability between and within individual single-cell derived progenies we used continuous life-cell imaging with and without the addition of lineage-specific markers to create genealogical trees, revealing directly the exact inter-division times of all individual cells as well as their ancestry during the first approx. 5 days after T cell activation. Thus we were able to show that even under strictly controlled and homogenous conditions CD8 T cells evolve a surprisingly high inter and intraclonal variability that is hardly reduced compared to complex in vivo conditions. Further we observed the emergence of slower and faster dividing subtrees within a single-cell derived progeny and connected the inter-division times to memory and effector-like phenotypes. Together our results indicate that adoption of distinct cell-cycle speed correlates with differentiation into memory and effector subsets.

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IL-2 complexed with anti-IL-2 mAb JES6-1 dramatically increases sensitivity to LPS induced shock and mortality

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It was reported that in vivo biological activity of IL-2 can be dramatically increased by association of IL-2 with anti-IL-2 mAbs and that these IL-2 complexes possess selective stimulatory activity determined by the clone of anti-IL-2 mAb used. IL-2/S4B6 mAb complexes were described to be highly stimulatory for NK and memory CD8+ T cells and intermediately also for Treg cells. Contrary to that, IL-2/JES6-1 mAb complexes are stimulatory solely for CD25+ cells. Thus, IL-2/JES6-1 mAb complexes highly selectively expand Treg cells in non-immunized naïve mice per se. Several groups shoved that these complexes could be effective in various models of autoimmune diseases and that they are capable to facilitate long-term acceptance of allogeneic graft in immunocompetent mice without any immunosuppression. This makes such complexes attractive for future clinical application. However, we have unexpectedly found that mice treated with IL-2/JES6-1 mAb complexes show dramatically increased sensitivity to LPS-mediated shock and mortality (10-50 times). Mice treated with IL-2/JES6-1 mAb complexes and subsequently challenged with 10 μg LPS have 5-10 times higher plasma concentration of TNF-α 90 min post LPS injection in comparison to control mice challenged with 200 μg LPS. Such mice thus very quickly develop profound hypothermia and 100 % mortality. On the other hand, IL-2/S4B6 complexes sensitize mice to LPS only very weakly. This shows that future development of IL-2-based immunotherapeutics should carefully check potential co-toxicity with LPS.

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Overcoming multidrug resistance with inhibitor of ABC transporters bound to HPMA copolymer carrier as a potential therapeutic approach in cancer treatment

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Multidrug resistance (MDR) is defined as the cross-resistance or insensitivity of cancer cells to the cytostatic/cytotoxic actions of various anticancer drugs which are structurally unrelated and have different molecular targets. The principal mechanism by which many cancers develop MDR is up-regulation of P-glycoprotein (P-gp) expression, a broad specificity trans-membrane efflux pump belonging to ATP-binding cassette (ABC) transporters which reduces intracellular concentration of wide variety of drugs below the effective cytotoxic threshold.
In the present study, we developed and tested HPMA copolymer conjugates bearing either cytostatic drug doxorubicin (Dox), P-gp inhibitor 5-methyl-4-oxohexanoyl reversin 121 (R121), or both drugs bound via pH-sensitive hydrazone bond for overcoming MDR. Such conjugates have significantly improved solubility, prolonged half-life in circulation and they allowed targeted delivery of their drugs into solid tumor due to the enhanced permeability and retention effect (EPR).
We demonstrated that co-delivery of Dox and R121 bound to the same polymeric carrier, P-R121(Dox) results in superior cytostatic and cytotoxic activity in doxorubicin-resistant P388/MDR cell line. P-R121(Dox) conjugate could induce strong cell cycle arrest with almost 60% of cells accumulated in G2/M phase with a concomitant decrease of cells in G1 and S phase after 24 h incubation. Moreover, P388/MDR cells treated with P-R121(Dox) resulted in 18-fold decrease of live cell proportion as compared to untreated cells.
Finally, antitumor activity in mouse tumor models of drug-induced (P388/MDR) or natural resistant (CT26) was evaluated. The treatment with P-R121(Dox) conjugate resulted in a significant tumor growth reduction and complete tumor regression in 6 out of 8 mice in CT26 model, whilst P-Dox/P-R121 mixture at equivalent dose reduced the tumor growth to a lesser extent, and cured one animal only. In parallel, significant inhibition of tumor growth and prolonged survival was observed in P388/MDR lymphoma.

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Time-resolved transcriptome and proteome landscape of human regulatory T cell (Treg) differentiation reveals novel regulators of FOXP3

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Regulatory T cells (Tregs) expressing the transcription factor FOXP3 are crucial mediators of self-tolerance, preventing autoimmune diseases but possibly hampering tumor rejection. Clinical manipulation of Tregs is of great interest, and first-in-man trials of Treg transfer achieved promising outcomes. Yet, the mechanisms governing induced Treg (iTreg) differentiation and the regulation of FOXP3 are incompletely understood. To gain a comprehensive and unbiased molecular understanding of human FOXP3 induction, we performed time-series RNA sequencing (RNA-Seq) and proteomics profiling on the same samples during human iTreg differentiation. To enable the broad analysis of universal FOXP3-inducing pathways, we used five differentiation protocols in parallel. Integrative analysis of the transcriptome and proteome confirmed involvement of specific molecular processes, as well as overlap of a novel iTreg subnetwork with known Treg regulators and autoimmunity-associated genes. Importantly, we propose 37 novel molecules putatively involved in iTreg differentiation. Their relevance was validated by: a targeted shRNA screen confirming a functional role in FOXP3 induction; discriminant analyses classifying iTregs accordingly; and differential expression in T cells from inflammatory bowel disease patients.
The data generated by this approach facilitate understanding the molecular mechanisms underlying iTreg generation as well as the concomitant changes in the transcriptome and proteome. Our results provide a reference map exploitable for future discovery of markers and drug candidates governing control of Tregs, which has important implications for the treatment of cancer, autoimmune and inflammatory diseases.

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The miR-26 family affects pre-B cell differentiation and expansion

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MicroRNAs (miRNAs) are short, non-coding RNAs of about 21-24 nucleotides length that regulate gene expression and thereby affect physiological as well as pathological processes such as cancer. Several miRNAs have been described to mediate pro-tumorigenic as well as tumor-suppressive functions. One miRNA family involved in cancerogenesis is the miR-26 family, which is aberrantly expressed in different types of cancer. However, little is known about the effect of altered miR-26 expression in the context of immune cell malignancies. Furthermore, despite its prominent expression in early lymphocytes, the role of miR-26 in hematopoiesis is still unknown.
By combining in vitro and in vivo approaches we could demonstrate an important role for the miR-26 family in early B cell development. Pre-B cells overexpressing miR-26a or b showed reduced differentiation, increased proliferation and a partial inhibition of apoptosis upon IL-7 withdrawal, eventually resulting in their oncogenic transformation. Conversely, a functional knockdown of the miR-26 family by a competitive sponge construct enhanced B cell differentiation. These observations are supported by findings in our miR-26 sponge mouse model. In analogy to our in vitro data, these mice displayed an increased number of immature B cells and a decrease in pre-B cells, indicating enhanced B cell differentiation. Furthermore, the miR-26 family not only plays a role in early B cell development but also in mature B cells, as splenocytes derived from miR-26 sponge mice showed reduced proliferation upon LPS stimulation.
Together, these results clearly indicate the importance of the miR-26 family in early B cell development and suggest a pivotal role in regulating pre-B proliferation versus differentiation. Furthermore, we suggest a potential oncogenic role of miR-26 in B cell leukemia by conferring a hyper-proliferative and anti-apoptotic pre-B cell phenotype.

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Dissecting the T cell-extrinsic requirements for Tfh cell fate decisions

Yinshui Chang and Dirk Baumjohann

T follicular helper (Tfh) cells are critical for humoral immune responses by initiating and maintaining the germinal center reaction to generate high-affinity antibodies and B cell memory. It is hypothesized that Tfh cell fate is a result of a combined action of continued interactions with antigen-presenting cells (APCs) such as dendritic cells (DCs) and B cells, T cell receptor signaling strength, transcriptional regulators (LEF-1, TCF-1, Bcl6), and miRNAs (miR-17~92, miR-155, miR-146). While there is a growing body of knowledge on the molecular mechanisms in T cells that drive Tfh cell differentiation and function, the T cell-extrinsic signals that induce Tfh cells remain largely unknown. We could previously show that at early stages of the immune response, DCs were potent inducers of the Tfh cell phenotype in activated CD4+ T cells, independently of cognate interactions with B cells. However, it remains largely unknown what composition of stimuli is required to initiate Tfh cell differentiation. Here, we investigated the costimulatory and coinhibitory surface molecules expressed on the DCs and their contribution to Tfh cell fate decisions. We used a hematopoietic progenitor cell line and modified its surface receptor composition using CRISPR/Cas9. Using the DCs derived from the modified cell line, we systematically evaluated changes in their ability to prime Tfh cells und thus shape their identity.

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PTBP1 ensures proper B cell development by guaranteeing precise processing of the transcriptome in Pro-B cells

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Accurate control of proliferation is crucial during B cell ontogeny. Pro-B cells are quiescent when rearranging the immunoglobulin heavy chain, while early Pre-B cells proliferate vigorously after successful recombination of the heavy chain. Today we understand the contributions of signalling pathways and transcription factors that control B cell development and proliferation. By comparison, our understanding of how RNA-binding proteins control B cell development is very limited.
Polypyrimidine Tract Binding Proteins (PTBPs) are a family of RNA-binding proteins that control alternative splicing, alternative polyadenylation, mRNA abundance and IRES-driven translation. PTBP1 is ubiquitously expressed and is necessary in germinal centre B cells to generate a high affinity antibody response (Monzon-Casanova et al. Nat Immunol 2018). If PTBP1 is deleted or absent from a cell, the neuronal paralog PTBP2 is expressed and compensates for many functions of PTBP1. To assess the functions of PTBP1 during B cell development we have generated a double conditional knock out (cKO) mouse model by deleting Ptbp1 and Ptbp2 using mb1-cre. Ptbp1 and Ptbp2 cKO mice have a complete blockade in B cell development at the Pro-B cell stage. Pro-B cells from Ptbp1 and Ptbp2 cKO mice show a dramatic deregulation of proliferation with an enhanced entry into S-phase and a block at the G2 stage.
To elucidate the molecular targets of PTBP1 in Pro B cells we have performed RNAseq on Pro-B cells from cKO mice and controls and used PTBP1-iCLIP to identify direct binding sites of PTBP1 in the whole transcriptome. This has revealed numerous direct targets of PTBP1 that are differentially alternatively spliced and show altered mRNA abundance in the absence of PTBP1 and PTBP2. Thus, in developing B cells, PTBP1 acts as a guardian of the transcriptome ensuring proper B cell development by controlling accurate alternative splicing and abundance of numerous transcripts necessary for promoting quiescence in Pro-B cells.

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Quantitative analysis of protein synthesis in naïve T cells reveals posttranscriptional mechanisms for rapid activation

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Resting naïve T cells are metabolically inert but need to be prepared for an immediate response to antigens by executing a complex activation program. By using a pulsed SILAC mass spectrometry approach, we estimated absolute protein synthesis rates (protein copies per minute) for the entire proteome of naïve and activated human CD4+ T cells. While the majority of the proteome of naïve T cells was stable, roughly 1 % of the proteins turned over rapidly, including proteins that shape the identity and function of CD4+ T cells. We further extended our dataset with an estimation of mRNA copy numbers leading to the discovery that naïve T cells contain large numbers of idle ribosomes and mRNA, which rapidly engaged upon activation to ramp up the activation program. Finally, we showed that despite the low glycolytic activity, naïve T cells contained large numbers of all glycolytic enzymes except the glucose transporter SLC2A1/3 and hexokinase 2, which is the initial enzymes of glycolysis. Collectively, our study reveals a minimal maintenance program in naïve T cells and identifies protein turnover, preformed stores of enzymes and an idling translational machinery as key elements that poise T cells for rapid responsiveness.

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Dendritic cell migration to CNS and gut is differentially dependent on alpha4-integrins

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Leukocyte migration into different immune compartments is a highly regulated process, licensed by the expression of specific molecular anchors. In the context of autoimmune pathogenesis in the CNS it has been well established that T cell infiltration is mediated by the alpha4 (a4) integrin heterodimer VLA-4 (a4b1) in both mice and men. Likewise, T cell homing to the gut has been shown to be dependent on its alternative heterodimer LPAM-1 (a4b7). However, aside from antibody blocking studies, the role of a4 integrins for the migration of myeloid antigen-presenting cells has not been addressed in detail. By genetically ablating Itga4 in dendritic cells (DCs) and macrophages via CD11c and Lyz2 promoters, respectively, we sought to elucidate its role in homeostasis and disease. While direct competition of a4-deficient and -sufficient cells in mixed bone marrow chimeras (MBMCs) revealed an infiltrative advantage of a4-expressing monocyte-derived dendritic cells over their KO counterparts in experimental autoimmune encephalitis (EAE), we found no alteration of the myeloid compartment in the CNS in steady state and EAE in the absence of a4-expressing DCs in a non-competitive setting. Accordingly, no clinical phenotpye was observed in conditional knock-out (cKO) mice as compared to their wild type (WT) littermates. These data indicate that CNS-targeting DCs can rely on other homing-factors in the absence of a4. In small intestine and colon, CD103+CD11b+ DCs showed a marked reduction compared to CD103+CD11b- DCs in naive cKO mice, a finding confirmed by direct competition in MBMCs. Subjecting these mice to a model of gut and systemic inflammation by repetitively injecting anti-CD3 antibody did not reveal an altered disease course compared to WT littermates. Likewise, clearance of citrobacter rodentium infection was similar in cKO mice and WTs. We conclude that while the intestinal DC compartment is altered in homeostasis, inflammatory conditions can promote an environment that is indifferent to a4 expression by DCs, allowing functional immune responses to proceed in its absence.

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Effector differentiation potential determined at the level of human CD8+ naïve T cell subsets

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Antigen recognition by CD8+ naïve T cells induces immune activation and rapid effector differentiation that ensures elimination of virally-infected and malignant cells. While signals from the extracellular milieu and downstream of the TCR have long been known to play a crucial role, it is currently unknown whether heterogeneity within the naïve CD8+ T cell pool shapes the potency of effector differentiation. We report the identification of two discrete subsets of human CD8+ TN cells, defined by positive and negative expression of the chemokine receptor CXCR3, with different potential to generate fully-differentiated effector T cells following antigen-specific stimulation. The CXCR3+ are more abundant than the CXCR3– naïve in peripheral blood and lymphoid tissues, retain enhanced expression of effector-related genes ex vivo and display features of the T cell receptor β chain repertoire indicating enhanced capacity to bind the peptide:major histocompatibility complex. Our findings imply that CD8+ T cell effector differentiation potential in humans is shaped by heterogeneity in the preimmune repertoire.

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Survival kinetics of innate lymphoid cells (ILCs) in explant cultures of normal human skin (NHS)

Natalia Alkon, Wolfgang Bauer, Georg Stingl

ILCs represent a relatively newly discovered cell type. They are defined as lineage-negative (Lin-), CD45+ cells of lymphoid morphology, which express the alpha-chain of the IL-7 receptor (CD127) and lack antigen-specific receptors. In NHS ILCs are present in very small amounts. Reportedly, their numbers are substantially increased in inflamed tissues. This phenomenon can be explained either by an inflammation-driven influx of ILCs from blood to skin, or by an expansion of ILCs/ILC precursors residing in the skin. To investigate this question, we used explant cultures of NHS as a model of tissue perturbation.
Sheets of NHS (up to 10x10 cm2), obtained from plastic surgery department, were dermatomed (0.3 mm) and cut into pieces. Then the tissue was either immediately subjected to enzymatic digestion (day 0 only), or alternatively, equal amounts of material were incubated in culture flasks for different time periods (day 2, 4, 6, 8, 10). Cell samples collected at different time-points were sieved and immunostained for the presence of ILCs.
Results obtained show that the presumed pan-ILC marker CD127 is not susceptible to enzymatic degradation. Thus, results from day 0 can be reliably compared to those from consecutive days. Our data indicate that viable CD45+, Lin-, CD127+ ILCs are dramatically increasing in culture over time. The percentages of ILCs among viable CD45+CD3- cells gradually increased from 2.6 (day 0) to 15.8 (day 2), to 44.9 (day 4), to 63.9 (day 6), and slightly declined until day 10. These data can be interpreted in different ways which are not mutually exclusive. First, ILCs of intact skin may be firmly anchored within collagen bundles and slowly released or, perhaps, actively crawling out from their hideaway upon perturbation. The second option, is that ILCs/ILC precursors undergo mitosis upon the delivery of stimuli generated in perturbed skin. Further investigations could possibly help to unravel the role of ILCs not only in cutaneous host defence, but perhaps also in the development and maintenance of homeostasis of the host's outmost organ.

