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Abstracts

Evolution of the Immune Systems of Metazoa

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Understanding the evolution of the immune systems of Metazoa by comparative immunology implies looking at homologies and divergences to trace the origin of the elements but also looking at analogies and convergence to understand the selection pressures and the constraints imposed onto their development. The arsenal of elements that have the properties to be included in efficient, specific and economic immune systems from sponges to mammals is large but limited because of these constraints. Therefore, it is not surprising that sharing and conservation of some successful elements and pathways can be seen across Metazoa (phagocytosis, signaling cascades, complement components, immunoglobulin superfamily, leucine rich repeat and lectin receptors), without being automatically accompanied by the conservation of whole systems. Whether innate or adaptive, the various arms of intra- or extracellular immune systems end up using diversified populations of receptors and effectors in all Metazoa. Duplication within the germline, with the creation of multigene families can increase the number of elements adapted to either recognition or regulation. But it is the somatic usage of combinatorial association at the peptide, RNA, DNA levels that allows the individual's immune systems to use a number of receptors and effectors much larger than the number of genes of one's genome. Some of these somatic adaptations can be used in invertebrate phyla. In vertebrates the random somatic generation of large repertoires of immunoreceptors via AID and RAG enzymes, plus the possibility to increase further the repertoires by gene conversion and somatic mutation, thereby increasing the risk of autoimmunity, made necessary the selection of effector cells. What made it possible was the uncommitted lymphocyte a cell type not present in all Metazoa, and allowing clonal selection. Its origin lies in some invertebrates hemocytes as seen from the sharing of transcription factors, but its modern T- B-like lineages can now been traced in the most ancient vertebrates, the agnathans (hagfish and lamprey) even though they use leucine-rich repeats receptors when gnathostomes (from shark to mammal) use immunoglobulin superfamily members. For selecting T cells the combination of proteosome processing, MHC class I and II presentation in the thymus has been the solution selected in gnathostomes. B-cell selection, also a conserved step, takes place via exposure to native determinants in various stromal environments. This basic unit has been conserved with minor variations. What can vary are the number of receptor isotypes, the ways to create somatic diversity and the architectures of the lymphoid organs, leading to variations in the immune responses. In addition variations are visible in the balances between the usage of innate and adaptive components that are modulated by the life-histories traits specific to each category of organism.

From Embryonic Stem Cells and Induced Pluripotent Cells to Lymphocytes

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The development of the cells and organs of the innate and the adaptive immune system of mice and their functions has traditionally been studied "in vivo" in wild-type mice and selected mutant mice with important alterations in their embryonic development and in the functions of their immune system at different postnatal times. Thus, the roles of many experimentally identified genes have been studied in transgenic mice that have been generated by targeted integration into appropriate, in many cases the proper, homologous sites of the mouse genome. Embryonic stem (ES) cells, but not hematopoietic stem cells, progenitor or differentiated cells of the innate and the adaptive immune system are capable of integrating exogenously introduced genes by homologous recombination. Whole genome transcriptome and proteome analyses have now identified such a large number of genes with potential functions at different stages of the development and functions of the system that the establishment and breeding of transgenic mice for each of these genes or combinations of them appears no longer possible. Alternative methods allowing a larger number of genes to be analysed from homologously recombined, mutant ES cells to cells of the immune system must be developed. Thus, one general strategy plans to develop wild-type and mutant ES cells "in vitro" to pluripotent, longterm-reconstituting

hematopoietic stem cells (pHSC) which are then transplanted into lethally irradiated recipient mice to regenerate their immune system with ES cell-derived donor cells. We have improved previously developed conditions for the "in vitro" differentiation of wild-type and mutant ES cells to myeloid and lymphoid cells that now allow a quantitative assessment of the time schedules and differentiation potentials of wild-type and mutant mouse ES cells. Mesodermal cells appear at day 5, pluripotent hematopoietic progenitors between day 9 and 11, and lymphoid progenitors from day 12 to 15 of "in vitro" differentiation. Although the differentiation to ES cells still needs to be improved to allow full reconstitution of all hematopoietic and lymphopoietic cell lineages in all lymphoid organs this "in vitro" development of ES cells is already good enough to analyse mutations leading to defects in early hematopoietic development. Thus, tal-$1^{-/-}$ and runx-$1^{-/-}$ ES cells show abortive differentiation after the formation of mesodermal cells, but not before. These differentiating mutant ES cells are compared with wild-type cells in microarray RNA expression analyses at these crucial, early times of hematopoietic differentiation to identify some of the candidate genes possibly involved in primitive and definitive erythropoiesis and in the development of pluripotent hematopoietic progenitor cells.

The "in vitro" differentiation protocol can also be used to analyze the hematopoietic potentials of induced pluripotent stem (iPS) cells generated by transduction of differentiated mouse cells with the transcription factor genes sox2, oct4 and klf4. iPS cells generated from mouse embryonic fibroblasts show qualitatively and quantitatively comparable lymphoid differentiation capacities, while iPS cells generated from 5-FU-treated bone marrow cells, or from fetal liver-derived preBI cells show deficiencies in this lymphoid differentiation capacities which are differently severe for different iPS cell clones—a defect that appears related to the levels of continued transduced transcription factor expression during "in vitro" differentiation used for the generation of the iPS cells. Such overexpression of iPS-inducing transcription factors should be controllable, i.e., downregulatable with tetracycline-controlled vectors.

In conclusion, such "in vitro" ES cell differentiation to transplantable, longterm-reconstituting pHSC will allow to test a larger number of candidate genes in the development of cells of the innate and the adaptive immune system "in vivo".

Divergence of Innate-Like and Adaptive T-Cell Precursors Occurs before Commitment to the $\alpha\beta$ and $\gamma\delta$ Lineages

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Besides conventional adaptive T cells, the thymus supports the development of unconventional T cells with innate characteristics, such as NKT cells and IELs. Their particular functional properties, tissue localization, antigenic specificity and the requirement for agonist-mediated positive selection distinguish them from adaptive T cells. It is believed that both innate-like and adaptive T cells develop from common precursors undergoing instructive T cell receptor (TCR) mediated lineage fate decisions. However, we show that in contrast to adaptive $\alpha\beta$T cells, innate-like $\alpha\beta$T cells are not selected against functional TCRγ rearrangements and express TCRγ mRNA. Likewise, in contrast to the majority of $\gamma\delta$T cells, thymic $\gamma\delta$NKT cells are not efficiently selected against functional TCRβ chains. Furthermore we show, that $\gamma\delta$NKT cells carry auto-reactive TCRs suggesting that they require agonistic TCR signals for lineage commitment similarly to $\alpha\beta$NKT cells. These data suggest that innate-like and adaptive T cells develop from distinct precursors, which respond differently to agonistic and autonomous TCR signals and commit independently to the $\alpha\beta$ or $\gamma\delta$ lineages. Our findings outline a new framework for the understanding of early thymocyte differentiation and TCR-driven $\alpha\beta/\gamma\delta$ T cell lineage commitment.

Immunoreceptor Signaling in Innate and Adaptive Immune System Cells

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Antigen receptor engagement in T and B lymphocytes leads to activation of immunoreceptor tyrosine-based activation motif (ITAM)-containing transmembrane adapters including CD3, T cell receptor (TCR)ζ, Igα, and Igβ chains that initiate formation of signaling microclusters and induction of rapid and