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Novel inborn error of immunity linking aberrant cytoskeletal dynamics and severe immune dysregulation

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Disorders of immune regulation comprise a poorly characterized group of inborn errors of immunity with predominant autoimmunity and/or autoinflammation. Recently, study of patients with autoinflammatory syndromes carrying mutations in actin regulators have suggested that actin cytoskeleton integrity is key to prevent the onset of autoinflammation. However, discovery of additional molecules linking actin cytoskeleton with specific inflammatory pathways will be necessary to decipher the complex network controlling immune homeostasis in humans.
We discovered a novel gene defect affecting actin cytoskeleton dynamics in a patient with impaired immune regulation. Clinically, the patient suffered from recurrent fever, hepatosplenomegaly, infections with unknown etiology and severe amyloid A amyloidosis very early in life. Using whole exome sequencing, we identified a missense mutation in a gene encoding a guanine nucleotide exchange factor. We were able to show that this mutation leads to impaired downstream signaling indicating a loss-of-function mutation. Moreover, patient primary cells showed morphological abnormalities, including reduced polarization and decreased formation of actin-rich protrusions. Our study identifies a hitherto poorly studied protein as a key regulator of actin dynamics. Naturally occurring variants in actin-related genes will allow us to dissect the molecular mechanisms by which the highly controlled cytoskeleton meshwork interacts with the inflammatory machinery. To complement our findings from these monogenic diseases, we are using CRISPR/Cas9-based screens to discover additional actin-related genes involved in immune homeostasis.

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Antigen-dependence of cell-cycle speed during priming shapes CD8 T-cell memory

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While antigen-primed T cells proliferate at speeds close to the physiologic maximum of mammalian cells, T-cell memory is maintained in absence of antigen by exceedingly rare cell divisions. The transition between these fundamentally distinct proliferative programs has been difficult to resolve via population-based analyses. Here, we computationally reconstructed the proliferative history of single CD8 T cells upon vaccination and implemented a novel approach to directly measure cell-cycle speed in vivo. We found that the transition between acute proliferation and memory quiescence occurred gradually through changes in cell-cycle speed rather than abrupt division cessation. Slower cycling central memory precursors, characterized by an elongated G1 phase, segregated early from the bulk of rapidly proliferating effector subsets and selectively elongated their cell-cycle as soon as antigenic stimuli were removed. In line with these findings, we demonstrate that the duration of primary antigen availability but not that of inflammatory stimuli determines the number of cells entering the memory compartment. By identifying an antigen-dependent hierarchy of cell-cycle speeds among emerging CD8 T cell subsets, we provide crucial mechanistic insights important to guide future vaccination strategies.

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Sequence Analysis of Immunoglobulin Genes from Human Monoclonal Acetylcholine Receptor Specific Antibodies to Investigate the Development of Autoreactive B cells

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The central question of autoimmune diseases is how the normal tolerance mechanisms fail. In the case of autoantibody mediated diseases, such as myasthenia gravis, caused by antibodies against the acetylcholine receptor (AChR) or related proteins, it is well established that numerous potentially auto reactive B cells escape the central tolerance checkpoint during development and populate the periphery. The requirement for T cell help, combined with negative selection of auto reactive T cells in the thymus, ought to prevent these auto reactive B cells from class switch and high affinity antibody production. Two possible ways that this checkpoint could fail are (i) auto reactive B cells bind to and receive T cell help for a non-self antigen such as a pathogen, and the recognition of the self antigen is a coincidence; or (ii) the only antigen bound by the autoreactive B cell is the self antigen, and T cell help is obtained either via antigen co-capture or from auto reactive T cells that have escaped tolerance. The first of these hypotheses predicts that high affinity AChR-reactive B cells will develop from naive B cells whose germline B cell receptor (BCR) could be of higher or lower affinity for AChR than the mature BCR, because affinity for AChR does not contribute to obtaining T cell help. In this scenario the germline BCR need not bind AChR, since this autoreactivity could be acquired during somatic hypermutation. The second hypothesis requires the existence of naive B cells whose germline BCR has a high enough affinity for AChR to enable antigen capture, and predicts that subsequent mutations should increase this affinity.

To test between these hypotheses, we isolated AChR-reactive B cells from patients with myasthenia gravis by antigen-capture activation fluorescence cell sorting. We expanded single cells in vitro, deep-sequenced their transcriptomes, and examined the sequences of their expressed immunoglobulin genes. From 5 patients whose serum contained detectable titres of anti-AChR IgG, we isolated 34 IgM clones, and 5 IgG clones. From 3 healthy donors, we obtained 1 IgM clone. Sequences of the IgM clones examined so far involve germline V(D)J genes of both heavy and light chains. Testing between the two hypotheses described above will require analysis of the IgG clones, and the relationships between IgM and IgG, which are ongoing.

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Loss of NIK in thymic epithelial cells leads to fatal autoimmunity through aberrant Treg development

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Medullary thymic epithelial cells (mTECs) mediate central T cell tolerance through negative selection. mTecs are, however, also critical for the formation of peripheral tolerance by instructing the development of nTregs. Which of these two functions dominate is a matter of some debate.
We have generated a mouse model in which the development and function of mTECs are compromised by conditional ablation of the NF-κB inducing kinase (NIK, encoded by Map3k14) restricted to the TEC compartment (mTEC-NIK). In contrast to germ-line deficient NIK-/- mice, which develop largely normally and show no overt pathology, mTEK-NIK mice rapidly developed severe and fatal multi-organ autoimmunity shortly after birth, characterized by infiltration of effector T cells in various organs. The observed autoimmunity was clearly T cell mediated, as the survival of the mice could be significantly improved upon antibody mediated T cell depletion. However, whereas there was no obvious indication of augmented auto-reactivity through the loss of negative selection, Tregs, although emerging from the thymus, were dysfunctional as indicated by high-parametric single cell analysis of Treg markers and in vivo suppression assays. Conversely, adoptive transfer of normal Tregs prevented autoimmunity. Most strikingly, in thymic co-transplantation, Tregs primed by wildtype thymi were able to prevent autoimmune pathology by mTEC-NIK educated T cells. We thus postulate that Treg induction rather than negative selection of autoreactive T cell clones is the superior duty of mTECs in thymic T cell education.

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RelB deficiency in DCs protects from autoimmune inflammation due to spontaneous accumulation of tissue Tregs

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Foxp3+ regulatory T cells are well-known immune suppressor cells in various settings. We provide evidence that knockout of the relB gene in dendritic cells (DCs) results in a spontaneous and systemic accumulation of Foxp3+ regulatory T cells (Tregs) partially at the expense of microbiota reactive Tregs. Deletion of nfkb2 does not fully recapitulate this phenotype indicating that alternative NF-kappaB activation via the RelB/p52 complex is not solely responsible for Treg accumulation. Deletion of RelB in DCs results in an impaired oral tolerance induction and a marked type 2 immune bias among accumulated Foxp3+ Tregs reminiscent of a tissue Treg signature. Tissue Tregs were fully functional, expanded independently of IL-33 and led to an almost complete protection from experimental autoimmune encephalomyelitis. This protection was not due to inefficient DC priming in the absence of RelB as antibody-mediated ablation of Tregs was able to fully restore the disease. Thus, RelB-dependent pathways in DCs are an attractive target for treatment of autoimmune diseases but may come at risk of reduced immune tolerance in the intestinal tract.

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Cell-intrinsic stimulation of the cGAS STING axis as a trigger of systemic autoimmunity

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Aberrant activation of type I interferon (IFN) responses is increasingly recognized as a cause of autoimmunity. In SLE, a multi¬factorial, polygenic autoimmune disease, overproduction of type I IFN can be triggered by diverse pathomechanisms.
Two of these are deficiency for the cytosolic nucleases RNase H2 or Trex1, as illustrated by the rare monogenic SLE-like condition Aicardi-Goutières syndrome (AGS). Loss of function mutations in RNase H2 or Trex1 result in spontaneous genome damage and pathogenic type I IFN responses. Defects of RNase H2 result in impaired ribonucleotide excision repair, genome damage, cancer and induction of inflammatory responses by exposure of micronuclear DNA in the cytosol, activating the dsDNA-specific sensor cGAS. The IFN response of RNase H2-deficient cells is potentiated upon additional loss of p53, due to inefficient elimination of damaged and micronucleated cells.
Also upon loss of Trex1, the major cytosolic 3'-5' DNase, cGAS activation triggers disease. Loss of Trex1 is associated accumulations of cytosolic single strand DNA and spontaneous genomic DNA damage. We aim to understand the connection of DNA damage and the inflammatory response and try to identify the unknown nu¬cleic acid ligand that triggers cGAS in Trex1-/- cells. We found a mild increase in numbers of micronucleated cells in Trex1-/- mice but do not see enhanced IFN responses upon additional inactivation of p53 arguing against a major role of damaged cells as a source of the pathogenic IFN. Alternatively, oligonucleotide waste from DNA replication or repair might constitute cGAS ligands upon leakage into the cytosol of Trex1-/- cells. Inactivation of nucleotide excision repair in Trex1-/- cells and mice did not result in amelioration of the phenotype and we are now addressing end resection as a source of the immunostimulatory DNA and will also determine whether the IFN response of Trex1-/- cells is dependent on cell division.

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Accumulation of functional multi-potent hematopoietic progenitors in peripheral lymphoid organs of mice over-expressing IL-7 and Flt3-ligand

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Interleukin-7 (IL-7) and Flt3-ligand (FL) are two cytokines important for the generation of B cells, as manifested by the impaired B cell development in mice deficient for either cytokine or their respective receptors, and by the complete block in B cell differentiation in the absence of both cytokines. In order to further study their synergistic effect in lymphopoiesis in vivo, we generated mice constitutively over-expressing both IL-7 and FL. These double transgenic mice developed splenomegaly and lymphadenopathy characterized by tremendously enlarged lymph nodes even in young animals. Lymphoid, myeloid and dendritic cell numbers were increased compared to mice over-expressing either of the two cytokines alone, and for lymphoid cells the effect on their expansion was synergistic, rather than additive. We also found a strong effect of the two cytokines in the expansion of bone marrow hematopoietic progenitors, including Lineage-kit+Sca1+ (LSK), Common Lymphoid Progenitors (CLP) and pro/pre B cells, whereas Hematopoietic Stem Cells (HSC) were reduced by FL over-expression. Analysis of peripheral organs of these mice identified the presence of increased numbers of these progenitors in spleen and lymph nodes. When transplanted into irradiated wild-type mice, double transgenic lymph node cells showed long-term multi-lineage reconstitution of hematopoietic lineages, further confirming the presence of functional hematopoietic progenitors therein. Our double transgenic mouse model shows that sustained and combined over-expression of IL-7 and FL leads to a massive expansion of most bone marrow hematopoietic progenitors and to their associated presence in peripheral lymphoid organs where they reside and potentially differentiate further, thus leading to the synergistic increase in mature lymphoid and myeloid cell numbers. The present study provides further in vivo evidence for the concerted action of IL-7 and FL on lymphopoiesis and suggests that extramedullary niches, including those in lymph nodes, can support the survival and maintenance of hematopoietic progenitors that under physiological conditions develop exclusively in the bone marrow.

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Myeloid-derived suppressor cells control B cell accumulation in the CNS during autoimmunity

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PMN-MDSCs (polymorphonuclear myeloid-derived suppressor cells) have been characterized in the context of malignancies. Here we found that PMN-MDSCs had the unique ability to restrain B cell accumulation during central nervous system (CNS) autoimmunity. Ly6G+ cells were recruited to the CNS during experimental autoimmune encephalomyelitis (EAE), interacted with B cells that produced the cytokines GM-CSF and IL-6, and acquired properties of PMN-MDSCs in the CNS in a manner dependent on the signal transducer STAT3. Depletion of Ly6G+ cells or dysfunction of Ly6G+ cells through conditional ablation of STAT3 resulted in the selective accumulation of GM-CSF-producing B cells in the CNS compartment, which in turn promoted an activated microglial phenotype and failure to recover from EAE. The frequency of CD138+ B cells in the cerebrospinal fluid (CSF) of human patients with multiple sclerosis negatively correlated with the frequency of PMN-MDSCs in the CSF. Thus, PMN-MDSCs might selectively control the accumulation and cytokine secretion of B cells within the inflamed CNS.

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Antitumor activity of IL-2 and IL-7 immunocomplexes in combination with anti-CTLA-4 and anti-PD-1 mAbs

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Biologic activity of IL-2 and IL-7 in vivo is significantly increased when complexed with some of the respective anti-cytokine mAb. Different immune cell subsets can be preferentially stimulated depending on the anti-IL-2 mAb used to complex IL-2. IL-2/S4B6 mAb immunocomplexes (ic) induce preferential expansion of CD122high cells whereas IL-2/JES6-1 mAb ic highly selectively expand CD25high cells in mice. Similarly, IL-7/M25 mAb possess higher stimulatory activity for both naive and memory T cells in comparison to free IL-7. The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) molecules are inhibitory receptors which negatively regulate immune functions of T cells. Antibodies against these molecules represent promising immunotherapeutic tool for treating various malignant diseases. Regarding the fact that IL-2/S4B6 mAb ic show remarkable antitumor activity in numerous mouse tumor models, we examined possible synergism between IL-2 or IL-7 ic and CTLA-4 plus PD-1 blockade in tumor-bearing mice. We found out the expansion of recently activated CD8+ T cells mediated via IL-2 ic was further augmented by CTLA-4 plus PD-1 blockade. However, these two immunotherapeutic approaches didn´t show co-operative antitumor effects in mouse tumor models. Next, we figured out that IL-7/M25 mAb ic displayed higher biological activity in terms of promoting proliferation of peripheral CD4+ and CD8+ T cells in vivo compared to the cytokine alone. Thus, it makes IL-7/M25 mAb ic promising antitumor agent, together with the fact that it didn´t expand Treg cells,. Unexpectedly, IL-7/M25 mAb ic, but not free IL-7, worsened the antitumor activity of αanti-CTLA-4 plus anti-PD-1 mAbs in several mouse tumor models. This result demonstrate that administration of IL-7 ic might have immunosuppressive feature which needs to be further investigated.

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Enforced PGC-1alpha expression promotes CD8 T cell persistence and anti-tumor immunity

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CD8 T cells mount protective responses against viral/bacterial infections and cancers. Memory CD8 T cells can provide long-term protection against tumors, which depends on their enhanced proliferative capacity, long-term self-renewal and unique metabolic machinery to sustain cellular fitness. Memory CD8 T cells harbor higher mitochondrial mass and rely on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) to fulfill their metabolic demands. However, it remains unknown whether enforced mitochondrial biogenesis promotes CD8 T cell memory formation and persistence in vivo. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha) is a master regulator of mitochondrial biogenesis and controls pathways known to be crucial in memory formation, we thus hypothesized that overexpression of PGC-1alpha in CD8 T cells might improve cell fitness and memory lineage differentiation in tumor and infection settings. Indeed, PGC-1alpha overexpressing CD8 T cells persist better upon transfer and could mediate more robust recall responses to peptide vaccination or bacterial infection. More importantly, CD8 T cells with enhanced PGC-1alpha expression mediate better anti-tumor immunity and have an additive effect with anti-PD1 checkpoint blockade treatment. Altogether, our study provides important insights into rational design of effective therapeutic strategies against cancer by combining checkpoint blockade and adoptive transfer of CD8 T cells with better metabolic fitness.

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Tracking of CD4+ T cells during CNS autoimmunity – Using novel optogenetic tools

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During autoimmunity, the communication between epithelial surfaces and remote organs is mediated by immune cells, which are likely to relay environmental cues gathered at body surface areas to remote organs, including the central nervous system (CNS). Interference with immune cell trafficking remains one of the most powerful therapeutic strategies in chronic inflammation and autoimmunity. However, the impact of the origin of an immune cell on its functional phenotype in the remote target organ is unknown. Here, we use in vivo labeling at defined anatomical sites for "provenance" tracking of immune cells across compartmental borders in the context of experimental autoimmune encephalomyelitis (EAE). Specifically, we labeled T cells in mesenteric or inguinal lymph nodes (LNs) of T cell conditional mitoDendra2 reporter mice via photoconversion at disease onset and re-isolated photoconverted T cells from the CNS two days later. We found that antigen experienced CD44highCD62Llow T cells did not migrate between inguinal (draining) and mesenteric LNs (non-draining) while both homed to the spleen and the CNS. Interestingly, CNS-infiltrating effector T cells from mesenteric LNs were exclusively positive for alpha-4 integrin whereas effector T cells from inguinal LNs could either be positive or negative. Our experimental system will help to better understand the phenotype and function of T cells migrating into the CNS as a consequence of priming in a distinct peripheral immune compartment.

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Proximal Lck promoter-driven Cre expression is inefficient in gamma delta T cells and varies between neonatal and adult gamma delta T cells development

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During T cell development, Lck gene expression is temporally controlled by its proximal and distal promoters. The pLckCre transgenic mouse available from Jackson Laboratory, in which the proximal promoter of Lck drives Cre expression, is a commonly used Cre driver line to recombine floxed genes in T cells. pLckCre drives recombination early in thymocyte development, and is frequently used to delete genes in alpha beta and gamma delta T cells. We found that pLckCre failed to efficiently delete floxed genes during adult gamma delta T cell development, in contrast to a complete deletion in conventional as well as unconventional alpha beta T cells. Mechanistically, gamma delta T cells inefficiently transcribed the endogenous proximal Lck promoter compared to alpha beta T cells during adult thymic development. Still, a population of gamma delta T cells which had activated pLckCre was detected; many of which were located in non-lymphoid organs as well as pre-committed IL-17- or IFN-g-producing gamma delta T effector cells. We show that in newborn thymi both pLckCre and endogenous Lck proximal promoter expression were substantially enhanced. Thereby giving rise to gamma delta T cells with recombined floxed genes, which were increased in unique gamma delta T cell subsets, such as the IL-17-producing gamma delta T cells. Our data point out striking differences in Lck transcription between perinatal and adult gamma delta T cell development. Taken together, the data presented here sheds new light on gamma delta T cell development and stimulates a reanalysis of data generated using these pLckCre transgenic mice.

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Toll-like receptor signaling drives Btk-mediated autoimmune disease

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Bruton's tyrosine kinase (Btk) is a signaling molecule involved in development and activation of B-cells through B-cell receptor (BCR) and Toll-like receptor (TLR) signaling. We have previously shown that transgenic mice that overexpress human Btk under the control of the CD19 promoter region (CD19-hBtk) display spontaneous germinal center formation, increased cytokine production, anti-nuclear autoantibody formation and systemic autoimmune disease upon aging. As distorted TLR and BCR signaling are both implicated in autoimmune diseases, we studied the impact of TLR and BCR signaling on splenic B-cell characteristics.
We observed that phosphorylation of S6, a downstream target of Akt, was increased in CD19-hBtk B cells following BCR stimulation, but decreased following TLR stimulation, when compared with wild-type (WT) B cells. Likewise, the CD19-hBtk transgene enhanced B cell survival and proliferation upon anti-IgM stimulation, but had an opposite effect following CpG or CpG/anti-IgM stimulation. In splenic follicular and marginal zone B cells from aging CD19-hBtk mice BCR signaling stimulated in vitro IL-10 production in synergy with particularly TLR9 stimulation. The enhanced capacity of CD19-hBtk follicular B cells to produce the pro-inflammatory cytokines IFN-γ and IL-6 compared with WT B cells, was however not further increased upon in vitro stimulation. Finally, we used crosses with mice deficient for the TLR-associated molecule myeloid differentiation primary response 88 (MyD88) to show that TLR signaling was crucial for spontaneous formation of germinal centers, increased B cell cytokine production and the induction of anti-nuclear autoantibodies in CD19-hBtk mice.
Taken together, we conclude that high Btk expression does not only increase B cell survival following BCR stimulation, but also renders B cells more sensitive to TLR stimulation, resulting in increased IL-10 expression in activated B cells. Although BCR-TLR interplay is complex, our findings show that both signaling pathways are crucial for the development of pathology in a Btk-dependent model for systemic autoimmune disease.

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Investigating the molecular basis of Roquin-mediated control of T cell differentiation

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Post-transcriptional gene regulation by RNA-binding proteins (RBPs) controls T cell fate decisions. The RBPs Roquin-1 and -2 (genes Rc3h1 and Rc3h2, respectively) serve redundant functions. They bind via ROQ domains to 3'-untranslated regions (3'-UTRs) of mRNAs and control mRNA stability and translation. In T cells, Roquin proteins become inactivated by MALT1 cleavage downstream of T cell receptor (TCR) signaling and Roquin-1/2 double knock-out mice revealed accumulations of Tfh and Th17 effector T cells. We hypothesize that different degrees of TCR signal strength and MALT1-dependent cleavage of Roquin may be involved in the regulation of specific mRNA subsets and the molecular control of T cell differentiation.
Therefore, we investigate the effects of (i) graded Roquin reduction by increasing the deletion of Rc3h1 and Rc3h2 alleles, by (ii) introducing ROQ domain mutations to attenuate mRNA affinities and (iii) by stimulating T cells with increased TCR signal strength that correlates to Roquin cleavage. Finally, (iv) a CRISPR-Cas9-based MALT1-insensitive Roquin-1 knock-in mouse model will uncouple TCR signaling from Roquin inactivation.
Indeed, preliminary in vitro analyses of graded Roquin deletion suggested differential derepression for ICOS and IKBNS that remained repressed even at low Roquin abundance next to Ox40 which already responded to moderately decreased Roquin levels. Moreover, minimal Roquin amounts were still effective to suppress Th17 differentiation. Lastly, decreasing the affinities of Roquin for RNA binding by individual and combined ROQ domain mutations again revealed differential derepression of for example Ox40 or Regnase-1.
In future studies, we will decipher the gain-of-function effects of the established MALT1-insensitive Roquin-1 mouse model and we will seek to uncover how graded T cell stimulation regulates differential Roquin activities that mediate T lymphocyte fate-specifiying mRNA repression.

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TCR self-reactivity predispose CD8 T cells for virtual memory formation and modulate strenght of effector response

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CD8 virtual memory (VM) T cells have been recently established as an antigen inexperienced population but express markers reminiscent of central memory population (CD44+ CD62L+). It has been shown that VM cells can mount faster response towards a cognate antigen and produce IFNg compared to naïve cells. We have recently showed that formation of VM cells is intrinsically dependent on self-reactivity of their respective TCR where higher self-reactivity leads to formation of this population. Biological role of VM cells is still largely enigmatic. Therefore, we took advantage of our previously established model of retrogenic mice with ovalbumin reactive CD8 T cell clones to FACS sort naïve and VM cells of the same T cell clones and compared them in experimental model of autoimmune diabetes by adoptive transfer of limiting number of cells into RIP.OVA mice where ovalbumin is expressed under insuline promoter. Mice can then develop diabetes mediated by activated T cells after infection with Listeria expressing ovalbumin (Lm-ova). VM cells showed to be less efficient in destruction of pancreatic beta cells (i.e. more self-tolerant), than their naive counterpart in this experimental setup. Complementary, we measured effector response upon Lm-ova infection in congenic recipients and observed weaker upregulation of CD49d, core 2 O-glycosylated CD43, CD25 and PD-1, markers associated with efficient tissue infiltration and activation, but stronger expression of KLRG1. These results would indicate mechanism of peripheral self-tolerance for highly self-reactive clones to prevent autoimmunity but still worthy to keep them for fast effector response.

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Long-lived and stable evolution of a human T cell system by a putative single T cell precursor

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Background: We study the minimal requirements of human T cell immunity by analysis of patients with primary immunodeficiencies. In a patient with X-SCID1 due to a severe germline mutation in IL2RG we observed an attenuated phenotype due to autologous wild-type T cells. This can be explained by a somatic reversion in a (putatively) single T-cell progenitor.

Objectives: To study diversity and stability of the human T cell repertoire in this patient.

Methods: Serial Vβ analysis, TCR deep sequencing and Mass cytometry phenotyping of T cell subpopulations over 8 years.

Results: The patient had constant levels of ~400 CD8 and 200 CD4 T cells/µl (<5% naïve). T cell differentiation was stable with no phenotypic evidence of increased exhaustion or senescence over time. TCR deep sequencing showed increased clonality with about 3-fold reduced unique sequences compared to healthy donors. However, diversity was significantly higher than in a RAG-deficient Omenn syndrome patient and similar to a DiGeorge syndrome patient with severe thymic hypoplasia. While the CD4 repertoire remained stable over time, alterations in the CD8 repertoire were associated with dramatic expansions and contractions of single T cell clones. However, the 10-year longitudinal analysis revealed surprisingly stable overall repertoire diversity.

Conclusion: The putative single precursor provides a limited T cell system that has been surprisingly stable in phenotype and diversity, keeping the patient asymptomatic over a 10 year period. This longitudinal analysis provides a better understanding of human T cell immunity under limiting conditions.

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Investigating cell extrinsic effects of Roquin - mediated post – transcriptional gene regulation

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Post – transcriptional gene regulation by RNA – binding proteins is important for controlling a number of cell fate decisions during an immune response. The two paralogues Roquin – 1 and Roquin – 2 play a pivotal role in T cell activation and differentiation (Vogel et al., 2013). At steady state, Roquin proteins bind cis – elements in the 3' UTR of their target mRNAs conferring translational silencing and mediating mRNA decay. Upon T cell receptor (TCR) engagement, Roquin proteins are cleaved by the paracaspase MALT1, thus, de – repressing their target genes in a TCR signal strength dependent manner (Jeltsch et al., 2014). Regulation of Roquin targets such as Icos, Ox40, Ctla4 and Irf4 may drive cell fate decisions and effector functions of T cells. Loss or impairment of Roquin – 1 and Roquin – 2 function in murine CD4+ T cells leads to the development of severe autoimmune and auto - inflammatory pathologies, marked by a spontaneous activation of effector T cells and accumulation of T follicular helper cells (Tfh). Our preliminary data indicate that conditional loss of function of Roquin proteins in CD4+ T cells in addition to previously described cell intrinsic effects, also drives activation and Tfh differentiation of wild type T cells in a cell extrinsic manner. We hypothesize that Roquin – deficient cells produce one ore more factors that act on bystander T cells in trans. We aim to identify and characterise the trans – acting factor(s), elucidate its role in the development of autoimmune diseases and evaluate the trans – acting factor as a diagnostic and/or therapeutic tool.

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mTORC1-dependent RNA synthesis in cycling T cells

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Adaptive immune responses to infections are marked by rapid proliferation and expansion of lymphocytes. T cells have to briskly increase their biosynthesis of nucleic acids, ribosomes, fatty acids and proteins which in sum makes up blast formation. However, it is not clear how RNA synthesis and cell cycle regulation are connected and what the role of RNA polymerases in this process is. We show that mTORC1-deficient T cells expanded around three times slower in response to persistent antigen. We have also seen that the amount of total RNA in activated T cells is largely controlled by the mechanistic target of rapamycin complex 1 (mTORC1), but not by mTORC2. RNAseq data indicated that these quantitative differences apply to the transcription of all RNA biotypes, with rRNA comprising 83-87% of the T cells‘ RNA while its processing is not affected by mTORC1. Accordingly, RNA extraction and FISH experiments visualizing 47S precursor rRNA and polyA mRNA levels indicated that mTORC1-deficient cells express reduced 47S rRNA and mRNA levels. We have then shown that the transition between the G1 and S cell cycle phases is obstruced in mTORC1-deficient cells and thus their division rate is three times slower. Several metabolic inhibitors had similar effects on RNA synthesis. Altogether our data suggest that global anabolic biomass production by proliferating T cells is under mTORC1 control.

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Staphylococcus aureus-induced tissue resident memory T helper 17 cells (TRM17 cells) drive renal autoimmune disease

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Tissue resident memory T (TRM) cells represent a new type of memory cells that reside in peripheral organs without recirculating. They provide rapid on-site immune protection against previous exposed pathogens. However, it remains to be clarified whether TRM cells also interfere with responses unrelated to the primary infection, such as organ-specific autoimmunity.
Here, we combined flow cytometry and droplet-based single cell RNA-sequencing with epitope measurement (CITE-seq) to examine these TRM cells in human kidneys. We found high frequencies of CD4+ CD69+ TRM cells of the TH17 phenotype, which we identified based on homology to published core transcriptional and protein data sets. We operationally named them TRM17 cells. CD4+ TH17 cells are involved in the response to major human pathogens such as S. aureus, and play a critical role in autoimmunity such as crescentic glomerulonephritis (cGN). We established a mouse model of S. aureus infection that resulted in initially high renal bacteria titres and a profound accumulation of TH17 cells in the kidney. Renal TH17 cells persist long-term (>100 days) after clearance of the infection, present with the phenotype of TRM cells and partially protect against re-infection. Induction of autoimmune kidney disease (cGN) in mice which recovered from S. aureus infection, resulted in a more rapid and aggravated renal TH17 response and consequently developed a more severe course of cGN. By labelling renal cells in photoconvertible Kaede-transgenic mice, we were able to demonstrate that S. aureus induced TRM17 cells contribute significantly to the enhanced local IL-17 immune response in cGN.
Thus, pathogen-induced TRM17 cells in peripheral tissues are capable of rapidly responding to an antigenic unrelated challenge thereby driving renal autoimmune diseases. Our data suggest that TRM cells might have a previously unknown role in amplifying organ-specific autoimmunity.

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Role of Histone Methylation in Foxp3+ Treg Cell Development and Auto-immunity

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CD4+Foxp3+ regulatory T (Treg) cells develop both in thymus (tTreg) and peripheral lymphoid tissues (pTreg) and prevent catastrophic autoimmunity throughout life. A common hallmark of Treg cell development is the induction of Foxp3 expression upon appropriate antigen and cytokine stimulation, with Foxp3 acting as a stabilizer/amplifier of Treg cell-specific gene expression, including the Foxp3 gene itself. Besides several conserved non-coding regions (CNS1-3) that fine-tune Foxp3 gene expression, efficient DNA demethylation of CNS2 and promoters of Foxp3 targets are essential for the stability of a Foxp3+ Treg cell phenotype. Global mapping studies further indicated the establishment of a Treg cell-specific epigenetic landscape during development, but the functional relevance of such epigenetic regulation, in particular histone methylation, has remained poorly understood. Here, we have analyzed the role of two members of the H3K4 methyltransferase family, Mll1 and Mll2, in the biology of Foxp3+ Treg cells. Constitutive deletion of Mll1/Mll2 in adolescent BAC.Foxp3GFP-Cre x Mllfl/fl mice resulted in moderate aberrations in thymic and peripheral Foxp3GFP-Cre+ tTreg cell compartments. In Mll1/Mll2-double-deficient mice, peripheral Foxp3GFP-Cre+ tTreg cells were severely abrogated during age progression, with individual lymph nodes being enlarged due to increased effector T cell numbers, but immune tolerance was largely maintained by Foxp3+ Treg cells that escaped Mll deletion due to the lack of transgenic BAC.Foxp3GFP-Cre expression.
Strikingly, heterozygous Foxp3-driven deletion of Mll1 & Mll2 promotes aggressive autoimmune diabetes in adult NOD BAC.Foxp3GFP-Cre x Foxp3IRES-RFP x Mll1/2fl/wt mice, whereby diabetes incidence strongly correlates with homozygous MHC class II I-Ag7 expression. Ongoing studies focus on the molecular mechanisms underlying this novel function of Mll1 & Mll2 in Foxp3+ Treg cell development and autoimmunity.

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Antigen-exhausted CD4+ T cells deviate towards multiple states of anergy

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Persistent antigen presentation by tumor cells and in chronic infections functionally impair T cells over time. This hampers progress of adoptive T cell therapies for such diseases. To address how different kinetics and dosage of antigen presentation affect CD4+ T cells, we followed TCR transgenic T cells transferred into antigen-transgenic recipients where they received transient or chronic TCR signals of varying strengths, in the absence of confounding pathologies of chronic infections. We show that CD4+ T cells exposed to persisting antigen presentation at three different levels display phenotypes with varying kinetics and consequences. Chronically antigen-exposed CD4+ T cells showed impaired cytokine production upon re-stimulation, dose-dependent up-regulation of exhaustion- associated markers such as PD-1, Lag-3, 2B4, and TIGIT, anergy markers FR4 and CD73, Tfh-associated genes such as CXCR5, ICOS, Bcl-6, IL-21, and transcription factors Tox and c-Maf. Continued antigen presentation was sensed by the T cells, as indicated by Nur77- driven GFP-reporter expression and NFATc1 nuclear translocation, even at the highest dose where the TCR, and LAT, get chronically down-regulated. When challenged, signaling pathways responded to strong TCR signals with Ca2+ fluxes being the most robust one while the MAPK and Akt pathways were more easily tuned by persisting antigen. The cells' transcriptional profiles reflected the qualitative and quantitative changes in antigen presentation by dose-dependent up-regulation of exhaustion-, anergy- and Tfh-associated genes and down-regulation of memory-associated ones. Comparisons with naturally anergic and LCMV clone13-exhausted CD4+ T cells highlighted a common signature describing antigen-induced T cell anergy and exhaustion. Despite up-regulation of Tfh- associated markers, antigen-exhausted CD4+ T cells were losing their ability to provide help to B cells over time. Our results demonstrate that dose and timing of antigen presentation beyond the expansion phase reveal the plasticity of CD4+ T cells and determine their range of dysfunctionalities within an otherwise sterile environment.

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Control of self-renewal and identity of tissue-resident leukocytes by the transcription factors Bhlhe40 and Bhlhe41

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In tissues that undergo permanent self-renewal this capacity is usually restricted to a dedicated population of stem cells. In the hematopoietic system self-renewal has a much broader distribution as many mature leukocyte subsets regain the ability to self-renew as a part of their differentiation program. This unique feature of the hematopoietic system is exemplified by several innate-like lymphocyte subsets as well as by multiple tissue-resident macrophage populations. We previously identified the transcription factors (TFs) Bhlhe40 and Bhlhe41 as key regulators of the self-renewal of B-1a cells – a tissue-resident innate-like B lymphocyte subset that is predominantly generated during fetal and neonatal hematopoiesis. We now investigated whether these TFs may play a broader role in the regulation of tissue-resident self-renewing leukocyte populations such as tissue macrophages. Tissues in multicellular organisms are 'serviced' by resident macrophages which perform both generic and tissue-specific functions. The latter relies on unique tissue-specific molecular programs induced by signals from the microenvironment and executed through a combinatorial action of tissue-specific and broadly expressed transcriptional regulators. We now identified the TFs Bhlhe40/41 as novel regulators of alveolar macrophages (AMs) – a population that provides the first line of immune defense and executes homeostatic functions in lung alveoli. In the absence of these factors, AMs exhibited decreased proliferation that resulted in a severe competitive disadvantage of knockout AMs. Gene expression analyses revealed a broad cell-intrinsic footprint of Bhlhe40/41-deficiency manifested by a downregulation of AM signature genes and induction of signature genes of other macrophage lineages. Genome-wide characterization of Bhlhe40 DNA binding suggested that these TFs directly repress expression of lineage-inappropriate genes in AMs. Taken together, these results identify Bhlhe40/41 as key regulators of AM self-renewal and guardians of their identity.

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Non-hematopoietic Antigen Presenting cells of the Lymphoid organs in acute Graft-versus-host disease

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Hematopoietic stem cell transplantation is a curable therapy for hematopoietic malignancies. Allogeneic T-cells residing within the graft have the potential to eliminate remaining malignant cells by the Graft versus leukemia effect. Nevertheless, in 30 – 60% of HSCT cases allogeneic T-cells become alloreactive and attack host tissues resulting in acute Graft versus host diseases (aGvHD). Dendritic cells are antigen presenting cells (APCs) and have been thought to be the prime candidate for presenting self-antigens to the allogeneic T-cells. However, recent findings claim that even in absence of hematopoietic APCs GvHD cannot be prevented, suggesting the crucial role of non-hematopoietic APCs in the activation of allogeneic T-cells.
In this project, we investigate the role of lymph node stromal cells (LNSCs) in the initiation phase of aGvHD and their potential role as non-hematopoietic APCs.
Here we show that allogenic T-cells are activated in the lymphoid organs and not in the intestinal lamina propria in the initiation phase of aGvHD. In MHCIIΔ/Δ bone marrow chimeras as well as in TgCD11c-Cre+MHCIIΔ/Δ mice, allogenic CD4+ T-cells are activated with +3 days of allo-HCT in the SLOs (spleen, lymph nodes and Peyer's patches) and the TgCD11c-Cre+MHCIIΔ/Δ mice shows late aGvHD onset compared to the TgCD11c-Cre- mice.
Furthermore, LNSCs upregulates co-stimulatory receptors early after conditioning (in vivo and in vitro) suggesting that they may contribute as active antigen presentation cells. Moreover, depletion of fibroblastic reticular cells in Ccl19-Cre.iDTR mice skews the activation and proliferation of alloreactive CD4+ T cells early after allo-HCT whereas the DTR+Cre+ mice showed similar overall survival to DTR+Cre- mice.
In MHCIIΔ/Δ mice transplanted with CD11c.DOG mLNs, alloreactive CD4+ T cells in the spleen and LNs shows effector phenotype (CD44hiCD62Llow).
Conclusively, we show that alloreactive T-cells are activated within the SLOs even in the absence of hematopoietic APCs, but still it remains elusive, which non-hematopoietic cell initiates the alloreactive T-cell response.

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T cell homeostasis versus thymic output – unveiling the distinct survival signaling in peripheral T cells maintained by coronin 1

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Naïve T cells in peripheral lymphoid organs are derived from the thymus, where preparatory pool of various clones of T cell receptors (TCRs) against incoming pathogens or arising tumors are selected. T cells developed in the thymus will egress and migrate into the peripheral lymphoid organs, where they proliferate and survive to maintain the long-term homeostasis. However, it has been unclear whether the two major signaling pathways elicited by interleukin-7 (IL-7) and endogenous ligands for TCR are sufficient for peripheral T cell homeostasis.
We have defined an essential role for the tryptophan-aspartate repeat containing protein coronin 1 in the maintenance of peripheral T cells. In both mice and humans, deletion or mutation of coronin 1 results in a severe suppression of peripheral T cell numbers, without any defect in thymic T cell development nor restrained migration into the periphery. In fact, both IL-7 and TCR signaling in thymic T cells occur normally in the absence of coronin 1. Here we aim to understand how coronin 1 specifically maintains peripheral naïve T cells.
First, we investigated the critical stage of T-cell loss by coronin 1 deficiency, using Rag2-GFP reporter mice to analyze the recent thymic emigrants and mature naïve T cells. We found a specific and drastic reduction of mature naïve T cells in coronin 1-deficient mice while recent thymic emigrants were comparable. Second, survival and proliferation of peripheral naïve T cells in vitro were examined by treatment of naïve T cells with IL-7 or by stimulation of antigen-specific T cell responses. Separately, to assess the homeostatic proliferation of T cells in vivo, Rag2-deficient mice were adoptively transferred with wild-type or coronin 1-deficient T cells. Both in vitro and in vivo results suggest that coronin 1 is dispensable for proliferation signaling but essential for survival signaling.
Our latest challenge is to identify the peripheral T cell-specific survival signaling pathway via coronin 1.

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MicroRNA-mediated control of T-cell selection in the thymus

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T cell receptor (TCR) signal strength determines selection and lineage fate at the CD4+CD8+ double positive (DP) stage of intrathymic T cell development. Here we investigated the role of miR-181a/b-1, a microRNA that serves as a rheostat of TCR signal strength, in thymic selection. Loss of miR-181a/b-1 resulted in profound deficiency of T cell subsets undergoing agonist selection, including iNKT cells, MAIT cells, and Treg cells. Notably, Treg cells were present at normal numbers in the periphery due to homeostatic expansion, overall resulting in a Treg cell pool with limited TCR diversity. Surprisingly, miR-181a/b-1-deficient Treg cells were more efficient suppressors than their wild-type counterparts, even though expression of miR-181a/b-1 is virtually absent in wild-type peripheral Treg cells and peripheral miR-181a/b-1-deficient Treg cells were virtually transcriptionally indistinguishable from their wild-type counterparts. Elevated suppressive capacity was most likely due to transcription-independent accumulation of CTLA-4. Thus, we propose that agonist selection imposes a post-transcriptional legacy on Treg cell function.

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Elucidating Natural Killer T Cell Functional Differentiation and Diversification in a Timed Developmental Wave

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Natural killer T (NKT) cells are a small subset of glycolipid-recognizing T cells that incorporate features of both adaptive and innate cells. NKT cells provide anti-microbial defense and immune surveillance of malignancies; however, they are also involved in the pathogenesis of allergies, autoimmune and inflammatory diseases as well as certain types of cancer. One of the most striking hallmarks of NKT cells is the acquisition of unconventional memory-like cellular states during their development.
Inspite of seminal progress in the understanding of NKT cell functionality in recent years, the molecular mechanisms that drive the developmental acquisition of their peculiar phenotypes are only incompletely understood. This applies especially to the earliest developmental time-points, which are hard to characterize due to the rarity of the cells.
Therefore, we established a genetic model to induce a timed wave of synchronous NKT cell generation in order to elucidate early developmental phases and functional differentiation of NKT cell subsets in the mouse thymus. We extensively analysed the kinetic developmental changes in cell-surface marker as well as transcription factor make-up of thymic and peripheral NKT cells. We characterised cellular proliferation as well as the timing acquisition of the ability to produce various cytokines. By means of a Nur77eGFP reporter mouse, we monitored the TCR signal dynamics during the early developmental phases. Furthermore, we performed bulk RNA-seq and ATAC-seq of early NKT cell development in the thymus. Ultimately, we aim to compare gene-expression profiles as well as epigenetic modifications with a focus on enhancer landscapes.
Our overall goal is to obtain a systems biological overview of the developmental acquisition of functional memory-like states by NKT cells.

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T cell-specific Bcl6 is required for the maintenance of T follicular helper cells and germinal centers

Dominik Alterauge and Dirk Baumjohann

T follicular helper (Tfh) cells provide help to B cells, resulting in the generation of high affinity antibodies. Various factors orchestrate Tfh cell differentiation, including the continued interactions with APCs (DCs and B cells), TCR signaling strength, miRNAs (miR-17~92, miR-155, miR-146), and transcriptional regulators (LEF-1, TCF-1, Bcl6). The transcriptional repressor Bcl6 promotes the Tfh phenotype through the regulation of genes associated with migration, metabolism and alternative T helper cell fates. While Bcl6 is indispensable for Tfh cell generation, its role in the maintenance of Tfh cells remains to be elucidated. Here, to investigate the impact of Bcl6 on the maintenance of Tfh cells and ongoing GC responses, we analyzed the impact of temporally guided deletion of Bcl6 specifically in CD4+ T cells. We found that T cell-intrinsic Bcl6 expression was required for the maintenance of Tfh cells. In addition, CD4+ T cell-specific deletion of Bcl6 resulted in decreased GC B cell responses. In conclusion, our results highlight Bcl6 as a critical regulator of Tfh cell and GC maintenance.

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Lipid droplet formation fuels pathogenic ILC2 responses in airway inflammation

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Survival of the host relies on the establishment of a functional barrier immune defense that must be maintained in fluctuating states of food availability. Innate lymphoid cells (ILC) are an important component of tissue immunity involved in the maintenance and repair of tissue barriers such as the lung and intestine. However, chronic activation of ILC result in immune pathology. Recently, we revealed that lipids are essential to fuel type 2 ILC (ILC2)-mediated anti-helminth immunity. Whether both protective and pathogenic ILC responses underlie a similar metabolic control is completely unknown. Here we show that pathogenic ILC2 display a distinct metabolic phenotype upon chronic activation. In the context of allergen-driven airway inflammation ILC2 increase the uptake of both, external lipids and glucose. This metabolic adaptation of ILC2 to chronic inflammation is regulated on a cellular level by an increased expression of genes involved in fatty acid metabolism and glycolysis. Strikingly, both cellular fuels unite to allow for chronic activation of ILC2. Externally acquired fatty acids are transiently stored in lipid droplets to prevent lipotoxicity and promote the proliferation of ILC2. Strikingly, restricting dietary glucose by feeding mice a ketogenic diet largely ablated ILC2-mediated airway inflammation by impairing fatty acid metabolism and the formation of lipid droplets. Together, these results reveal the metabolic requirements of pathogenic ILC2 responses and suggest that abundant dietary availability of fat and glucose could be responsible for an overall increase in chronic airway inflammation in the Western World. As a correlative ketogenic diets may represent a potent dietary intervention strategy for the treatment of allergic airway inflammation in the future.

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Molecular architecture and regulation of BCL10-MALT1 filaments

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The CARD11-BCL10-MALT1 (CBM) complex triggers the adaptive immune response in lymphocytes and lymphoma cells. It serves as a scaffolding platform that activates downstream signaling events via association of mediators such as ubiquitin ligases and protein kinases. Beyond its scaffolding function, MALT1 contributes proteolytic activity to the CBM complex, which is key for optimal lymphocyte activation and differentiation. CARD11 acts as a molecular seed for inducing BCL10 filaments, but the dynamics of MALT1 integration and CBM complex assembly have remained elusive.
Using cryo-EM, we solved the helical structure of the BCL10-MALT1 filament. The structural model of the filament core solved at 4.9 angstrom resolution identifies the composition of the BCL10 inner core filament, as well as the interface between the N-terminal MALT1 death domain and the BCL10 caspase recruitment domain. The C-terminal MALT1 Ig and paracaspase domains protrude from this core, resembling a "paddle wheel-like" shape, which allows for coordination of mediators and substrates at the filament periphery.
Site-directed mutations within the BCL10 core filament abrogate the ability of the BCL10-MALT1 complex to oligomerize, highlighting the importance of BCL10-BCL10 interfaces for BCL10 filament assembly. Moreover, these interface mutations impede stimulus-dependent CARD11 recruitment, and thus CBM complex assembly, NF-kappaB DNA binding, as well as MALT1 protease activity. Further, expression of BCL10-MALT1 interface mutants in BCL10 or MALT1 KO T-cells completely abolishes the constitutive binding of BCL10 to MALT1, underscoring the importance of the newly identified BCL10-MALT1 interaction site for CBM complex formation, MALT1 protease activation, and NF-kappaB signaling.
Collectively, we present a model for the assembly and architecture of the CBM signaling complex and how it functions as a signaling hub in T-lymphocytes. Future studies could elucidate the mechanism of MALT1 protease activation in the complex, and investigate how the BCL10-MALT1 platform integrates other factors to initiate downstream processes.

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Innate Immune Activation in Dendritic Cells by Complement-Opsonized HIV

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Successful viral infection depends on host and viral factors. Activation of the immune system is detrimental for the virus, therefore viruses develop ways to avoid it. HIV activates the classical complement pathway by direct binding of the complement component C1q to the viral envelope glycoproteins and is then coated by C3 fragments. Thus, we can find complement-opsonized HIV (HIV-C) in infected individuals. HIV-C can bind to the complement receptors (CRs) expressed in DCs infecting them better than non-opsonized HIV. This leads to a higher type I interferon (IFN-I) expression. However, the intracellular pathway activated upon HIV-C infection is unknown. We tested if TBK1 and MAVS were implicated in the upregulation of IFN-I expression triggered by HIV-C infection. TBK1 plays an essential role in regulating inflammatory responses. To establish an antiviral state, TBK1 form several different complexes whose composition depends on the cell type and stimuli, MAVS being one of the components. Non-opsonized HIV infection blocks IFN-I expression. However, it is unknown how HIV-C avoids that block.
We confirm that HIV-C infection of DCs led to a higher IFN-I expression and ISGs, such as CXCL10 or RSAD2, compared to non-opsonized HIV. We show TBK1 phosphorylation and MAVS aggregation, indicating activation, upon HIV-C infection. Lastly, HIV-C infection phosphorylated STAT-1 and activated NFκB.
HIV-1 infection is not sensed in DCs, however when HIV is complement-opsonized there is an innate immune response leading to IFN-I and ISGs expression. Contrary to non-opsonized HIV-1 infection, HIV-C infection activates TBK1 and MAVS explaining the higher IFN-I expression. Further, STAT-1 phosphorylation and NFκB activation indicate that complement-opsonization of HIV-1 activates an antiviral state in DCs. Understanding the intracellular pathways leading to a better immune defense against HIV will help develop new therapeutic strategies.

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Unraveling the functional connection of Roquin and Regnase-1 in regulating Zc3h12a mRNA

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The regulation of gene expression on the post-transcriptional level exerts an essential function in immune cells as it controls the concerted expression of inflammatory mediators. In T cells, the RNA-binding proteins (RBPs) Regnase-1 and the paralogs Roquin-1 and Roquin-2 are shown to be critical regulators of cell activation and differentiation. Accordingly, mice with either a T cell-intrinsic deletion of Roquin or Regnase-1 encoding genes Rc3h1/2 or Zc3h12a, respectively, or with a single M199R point mutation in Rc3h1 develop severe autoimmune or auto-inflammatory diseases. Roquin and Regnase-1 mediate their function by binding to specific cis-elements in the 3´-UTR of immune-related mRNAs and thereby suppress the expression of their targets via mRNA degradation and/or translational silencing. The activation of T cells after TCR stimulation leads to degradation of Roquin and Regnase-1 via MALT1 paracaspase activity which finally triggers the release of previously repressed target mRNAs. Intriguingly, Roquin and Regnase-1 regulate an overlapping set of target mRNAs including Icos, Ox40 and CTLA-4. In addition, the Zc3h12a mRNA, shown to be feedback-regulated by its encoded protein Regnase-1, also appears to be a Roquin target. Thus far, the question whether Roquin and Regnase can act redundantly, in an additive/ cooperative or in a spatiotemporal distinct manner is under controverse debate. This project aims to elucidate the connection between Roquin and Regnase-1 by unraveling their interdependence in the regulation of Zc3h12a mRNA.

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CD301b+ SIRPα+ dendritic cells play a non-redundant role in generating self-tolerance in the thymus medulla

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The thymus contains a diversity of antigen presenting cells (APC) that exist in different locations and have distinct antigen processing capabilities. This suggests that they play non-redundant roles in mediating thymocyte selection. Many of these subsets were shown to be capable of driving clonal deletion, but these studies relied predominantly on T cell receptor transgenic models. We therefore still do not understand the extent to which distinct APC contribute to clonal deletion in the polyclonal repertoire. Here, we assessed the contribution of different APC subsets to clonal deletion using a cleaved caspase 3-based assay paired with cell type ablation or deficiency. We found that both cortical and medullary deletion were dependent on CD80/CD86 co-stimulation and that bone marrow derived APC mediated approximately half of clonal deletion events. Total deletion frequencies were not altered in the absence of B cells, pDC, or XCR1+ cDC1. In an effort to eliminate SIRPα+ cDC2, we discovered that a substantial proportion of thymic SIRPα+ DC express the lectin CD301b. CD301b expression was localized exclusively within the thymus medulla and depended on IL-4Rα expression. Deficiency of these IL-4/IL-13 signaled DC caused a measurable reduction in clonal deletion events. These data suggest that CD301b+ SIRPα+ DC represent a distinct population of antigen presenting cells within the thymus and that they are essential for non-redundant deletional tolerance in the polyclonal repertoire.

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Mouse Th1 cells respond with strong systemic cytokine release upon superagonistic anti-CD28 monoclonal antibody stimulation in vivo

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Superagonistic anti-CD28 monoclonal antibodies (CD28-SA) were developed to selectively target CD4+ Foxp3+ regulatory T cells (Treg) for therapeutic purposes. While CD28-SA treatment of autoimmune/inflammatory disease in mice led to Treg-mediated protection, healthy human volunteers injected with saturating amounts of CD28-SA developed a severe cytokine release syndrome (CRS) due to activation of effector/memory CD4+ T cells. Apart from a higher suppressive potency per cell of Treg from mice compared to humans, the lack of a true memory CD4+ T cell compartment in cleanly housed mice best explains the failure of mouse models to predict CD28-SA-induced CRS in humans. We, therefore, transferred in vitro differentiated TCR-transgenic OT-II Th1 cells into diphtheria toxin-treated wild-type or DEREG-C57BL/6 recipient mice. Injection of saturating amounts of CD28-SA into Treg-depleted recipient mice led to full activation of the OT-II Th1 cells releasing high amounts of IFNγ into the circulation. The comparison of Treg-sufficient and -deficient animals confirmed that in mice Treg are very potent in limiting effector/ memory CD4+ T cell activation in vivo. Transfer of Th1 cells and other T helper cell subsets into cleanly housed recipient mice will, thus, facilitate pre-clinical testing of immunomodulatory reagents in general and will help in better understanding the molecular requirements for CD28-SA-mediated effector/memory CD4+ T cell activation in vivo in particular.

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Investigating immunological mechanisms preceding osteoporosis development in patients with rheumatoid arthritis

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Background
Rheumatoid arthritis (RA) is an autoimmune disease characterized by joint inflammation and progressive destruction of cartilage and bone. In addition to periarticular bone loss of affected joints, RA is also associated with development of generalized osteoporosis, resulting from an imbalance between bone-resorbing osteoclasts and bone-forming osteoblasts. Osteoclasts are cells of hematopoietic origin that circulates in blood as osteoclast precursor cells (preOCL) until they form mature osteoclasts at bone surfaces. The aim of this study was to elucidate whether levels of circulating preOCL in blood from untreated early RA (ueRA) patients can be used to predict development of osteoporosis in later stages of disease progression.

Method
38 patients with ueRA fulfilling the ACR/EULAR 2010 classification criteria were included and compared to 23 age- and sex matched healthy controls (HC). Peripheral blood was obtained and analyzed by flow cytometry. Disease activity was assessed using DAS28, CDAI, SJC, TJC, CRP and ESR. Bone mineral density (BMD) was measured at inclusion and at the 1-year follow-up using bone densitometry.

Results
Patients with ueRA display an increased frequency of preOCL (CD14+CD16+ monocytes) in blood compared to HC. The levels of preOCL also correlates positively with the frequency of CXCR3+Th17 cells in blood. Interestingly, the frequency of preOCLs is negatively associated with several clinical disease activity parameters, while no association with bone mineral density is detected at inclusion. Furthermore, preliminary experiments show that preOCL isolated from peripheral blood of RA patients have increased capacity to differentiate into osteoclasts in vitro as compared to preOCL isolated from HC.

Conclusions
We show that a population of circulating monocytes, which are more prone to develop into mature osteoclasts, are increased in ueRA patients as compared to HC. Ongoing analyses of BMD at the 1-year follow-up will reveal whether this population of preOCL can be used to predict development of osteoporosis in RA patients.

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Designing novel human 3D cell models to study innate immune interactions

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Adding new dimensions to human primary barrier/immune cell models offers big advantages with respect to drug screening or host/pathogen interactions due to mimicking an in vivo-like situation. Therefore, we created a more complex scenario to monitor entry, spread and signaling of differentially opsonized HIV-1 or Aspergillus spp. to improve the understanding about host parameters during these processes.
In vivo, HIV-1 spontaneously activates complement (C) in seminal fluid, plasma and at mucosal sites. However, whether the viral opsonization pattern is involved in viral transmission is unknown. Here we studied the role of complement in HIV-1 transmission by Langerhanc Cells (LCs) using a human skin model. We found differences with respect to LC infection, viral fusion and transmission between non- and C-opsonized HIV-1. Our study provides novel insights at first steps of viral pathogenesis within a realistic setting and might lead to novel strategies to prevent HIV-1 infection. Further we studied invasive fungal infections such as Aspergillosis within a perfused respiratory tract model containing primary polarized epithelial cells and relevant immune cells such as dendritic cells and macrophages. Remarkably, culturing cells under perfusion significantly accelerated polarization and differentiation of epithelial cells compared to static conditions. After addition of antigen-presenting cells, Aspergillus fumigatus conidia and hyphae were efficiently sensed by dendritic cells and macrophages, which were fully functional in these systems. Thereby, adding new dimensions offers novel possibilities to not only study infections, spread, signaling under conditions mimicking the in vivo situation, but also to find new tools to combat these infections.

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A20 gene dose effects in B cell-mediated autoimmunity in mice

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The ubiquitin-editing enzyme Tnfaip3/A20 is a key negative regulator of NF-κB signaling. While activation of NF-κB signaling is essential for physiological immune responses, its deregulation is implicated in autoimmunity. Single nucleotide polymorphisms (SNPs) near the Tnfaip3 gene locus are linked to various autoimmune diseases and a B cell-specific loss of A20 is sufficient to induce autoimmune symptoms in mice. Strikingly, the heterozygous loss of A20 in murine B cells induces a much stronger autoimmune phenotype than its homozygous ablation. The pathology is characterized by B cell-activation, splenomegaly and the development of anti-nuclear auto-antibodies. Heterozygous reduction of A20 in B cells induces strongly increased numbers of germinal center B cells and plasma cells in secondary lymphoid tissues, which coincides with enhanced isotype switching.
To understand the effect of A20 depletion on terminal B cell differentiation, the development of antibody-secreting plasma cells was investigated by use of the in vitro-derived germinal center B (iGB) cell culture system. Proliferation, differentiation and isotype switching of B cells in this system are inversely correlated to A20 levels in a gene dose-dependent manner. This indicates that the autoimmune phenotype induced by heterozygous loss of A20 is not due to B cell-intrinsic hyperactivity, but rather depends on yet unidentified factors in the microenvironment in vivo.
Using competitive bone marrow transfer of cells from A20 homo- and heterozygous donors into one recipient mouse, we could show that the mere presence of A20-homozygous B cells in the body dampens the hyperactivity of the heterozygous B cells, further supporting an extrinsic influence from other players of the immune system.

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Enolase 1 of C. albicans and A. fumigatus directly bind and activate mouse and human B cells

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Of great relevance for immunocompromised patients, the incidence of life-threatening fungal infections by Candida albicans or Aspergillus fumigatus has increased in recent years. While the interaction of cells and molecules of innate immunity with these fungi has been studied to great depth, comparatively little is known about the modulation of adaptive immunity by secreted proteins of C. albicans and A. fumigatus. Thus, we here studied the cytosolic glycolytic enzyme Enolase 1 (Eno-1) which is also secreted and expressed on the cell surface. Eno-1 surface expression critically contributes to host adhesion and invasion. We now found that soluble Eno-1 preferentially binds to mouse and human B cells over other lymphocyte subsets. A more detailed analysis of B cell subpopulations revealed that Eno-1 bound particularly well to mouse marginal zone B cells. While the receptor for fungal Eno-1 on lymphocytes is still unknown, addition of Eno-1 to mouse splenocytes and human PBMCs upregulated CD86 expression, i.e. induced activation, and proliferation of all B cell subsets but not of CD4+ or CD8+ T cells. Concomitantly, Eno-1 induced IgM and IgG2b or IgG1 secretion by mouse and human B cells, respectively. We are currently investigating the anti-fungal activity of the antibodies secreted by B cells after Eno-1 stimulation. Moreover, Eno-1 also induced pro-inflammatory cytokine secretion, i.e. IL-6 and others, of which monocytes, but not B cells, were the main source. Together, Eno-1 is on the one hand important for fungi to adhere and invade the host but on the other hand, as shown here, also induces putatively protective B cell responses.

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Triggering complement receptors on DCs induces cellular immune responses during HIV-1 infection

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Immediately upon pathogen entry, non-specific immune responses are triggered in the host and within a very short time innate immunity is fully activated. Among these first innate components activated is the systemic complement system. Viruses such as HIV-1, spontaneously activate the complement system and therefore HIV-1 is already coated with complement fragments at the initial stages of infection.
Here we described a novel way of dendritic cell (DC) modulation during acute HIV-1 infection by triggering integrin receptor signaling. We could show that complement-opsonized HIV-1 (HIV-C) is sensed by dendritic cells, the sentinels and most important antigen-presenting cells of our immune system, compared to non-opsonized HIV. This recognition goes along with higher activation and maturation of dendritic cells and therefore causes stronger induction of cytotoxic T cells as well as T helper cells. We furthermore found that complement opsonization of HIV-1 induces a T helper 17 polarizing cytokine profile in DCs and polarized naïve T helper cells into the T helper 17 lineage. Additionally stronger maturation of DCs exposed to HIV-C induced higher type-I interferon responses, increased production of inflammatory cytokine and cellular complement components. These observed inflammatory responses and DC maturation was completely abrogated when complement component 3a receptor (C3aR) was blocked.
Our data indicate that there is a clear relation between activation of complement receptors and DC maturation along with induction of cellular immune responses. Thus, investigating the complement receptor signaling pathway in more detail could lead to the development of a novel DC-based vaccination therapy against viral infections.

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Dissecting the role of interleukin 17 in the development of postmenopausal osteoporosis

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Reduced levels of endogenously produced estrogen (E2) is the major cause of osteoporosis in post-menopausal women and hormone therapy prevents osteoporotic fractures. It is known that postmenopausal women suffering from osteoporosis have increased serum levels of interleukin 17A (IL-17A), which negatively correlates with bone mineral density. In mice, treatment with neutralizing IL-17 antibodies was shown to protect against ovariectomy (OVX)-induced bone loss, while mice lacking the IL-17 receptor had increased susceptibility to OVX-induced bone loss. This contradiction shows that more research is necessary to elucidate the role of IL-17A in postmenopausal osteoporosis.

We used IL-17A knockout (KO) mice to dissect the role of IL-17A in post-menopausal osteoporosis. To mimic endogenous E2 loss, female mice underwent sham or OVX operations at the age of 8 weeks and were sacrificed 3 weeks later. Radiological characterization of the skeleton and flow cytometric analyses of bone marrow cells were performed at termination.

Results show that the OVX-induced loss of cortical-, but not trabecular bone, is mediated via IL-17A. Furthermore, flow cytometric analyses of bone marrow cells revealed that the OVX-induced increased frequency of RANK positive pre-osteoclasts and up-regulated surface expression of RANKL on T cells in bone marrow, is dependent on IL-17A. These findings were also associated with increased mRNA expression levels of Acp5 and RANKL/OPG ratio in cortical bone after OVX of WT mice, but not of IL-17A KO mice.

Interestingly, sham operated IL-17A KO mice displayed significantly increased numbers of pre-osteoclasts accompanied by a tendency towards higher RANKL expression on bone marrow T cells, as well as increased mRNA expression of Acp5 in cortical bone, when compared to sham operated WT mice. This indicates an initial cellular and molecular "priming" towards osteoclastogenesis in the IL-17A KO mice, which is not reflected in the bone mineral density but may be due to a compensatory mechanism possibly involving elevated IL-17F. This issue needs further investigation.

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Investigating the post-transcriptional cooperation of Roquin and Regnase-1 in T cell differentiation

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Gene regulation on the post-transcriptional level exerts essential control over immune responses and is needed to prevent autoimmune diseases. The RNA-binding proteins Roquin-1 and its paralog Roquin-2 were found to be essential for the prevention of autoimmunity and auto-inflammation by controlling T cell activation and differentiation. They recognize cis-elements in the 3' UTRs of mRNAs and thereby induce mRNA decay. This post-transcriptional gene regulation in T cells is tightly controlled by the paracaspase MALT1, which, upon TCR activation and co-stimulation, cleaves Roquin proteins. Interestingly, the endonuclease Regnase-1 is regulated in the same way by MALT1 cleavage in T cells. Moreover Roquin proteins and Regnase-1 share a set of mRNA targets that are associated with T helper cell differentiation and T cell effector functions. A conditional deletion of Regnase-1 in T cells leads to a similar autoimmune phenotype as the knockout of Roquin-1/2 in T cells. But, whether Roquin and Regnase-1 are cooperating in target mRNA regulation in T cells still remains controversial. By generating mice with either single conditional knockouts of Roquin-1/2 and Regnase-1 or a combined knockout in T cells, we identified that, in addition to T cell activation, they also control regulatory T cell development in the thymus. We further performed mRNA sequencing in the different knockout situations and found that the majority of mRNA targets is controlled in a cooperative manner by Roquin and Regnase-1. These findings suggest that Regnase-1 and Roquin proteins play an important role in the prevention of autoimmunity by cooperatively controlling post-transcriptional gene regulation in T cells.

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Post-transcriptional regulation of CD8+ T cell function by RNA-binding proteins

Stephanie Edelmann, Vigo Heissmeyer

In CD4+ T cells it is well established that post-transcriptional regulators like Roquin and Regnase inhibit T cell activation and differentiation by repressing several target mRNAs. Apart from a significant activation phenotype, it is still unclear, how the deficiency of these regulators influences CD8+ T cell functions.
Our first results demonstrate that Roquin-1/2-deficiency in CD8+ T cells leads to an upregulation of transcription factors (IRF4, T-bet), high proliferation and increased production of cytokines (IL-2, TNF-alpha). Interestingly, also detectable amounts of preformed cytotoxic molecules like granzyme B and perforin were found in Roquin-1/2-deficient CD8+ T cells, which resulted in a significantly higher cytotoxicity in a redirected lysis assay in vitro. In a mouse melanoma model, we will now analyze, if Roquin-deficient CD8+ T cells are indeed superior in controlling tumor growth in vivo and how the cells respond to checkpoint inhibition (anti-PD1, anti-CTLA-4). As these findings could have clinical implications, we are also developing approaches and tools to modify and detect Roquin activity in order to use this knowledge for the improvement of adoptive T cell therapy for tumor patients.

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Fate-mapping of GM-CSF in chronic inflammatory disease reveals the molecular landscape of pathogenic TH cells

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The cytokine GM-CSF differs from the other members of the CSF-family in that it is a pro-inflammatory mediator rather than a steady-state growth factor. Under non-inflammatory conditions GM-CSF is hardly detectable and the only cell type known so far to depend on GM-CSF are alveolar macrophages. During tissue-inflammation, GM-CSF production is pivotal and emerges as the main communication conduit between T helper (TH) cells and tissue-invading inflammatory phagocytes. TH cells have been shown to be an important source of GM-CSF during neuroinflammation. However, it is still unclear which TH cell subset expresses GM-CSF, or whether GM-CSF-production even marks a stable pathogenic TH cell subset. To study the phenotypic and functional properties of GM-CSF-producing TH cells, we generated a new mouse model to fate-map and report GM-CSF (FROG) expression.
The FROG system allows us to follow pathogenic GM-CSF-producing TH cells longitudinally in the course of neuroinflammation. Thereby, we are able to analyze their genetic, epigenetic and proteomic features using unbiased high-dimensional technologies. Preliminary data suggest that GM-CSF producing TH cells form long-term memory and their epigenetic modifications explain the recurrence and chronicity of inflammatory diseases such as multiple sclerosis (MS). The FROG system is next used to identify the transcriptional machinery regulating GM-CSF expression, which shall reveal the nature of this discrete TH subset critically involved in regulating the phagocyte activity in tissue inflammation.

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Regulation of the Inducible T-cell costimulator Icos by combinatoral activity of cis-elements and trans-acting factors on the mRNA level

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The Inducible costimulator Icos is expressed on T cells once they become activated. Icos plays an important role in the humoral immune response since Icos-deficient mice deal with autoimmunity and furthermore its loss-of function in humans is linked to immunodeficiency.
On the mRNA level, Icos is tightly post-transcriptionally controlled. The 3'UTR is the non-translated region of an mRNA, placed downstream of the translation termination codon. Such noncoding sequences often contain binding sites for regulatory proteins and miRNAs, allowing them in an independent, cooperative or exclusive manner to function in the regulation of the transcript. It has been demonstrated that the RNA-binding protein Roquin-1 and its paralog Roquin-2, the endonuclease Regnase-1 as well as several miRNAs are some of these trans-acting factors involved in the regulation of Icos mRNA. Roquin recognizes and binds to stem-loop structures, as for example the CDE element, a tri-loop hairpin that is also found in the Icos mRNA. Roquin binding initiates the degradation of the target transcript by interacting with components of the CCR4-NOT complex, resulting in deadenylation and decapping of the mRNA and further degradation. Roquin-regulated cis-elements can be complex and composite and involve several but also atypical binding sites. Bearing a relatively long 3'UTR, the Icos mRNA probably contains many other not yet identified cis-elements.
Performing a comprehensive mutagenesis screening approach of the mouse Icos 3'UTR will reveal important cis-elements in Icos mRNA. This will give us more insights into the regulatory network acting on Icos mRNA.

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The origin and relevance of Antigen inexperienced memory T cells

Alena Moudra, Jiri Novotny, Michal Kolar, Pavel Stopka, Ondrej Stepanek

Antigen inexperienced memory T (AIMT) cells form 10-20% of CD8+ T-cells under normal unperturbed conditions. We have found the AIMT cells in mice across the spectrum of hygienic conditions, ranging from germ free C57Bl/6J and Balb/c strains to the undomesticated house mouse and thus we have confirmed its physiological relevance.
Further, we wanted to ascertain whether the AIMT cells from young and aged animals share TCR repertoires. We have sorted CD8+ T-cells from mice with a fixed beta chain of TCR (Vb5) and created the TCRalpha libraries of naïve and the AIMT cells from young and aged animals. The PCA analysis of the TCR repertoires confirmed that naive cells and AIMT cells utilize distinctly different TCR repertoires and moreover that the TCR repertoires are shared among young and aged AIMT cells, indicating being of the same origin.

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Early-onset autoinflammatory disease in the context of gastrointestinal tract

Marini Thian, Rico Chandra Ardy, Elisabeth Salzer, Julia Pazmandi and Kaan Boztug

Purpose
Inflammatory bowel diseases (IBDs) comprise a heterogeneous group of diseases in which a disruption of immune homeostasis in the gut results in chronic intestinal inflammation. Early onset IBD (EO-IBD) denotes a subset of patients that present with a severe symptoms early in life (<10 years of age). Previous studies have identified monogenetic loss-of-function mutations in IL10 receptor, providing proof-of-concept for Mendelian subtypes of IBD. However, the genetic disease etiology remains enigmatic in most patients.

Methods
We performed whole exome sequencing for a cohort of EO-IBD patients to identify novel monogenetic causes of EO-IBD.

Results
We identified loss-of-function mutations in IL21 as a novel genetic etiology linking immunodeficiency and early-onset IBD. We also identified deleterious autosomal recessive mutations in CD55 leading to loss of protein expression in patients with protein-losing enteropathy (PLE) and bowel inflammation.

Conclusion
Data from our informative patient cohort supports the hypothesis that EO-IBD may be caused by disease genes inheritable in a Mendelian fashion, which may present concomitantly with an immunodeficiency or PLE. Identification of single-gene defects leading to EO-IBD will have implications for our mechanistic understanding of mucosal immune homeostasis and will identify critical signaling axis relevant to late-onset IBD that might represent attractive targets for potential therapeutic treatments.

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The positive selection of T cells with different degrees of self-reactivity

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Self reactivity has been shown to play a significant role in conventional T cell effector function, however functional differences only appear after positive selection in the thymus. During development, positively selected thymocytes avoid death by sensing and adjusting to homeostatic signals from self-peptide:major histocompatibility complexes (MHCs). As a result, these thymocytes experience a range of signals and mature into a diverse repertoire of T cells with different degrees of self reactivity and effector function. Here I investigate the positive selection of CD8 T cells across the spectrum of self reactivity, using three T cell receptor transgenic (TCRtg) models with relatively high, moderate, and low self reactivity. I show that cells with high self reactivity reach maturity more quickly than cells with lower self reactivity in ex vivo and in vivo systems. Using two-photon microscopy, I am also studying how T cell self reactivity correlates with TCR signaling kinetics during positive selection. Finally, I am using an experimental system where positive selection of a defined TCR is dependent on exogenous peptides. With this system, I explore how (1) peptide affinity for MHC, and (2) TCR affinity for peptide:MHC influence T cell self reactivity. Together, these experiments will illuminate how thymocyte peptide:MHC interactions inform the development and effector function of positively selected T cells with high and low self reactivity.

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Distinct T cell responses in early versus late viral rebounders after broad neutralizing antibody infusion in HIV-1 positive patients

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"Passive immunotherapy" with broadly neutralizing antibodies (bNABs) is currently being tested in clinical trials for anti-HIV-1 therapy. We investigated if and what type of host immune responses are induced after anti-HIV-1 bNAb infusion. The majority of patients showed a decrease in viral load after antibody infusion. After a single infusion, 7 patients were able to control the virus for more than 56 days (late rebounders) while 8 quickly returned to their original viral load (early rebounders). By high-dimensional cytometry we detected an innate immune response in all patients after bNAb infusion, in the late rebounders we saw a significant increase in T cell clonality by single cell sequencing and using the GLiPH.2 algorithm (which clusters T cell receptor sequences into groups with shared specificity), we detected significantly more HIV-1 specific T cells in the activated T cell compartment. We conclude that passive antibody infusion activates the innate immune compartment in all patients and that the induction of T cell responses correlates with prolonged viral control.

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Helicobacter hepaticus as disease driver in a novel CD40-mediated spontaneous colitis-model

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The mammalian gastrointestinal tract is shaped by microbiota. To maintain mucosal homeostasis, a balance between appropriate immune responses to invading pathogens and tolerance to food and commensal-derived antigens is essential. Disturbed balances can result in severe inflammatory disorders like Inflammatory Bowel Disease. Dendritic cells (DCs) play a key role in this regulation as they can induce both, immunity and tolerance. To investigate the role of the CD40L-CD40 axis in tolerance vs. immunity and the role of DCs therein, we generated a murine model with constitutive CD40-signaling in DC. CD40-signaling leads to migration of CD103+ DCs from the colonic lamina propria to draining lymph nodes, followed by DC-apoptosis. This loss of CD103+ DCs caused lack of RORγt+Helios- induced regulatory T cells, an increase of Th1/Th17 effector cells in the colon, resulting in breakdown of mucosal tolerance and severe colitis.
We used sera from these mice to isolate fecal antigens recognized by mice with colitis, but not control mice and studied changes of the microbiota during disease development. We detected Helicobacter hepaticus (H.h.)-specific antibodies in transgenic mice and could protect them from disease onset by rendering them H.h.-free. Upon H.h.-reinfection of transgenic mice, rapid disease onset was observed. Our data suggest that H.h. is the disease driver in a CD40-mediated spontaneous colitis-model, allowing us to study T cell specificities and differentiation plasticity during inflammation more in detail.

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MALT1 phosphorylation controls activation of T lymphocytes

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Engagement of the T cell receptor (TCR) by specific antigens induces several signaling pathways culminating in T cell activation, differentiation, and proliferation. Thereby, the CARD11-BCL10-MALT1 (CBM) complex bridges proximal TCR signaling to MALT1 protease activation and canonical NF-kappaB signaling. MALT1 exerts a dual function in the CBM complex upon TCR stimulation: the MALT1 scaffold recruits the E3 ligase TRAF6 to the CBM complex, resulting in activation of the canonical NF-kappaB pathway and JNK signaling, and MALT1 proteolytic activity modulates T cell activation by cleaving several important signaling and post-transcriptional regulators. To date, regulation of MALT1 function was shown to be mainly mediated by mono- or poly-ubiquitination. In this study, multiple novel phosphorylation sites were identified in the C-terminus of MALT1 upon T cell activation which support MALT1 scaffolding function.
In mass spectrometry experiments, three phosphorylated MALT1 peptides were identified, covering a total of five phosphorylated serine residues. Generation of phospho-specific antibodies allowed for detection of MALT1 phosphorylation in Jurkat T cells after stimulation, revealing that MALT1 is transiently hyper-phosphorylated upon TCR stimulation. Rescue experiments of MALT1-deficient Jurkat and CD4 T cells with phospho-defective MALT1 variants showed for the first time that MALT1 phosphorylation augments IkappaBalpha degradation and NF-kappaB activation. Furthermore, CK1alpha was identified as a MALT1 protein kinase catalyzing MALT1 phosphorylation, as identified by in vitro kinase assays using a phospho-specific antibody. Indeed, CK1alpha was shown to be essential for CBM assembly and NF-kappaB activation. In rescue experiments of CK1alpha-deficient Jurkat T cells, CK1alpha catalytic activity and CARD11 binding were not only required for its recruitment to CARD11, BCL10 and MALT1, but were also essential for overall formation of the CBM complex. Collectively, identification of MALT1 phosphorylations after TCR signaling has revealed a new level of regulation in the CBM-mediated NF-kappaB pathway.

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Impaired immune response to primary but not to booster vaccination against Hepatitis B in older adults

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Many vaccines are less immunogenic and effective in the elderly compared to younger adults due to immunosenescence. As most vaccines utilized in the elderly contain antigens, with which the target population had previous contact, most studies investigating vaccine-induced immune responses in the elderly do not analyze responses to neo-antigen but rather booster responses. It can be hypothesized that age-related differences in the immune response are distinct for primary and recall responses. We therefore aimed to investigate primary and recall responses using the same antigen in young and older adults using Hepatitis B vaccine as a model antigen.
Young (20-40y) and elderly (>60y) healthy volunteers received either a primary series or a single booster shot (primary vaccination >10 years ago) of Twinrix and antibody responses were monitored. After primary vaccination, antibody responses were lower and delayed in the elderly compared to young adults. Non-responders after the primary series were only observed in the older group. Maximum antibody concentrations after booster vaccination were similar in both age groups. Focused gene expression profiling was performed before and 1 day after the vaccination. We identified 29 transcripts that correlated with age at baseline and clustered in a network centered around type I interferons and pro-inflammatory cytokines. In addition, smaller 8- and 6-gene signatures were found at baseline that associated with vaccine responsiveness during primary and booster vaccination, respectively. When evaluating the kinetic changes in gene expression profiles before and after primary vaccination, a 33-gene signature, dominated by IFN-signaling, pro-inflammatory cytokines, inflammasome components, and immune cell subset markers, was uncovered that was associated with vaccine responsiveness. In contrast, no such transcripts were identified during booster vaccination.
Our results document that antibody responses and gene signatures are different in primary and booster vaccination in old age.

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An early wave of IFN-γ modulates the affinity breadth of the CD8 T cell response

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Truly effective responses to intracellular pathogens are characterized by clonal diversity and diversity of effector T cell types as well as long-lived memory cells. During the immune response against intracellular pathogens, a multitude of CD8+ T cell clones with distinct T cell antigen receptors of various affinity for pathogen derived peptides in complex with MHC molecules are recruited. This clonal diversity is consistently observed despite factors favoring the dominance of high affinity clones. Low affinity CD8+ T cells are particularly important to explain how immunity against one pathogen can protect against future infections with a different, but related pathogen, a phenomenon known as heterosubtypic immunity. Our lab recently discovered that direct T cell - T cell communication limits the expansion of antigen-specific T cells and modulates CD8+ T cell differentiation by skewing towards memory. Those effects are mediated by an early wave of the cytokine IFN-γ. IFN-γ is produced by activated CD8+ T cell clones and shared between them within 24 hours following infection. Based on preliminary data we hypothesise that this early wave of IFN-γ is also critically involved in shaping the affinity breadth of the CD8+ T cell response by allowing low affinity CD8+ T cells to emerge despite the competitive advantages of high affinity clones.

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CD8+ memory T cell subsets defined by CX3CR1

Carmen Gerlach

CD8 T cells are crucial for the protection against intracellular pathogens and cancers. They can be subdivided into functionally distinct subsets that exist even among CD8 T cells that bear the same T cell receptor. We have recently discovered that the chemokine receptor CX3CR1 is a very useful marker to study the heterogeneity within CD8 T cell responses. CX3CR1 expression levels can distinguish classical 'central memory' T cells (TCM; CX3CR1–) from 'effector memory' T cells (TEM; CX3CR1hi). In addition, this marker enabled the identification of a novel CD8 TMem subset, the 'peripheral memory' T cells (TPM), which are CX3CR1int and have unique functional, homeostatic and trafficking properties. Most importantly, by using the high-resolution CX3CR1 marker, we were able to show that, in contrast to the current dogma, conventional TEM do not surveil the extravascular space of peripheral tissues. Instead, TEM are migrationally restricted to blood and spleen, while the newly delineated TPM are the predominant migratory TMem subset surveilling peripheral tissues. We are currently studying the mechanisms responsible for their unique properties.

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LAMTOR2 Is Dispensable for T Cell Receptor-mediated Signaling of Thymocytes but Is Critical for Medullary Thymic Epithelial Cells

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LAMTOR2 is an endosomal adaptor protein that regulates ERK and mTORC1 signaling (Teis et al., 2006; Sancak et al., 2010). Patients with LAMTOR2-deficiency are characterized by severe congenital neutropenia, growth failure, partial albinism, as well as B and T cell deficiencies (Bohn et al., Nat Med 2007). To determine the role of LAMTOR2 in T cell development and homeostasis we used conditional knockout mouse models.

Interferon type 1-inducible deletion of Lamtor2 resulted in reduction of thymus weight and total thymocyte numbers in Mx1 Cre-Lamtor2fl/fl mice. FACS analysis revealed an impaired T cell development with a partial block at the DN (double negative) CD4-CD8- T cell precursor stage in LAMTOR-deficient mice after 7 and 21 days of poly I:C injection. To discriminate between T cell-intrinsic and -extrinsic effects, we generated pre-TCRα-iCre-Lamtor2fl/fl conditional knockout mice. In contrast to Mx1 Cre-Lamtor2fl/fl mice, T cell-specific deletion of Lamtor2 at DN stage 2-3 did not alter frequencies of total thymocytes and T cell progenitor subsets. Furthermore, TCR signaling (p-ERK, p-LAT, p-LCK, p-PLCγ, Nur77) and internalization of TCRβ upon stimulation with anti-CD3ε ± anti-CD28 was normal in pre-TCRα-iCre-Lamtor2fl/fl mice, suggesting that LAMTOR2 in T cells is dispensable for T cell development after DN stage 2-3.

To assess whether impaired T cell development in Mx1-Cre-Lamtor2fl/fl mice is caused by thymic epithelial defects, we analyzed thymic epithelial cells (TECs) 4 days post poly I:C injection by flow cytometry. Mx1-Cre-Lamtor2fl/fl mice showed a reduced ratio of CD45 EpCAM+UEA 1+Ly51- medullary TECs (mTECs) to CD45-EpCAM+UEA-1-Ly51+ cortical TECs. The proportion of mature (MHCIIhigh) mTECs was not altered. Further studies are underway to determine the role of LAMTOR2 in mTECs.

Taken together, our findings show that LAMTOR2 is not required for TCR-mediated signaling in T cell development but might play a critical role in controlling mTEC homeostasis.

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Inherited Loss-of-Function of RIPK1 Causes Immunodeficiency and Intestinal Inflammation

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Children with very early onset inflammatory bowel diseases (VEO-IBD; onset <6 years) often show life-threatening conditions refractory to conventional treatment. VEO-IBD may result from inborn errors of immunity, yet most patients still lack genetic diagnosis. To unravel novel genetic signatures of VEO-IBD, we have conducted whole exome sequencing of 600 patients. Our screen revealed RIPK1 loss-of-function mutations as a novel molecular cause for VEO-IBD in 8 patients from 6 unrelated families. While 2 patients expressing truncated RIPK1 presented with immunodeficiency and diarrhea, 6 patients with missense mutations in the death domain primarily showed signs of VEO-IBD. All patients showed increased susceptibility to infections.
Immunophenotypical analysis of patients' peripheral blood mononuclear cells exhibited impaired lymphocyte differentiation, as demonstrated by reduced frequency of CD45RO+CCR7- effector memory T cells, CD45RO+HLA-DR+ memory activated regulatory T cells, CXCR3+CCR6- and CXCR3-CCR6+ T helper cells as well as IgD-CD27+ class-switched B cells. Patient-derived monocytes and monocyte-like BLaER1 cells with overexpression of RIPK1 mutant variants showed increased IL-1beta secretion upon LPS priming, without requirement of second stimuli (e.g., ATP, nigericin). Blockade of NLRP3 and MLKL by small molecule inhibitors reduced the secretion of IL-1beta, suggesting that both pathways are implicated in the dysregulated proinflammatory responses. In addition to immune dysfunctions, we could also detect intrinsic defects in RIPK1-deficient epithelial cells. Coloncarcinoma cells with transgenic expression of patient-specific mutations exhibited impaired TNF-alpha-mediated NF-kappa-B signaling and TNF-alpha-induced cell death responses.
Our study demonstrates that RIPK1 deficiency is a life-threatening Mendelian disorder with defects in the adaptive and innate immune system as well as the intestinal epithelium. The characterization of rare patients with RIPK1 deficiency highlights the critical role of RIPK1 in human immune and intestinal homeostasis.

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Common gamma chain mutation is partially rescued by alternative splicing and results in IL-21R-like deficiency

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Mutations in interleukin 2 receptor, gamma (IL2RG) perturb signaling of the receptors for IL-2, IL-4, IL-7, IL-15, and IL-21 and cause X-linked severe combined immunodeficiency (X-SCID). Severe forms of SCID are caused by frameshift or splicing mutations in IL2RG. However, normal to low frequencies of T and NK cells were reported for atypical forms of SCID, which can result from different mechanisms such as hypomorphic mutations, spontaneous somatic reversion in T cell clones, or maternal engraftment of T cells. Here, we report two patients with a novel IL2RG frameshift mutation that is partially rescued by alternative splicing and results primarily in dysfunctional IL-4R and IL-21R signaling.
The IL2RG mutation was identified using whole exome sequencing. Expression of IL-2Rγ was analyzed by immunoblotting and flow cytometry. RNA isoforms were studied by cDNA sequencing and qRT-PCR. IL-2Rγ-mediated signaling was assessed by phospho-flow.
In two brothers born to non-consanguineous parents of Romani descent suffering from cryptosporidiosis, severe diarrhea, and cholangitis, we identified a novel mutation in the first exon of IL2RG (c.87delG) predicted to cause a frameshift using whole exome sequencing. Immune workup showed normal pan T, B, and NK cell numbers, but defective B cell differentiation. Flow cytometry and immunoblotting revealed normal expression of the mutated IL-2Rγ protein. Whereas phospho flow showed that IL-2-, IL-7-, and IL-15-induced STAT5 phosphorylation was mildly affected in both patients, STAT6 and STAT3 phosphorylation in response to IL-4 and IL-21 was significantly reduced. Sequencing of IL2RG cDNA revealed the usage of an alternative splice site downstream of exon 1 in both patients resolving the predicted frameshift. The alternative splice site combined with c.87delG causes 16 mutated amino acids in IL2RG. In silico modelling suggested alterations in the N-terminal domain of mutated IL-2Rγ, which might impair functional IL-4 signaling.
We present a novel frameshift mutation in IL2RG causing a hypomorphic SCID phenotype (T+NK+B-) that is partially rescued by alternative splicing. Our report exemplifies that alternative splicing mechanisms might be considered in patients with atypical SCID.

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New tricks for old dogs - Creation of deadramers from single MFG-E8 PS binding domains

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The quick clearance of apoptotic cells is an important task of phagocytes to prevent release of intracellular antigens, thereby maintaining self-tolerance. Target cells are recognized by exposure of an 'eat me' signal with phosphatidylserine (PS) being one the best studied.
Already established cell death detection tools mostly rely on Annexin V to bind PS on dying cells. Despite its efficiency in vitro, serious drawbacks occur with in vivo detection of apoptotic and dead cells due to the strong dependency of Annexin V function on the presence of Ca2+ ions, which also limits the use of Annexin V to a special binding buffer. Furthermore, considerable cell death and damage occurring during organ preparation for FACS analysis might give an obscured view on the actual amount of dead cells in an experimental setup.
We believe in the supremacy of MFG-E8, a calcium-independent PS-binding protein in terms of recognition of dying cells. This activity has been mapped to its C2 domain in previous reports, but it also features an additional homologous C1 domain. In order to harness the advantages of MFG-E8 and develop a flexible and cost-effective tool for the analysis of cell death, we multimerized bacterially expressed and functionalized single PS-binding domains using the Streptavidin-biotin interaction. The C1 domain also showed binding activity to dying cells and even proved to be superior over C2 in this setup. This research will find use in the implementation of future in vitro and in vivo experimental approaches, be it cell death as part of cell fate decisions, during infections or development.

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CCL17 promotes tumorigenesis by supporting regulatory tumor-associated macrophages

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Myeloid cells are critically involved in the pathophysiology of cancers. CCL17, a chemokine of the C-C family, is known to be expressed by CD11b+ dendritic cells (DCs) and to play a role in inflammatory processes, such as colitis. While expression of CCL17 is limited to DCs in the steady state, we found macrophage subsets upregulate CCL17 expression in the tumor microenvironment. Using a CCL17-GFP-reporter/knock-in mouse we investigated the role of CCL17 in colon- and melanoma-cancerogenesis and find that CCL17 promotes tumor initiation and -progression. CCL17–deficient mice developed fewer tumors in the AOM/DSS-induced model of colitis-associated cancer and in syngeneic subcutaneous tumor models (MC38, B16) tumor growth was reduced in the absence of CCL17. The observed effects in both the AOM/DSS and the syngeneic tumor models were conserved in Rag1-deficient mice, indicating T- and B-cell independent mechanisms. Intriguingly, the frequencies and phenotype of tumor associated macrophage (TAM) subsets were significantly altered in the absence of CCL17. Regulatory macrophages, marked by the expression of MMR (CD206) were less abundant in the tumors of CCL17-KO mice and MC38 tumors of CCL17 KO mice showed a reduced Arginase-1 expression, while TNFα secretion was increased in colon tumors of CCL17 KO mice, both indicating a shift in macrophage functionality.
Polarized CCL17-KO BMDMs expressed lower levels of Arginase-1, higher levels of iNos and showed increased TNFα secretion, confirming in vivo findings.
Moreover, co-injection of IL-4 polarized CCL17-KO BMDMs with MC38 tumor cells into WT mice led to a significantly reduced tumor growth, compared to the co-injection with CCL17-competent BMDMs, highlighting the central role of the TAMs for the observed effect in CCL17-KO mice. Thus, beyond its role as marker for TAMs, CCL17 might be a novel regulator of macrophage function with relevance for anti tumor immunity.

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Plasmacytoid dendritic cells regulate resolution of skin inflammation

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Topical application of the toll-like receptor (TLR) 7/8 agonist Imiquimod (IMQ) is used as an established model to induce psoriasis-like skin inflammation in mice. We showed previously that plasmacytoid dendritic cells (pDCs) are part of the immune infiltrate of IMQ treated skin. However, their contribution to IMQ-induced skin inflammation is unclear. Here we found that depletion of pDCs resulted in an exacerbated inflammatory response during prolonged IMQ application. Moreover, the recovery phase after IMQ withdrawal was significantly extended in the absence of pDCs, suggesting an immunosuppressive function of pDCs. The aggravated skin inflammation observed in pDC-depleted mice was accompanied by increased expression of cutaneous pro-inflammatory cytokines and an elevated accumulation of neutrophils and IL17-expressing dermal γδT-cells in the skin. Mechanistically we show that pDCs, which accumulate in draining lymph nodes and IMQ-treated skin, up-regulate the expression of programmed death-ligand 1 (PD-L1) while its cognate inhibitory receptor PD-1 is constitutively expressed by γδT-cells. IMQ activated pDCs actively suppress IL17-production in γδT-cells which can be reverted upon PD-L1 blockade. Collectively, our results demonstrate that pDCs are endowed with an immunosuppressive capacity during IMQ treatment due to the up-regulation of PD-L1, which prevents excessive IL17 production by PD1-expressing γδT-cells.

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The origin of plasmacytoid dendritic cell and the influence of TLR activation

Andrea Musumeci, Anne Krug

Plasmacytoid DCs (pDCs) are a subset of dendritic cells (DCs) highly specialized in the recognition of viral pathogens and the rapid production of large amounts of antiviral type I interferons (IFN). Being short lived and non-proliferative, these cells must be continuously replenished from undifferentiated progenitors in the bone marrow (BM). pDCs were shown to have dual ontogeny, originating from progenitors of the myeloid as well as the lymphoid lineage. Several intermediate subpopulations have been identified as committed precursors based on diverse sets of surface markers. Our interest is focused on a late pDC-like precursor population expressing Siglec H and BST2, but low levels of CCR9. Found predominantly in BM, these cells already possess functional pDC characteristics, such as the ability to respond to TLR-7 and -9 ligands and produce type I IFNs. However, a portion of these cells retains plasticity to differentiate into classical DCs (cDCs) in steady state conditions as well as under inflammation (EAE), upregulating markers such as CD11b and transcription factors (TFs) such as Id2 and Batf3. By means of single cell analysis techniques, including flow cytometry and single cell RNA sequencing, we investigate whether this plasticity is a result of oligopotency, whereby individual cells can be driven towards one or the other cell fate by external stimuli, or subset-specific commitment, where individual cells have either pDC or cDC fate. In line with previous findings, we observe a high degree of precommitment in DC precursors in the steady state. In addition, we find that endosomal TLR9 engagement affects a subset of precursors, increasing output of mature pDCs, by a combination of direct and indirect stromal-cell dependent effects. Our data show that pDC-like precursors are a heterogeneous population with dual fate, and that TLR9 activation increases pDC differentiation from these precursors.

IN MEMORIAM
OF
HARALD VON BOEHMER

Harald von Boehmer
1942 – 2018

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 July 20th 1944 Plotters – a group of oppositionists that planned and attempted to rid Germany of Hitler. The failed attempt led to Hasso being sentenced to death and executed when Harald was just two years old. The early loss of his father remained a challenging circumstance for Harald throughout his life.

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 2019 it is difficult to remember how confused immunologists were in the early 70's about T cells and what controlled their development and subsequent activation.

In the mid-1970s, Harald, together with Jonathan Sprent, showed that stable bone marrow chimerism is the result of deletion of donor cells with reactivity against recipient MHC. The presence of suppressor cells was ruled out in these studies by showing that the addition of chimera lymphocytes to normal donor-type lymphocytes did not prevent the latter from differentiating into cytotoxic lymphocytes. During the same period, Harald and Jonathan proved that in order to be stimulated by an antigen/MHC combination, the T cells had to be exposed to the MHC allele under study during development in the thymus.

In the early 1980s, the first T cell receptor (TCR) genes were found (Tak Mak and Mark Davis discovered the human and mouse TCR, respectively), and experiments transferring TCR alpha 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, a finding confirmed by crystallographic studies.

Curriculum Vitae

The next question Harald decided to tackle was the issue of immunological tolerance. How is it possible that T cells can respond to many different antigenic peptides, bound by MHC proteins, but do not attack self-antigens when functioning properly? Harald and his colleagues showed that self-reactive T cells are destroyed as they develop in the thymus. This was done through a very clever experiment in which mice expressing T cells with a single TCR were produced. The antigen target of these T cells was an HY-peptide expressed only in male mice. The male-specific T cells appeared as expected in the lymph nodes of female mice, but they disappeared during development in the thymus of male animals. In later years, Harald and his colleagues readdressed this issue and reported deletion of CD4+CD8+ thymocytes in the absence of TCR editing.

The TCR transgenic mice also served to answer questions related to positive selection and the matching of specificity and function. Harald's team demonstrated that the interaction between the TCR and peptide/MHC complex determines whether a thymocyte would differentiate along the CD4+CD8– T cell lineage or the CD4–CD8+ T cell lineage. They proved that there is, in fact, positive selection, as mice lacking the appropriate MHC allele to pair with the transgenic TCR failed to generate single-positive cells, and thus development was arrested at the CD4+CD8+ stage. This was then named "death by neglect" (inability to bind MHC) as opposed to "death by negative selection" which is the active induction of apoptosis in thymocytes with high affinity for self peptides or MHC. Harald summarized these findings very fittingly: "the thymus selects the useful (positive selection), destroys the harmful (negative selection), and ignores the useless (no MHC binding)".

Harald's later work continued to add basic insights to the understanding of early T cell development, including the identification of the pre-TCR and its roles in thymocyte survival, allelic exclusion, and commitment to the αβ or γδ T cell lineage. Harald and his team also studied the generation and function of regulatory T cells with the goal of utilizing these cells to prevent or interfere with unwanted immune reactions.

After his retirement, while already living here in Seefeld in Tirol, Harald wrote in his last review: "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".

Eulogy for Harald von Boehmer

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 the German

In Memoriam of Harald von Boehmer (1942–2018)

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On June 24, 2018, at the age of 76, 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 worldwide 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. Together 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
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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 ben-

efited 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 meeting. 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

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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

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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	Donor of responding T cells: C57L female			Donor of responding T cells: $\alpha\beta$TCR transgenic female			Donor of responding T cells: $\alpha\beta$TCR transgenic male		
		1/frequency		p*	1/frequency		p	1/frequency		p
C57BL/6 female	$CD4^-8^+$	>25,000			>25,000			>25,000		
C57BL/6 female + Con A	$CD4^-8^+$	2.3	(1.6–3.3)	0.66	1.8	(1.2–2.6)	0.87	2.3	(1.3–4.0)	0.18
	$CD4^+8^-$	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.

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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 (1.0–1.6×10^8 per thymus) transgenic mice.

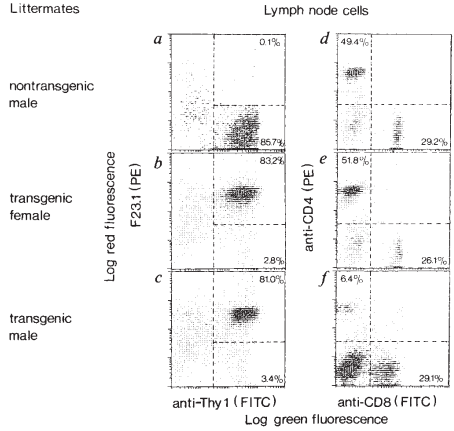

Fig. 1 Comparison of cell surface expression of $F23.1^+$ TCR β chain, CD4 and CD8 molecules on lymph node T ($Thy1^+$) cells from female and male $\alpha\beta$TCR transgenic mice and their nontransgenic male littermate as analysed by two-colour flow cytometry. Lymph node cells were stained with biotinylated 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

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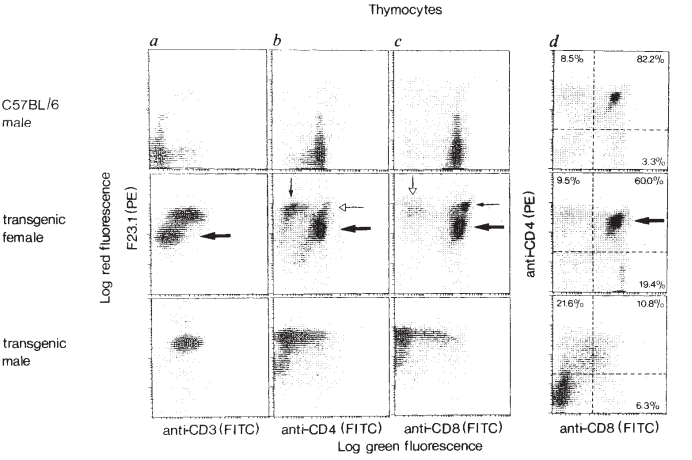

Fig. 2 Expression of F23.1$^+$ TCR β chain, CD3, CD4, and CD8 molecules on thymocyte subpopulations from normal C57BL/6 and from $\alpha\beta$TCR transgenic female and male mice. In panel *a*, cells were incubated consecutively with anti-CD3 mAb, FITC-conjugated goat anti hamster immunoglobulin, mouse 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

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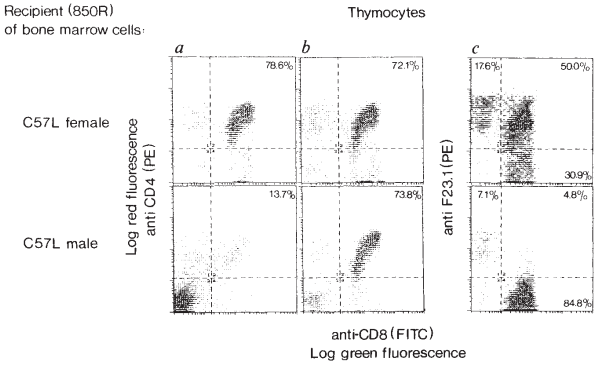

Fig. 3 Surface phenotype of thymocytes from C57L male and female irradiation chimeras reconstituted with bone marrow of $\alpha\beta$TCR transgenic female, either alone (*a*) or with normal bone marrow from C57L female (*b, c*). Thymocytes were stained with anti-CD4-PE (*a, b*) or biotinylated F23.1 (*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 auto-specific 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.

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

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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,

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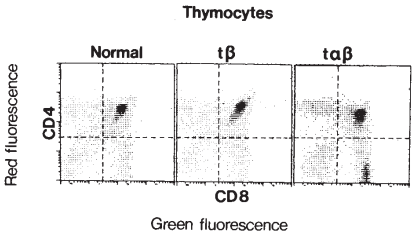

Fig. 1 The proportion of CD4/CD8 thymocyte subsets in female normal, β transgenic and $\alpha\beta$ transgenic mice. Thymocytes were stained by a mixture of phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibodies.

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.

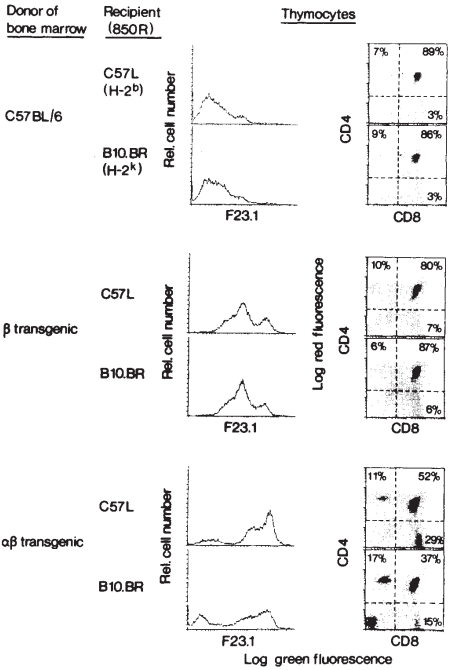

Fig. 2 The expression of transgenic β chain, CD4 and CD8 molecules on thymocytes obtained from various mice.

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

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thymuses of MHC-mismatched (H-2^k or H-2^d), but not partly mismatched (C57B1/6 × DBA/2)F_1 recipients, contained fewer thymocytes (10–20%) than thymuses of H-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-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-2^d/H-2^d homozygous but not $\alpha\beta$ transgenic H-2^d/H-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-2^b mice

If an interaction of the transgenic, heterodimeric TCR with thymic H-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 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 C57B1/6 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.

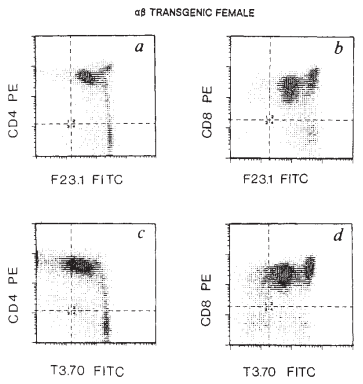

Fig. 4 Staining of thymocytes from female $\alpha\beta$ transgenic H-2^b mice by F23.1, T3.70, CD4 and CD8 antibodies. Thymocytes (yield 1×10^8) were doubled stained for F23.1 and CD4 or CD8 (*a* and *b*) and for T3.70 and CD4 or CD8 (*c* and *d*).
Methods. Thymocytes were double stained with F23.1 and 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: $CD4^+$, $F23.1^+ > 95\%$; $CD8^+$, $F23.1^+ > 95\%$; $CD4^+$, $T3.70^+ < 5\%$; $CD8^+$, $T3.70^+ > 90\%$.

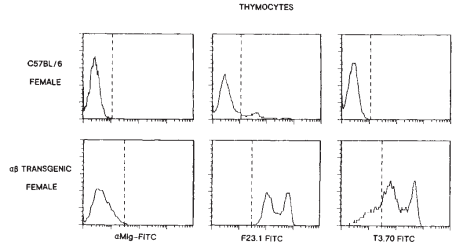

Fig. 3 Staining of thymocytes from female C57Bl/6 and $\alpha\beta$ transgenic H-2^b mice by F23.1 and T3.70.
Methods. Thymocytes from a female $\alpha\beta$ transgenic mouse were incubated with phosphate-buffered saline, F23.1 or T3.70 followed by incubation with FITC-labelled second antibody as described 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.

Single staining of thymocytes from female $\alpha\beta$ transgenic H-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-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

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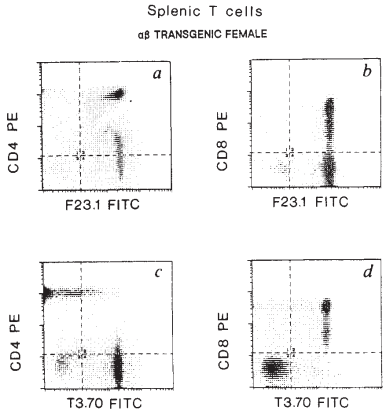

Fig. 5 Staining of peripheral, nylon-wool-purified splenic T cells from female $\alpha\beta$ transgenic H-2^b mice by F23.1, T3.70, CD4 and CD8. Nylon wool-nonadherent spleen cells from a female $\alpha\beta$ transgenic mouse were double stained with F23.1 and CD4 or CD8 (*a* and *b*) and with T3.70 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

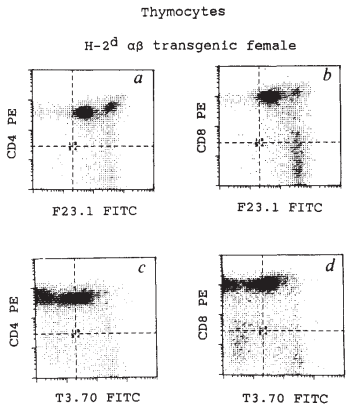

Fig. 6 Staining of thymocytes from female $\alpha\beta$ transgenic H-2^d mice by F23.1, T3.70, CD4 and CD8 antibodies. Thymocytes (yield 7×10^7) were double stained with F23.1 and CD4 or CD8 (*a* and *b*) and with T3.70 and CD4 or CD8 (*c* and *d*).
Methods. Female $\alpha\beta$ transgenic H-2^d mice 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: $CD4^+$, $F23.1^+ > 95\%$; $CD8^+$, $F23.1^+ > 95\%$; $CD4^+$, $T3.70^+ < 5\%$; $CD8^+$, $T3.70^+ < 5\%$.

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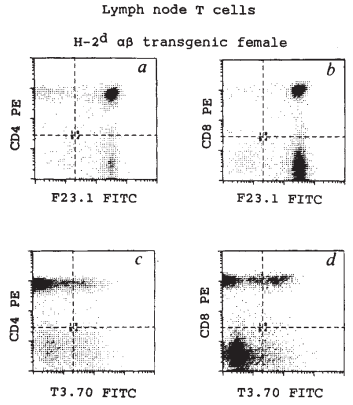

Fig. 7 Staining of lymph node cells (yield 2×10^7) from female $\alpha\beta$ transgenic H-2^d mice by F23.1, T3.70, CD4 and CD8 antibodies. **Methods.** Female $\alpha\beta$ transgenic H-$2^{d/d}$ mice were produced as described in Fig. 6. Lymph node cells were enriched for T cells by passing lymph node 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.

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

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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 $pT\alpha^{+/-}$

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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).

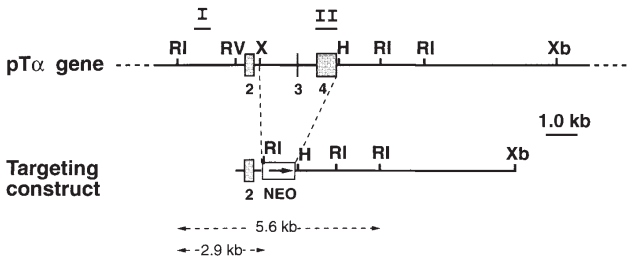

FIG. 1 Disruption of the pTα gene by homologous recombination: partial organization of the pTα locus[22] and structure of the targeting vector. The pTα gene was cloned from a 129/Ola-derived genomic library (kind gift of A. Berns, Amsterdam). The targeting vector was constructed by replacing approximately 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.

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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.

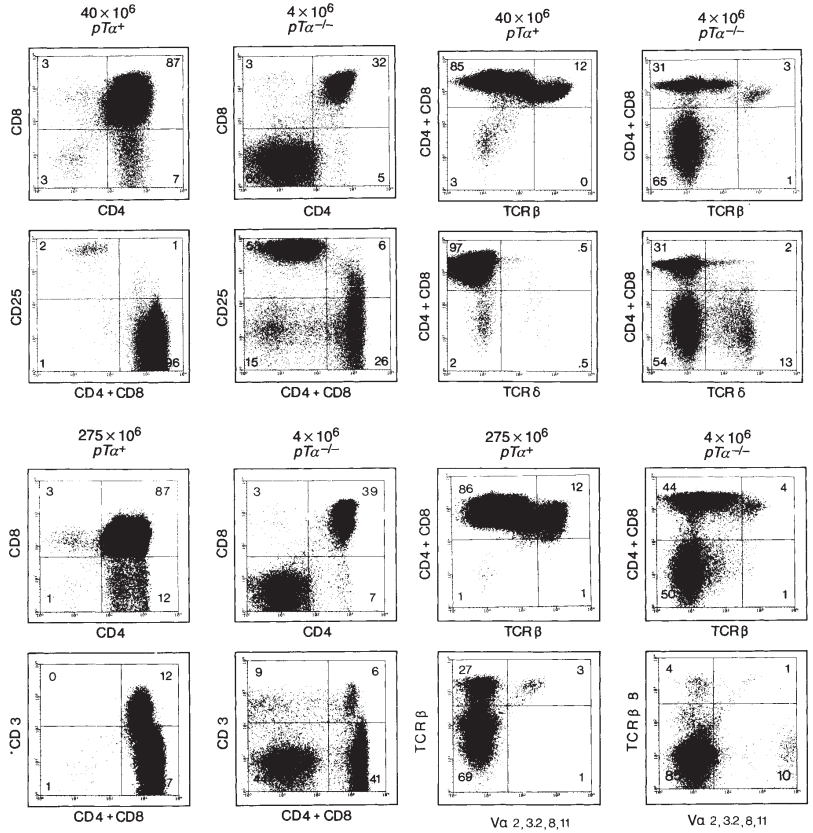

FIG. 2 Thymocyte subsets from $pT\alpha^+$ and $pT\alpha^{-/-}$ mice. Staining of thymocytes from 5-day-old (top two rows) and 28-day-old (bottom two rows) litters with CD4, CD8, TCRβ, TCRδ and TCR-V_α antibodies. All stainings were three-colour stainings with the following antibody-conjugates: anti-CD3–biotin 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 $\sim 4 \times 10^5$ 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.

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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]. □

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Poster Abstract Authors

(alphabetical order)

Speaker Schedule

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Becher, Burkhard	Sunday	20.01.19	11:30
Blander, Julie Magarian	Sunday	20.01.19	16:00
Davis, Mark	Saturday	19.01.19	18:00
Diefenbach, Andreas	Monday	21.01.19	09:00
Ellmeier, Wilfried	Wednesday	23.01.19	12:15
Förster, Reinhold	Wednesday	23.01.19	09:00
Griffiths, Gillian	Tuesday	22.01.19	11:00
Hogquist, Kris	Sunday	20.01.19	09:30
Hornung, Veit	Sunday	20.01.19	17:00
Iannacone, Matteo	Wednesday	23.01.19	11:00
Jameson, Stephen	Wednesday	23.01.19	14:30
Kastenmüller, Wolfgang	Wednesday	23.01.19	09:30
Kallies, Axel	Wednesday	23.01.19	17:00
Liston, Adrian	Wednesday	23.01.19	11:30
Littman, Dan	Sunday	20.01.19	16:30
McHeyzer-Williams, Michael	Tuesday	22.01.19	09:00
Prinz, Immo	Monday	21.01.19	11:00
Powrie, Fiona	Tuesday	22.01.19	17:00
Rajewsky, Klaus	Wednesday	23.01.19	14:00
Robey, Ellen	Sunday	20.01.19	11:00
Rodewald, HR	Sunday	20.01.19	09:00
Romagnani, Chiara	Monday	21.01.19	09:30
Rudensky, Sasha	Monday	21.01.19	17:00
Schlitzer, Andreas	Sunday	20.01.19	14:30
Turley, Shannon	Sunday	20.01.19	14:00
Victora, Gabriel	Tuesday	22.01.19	09:30

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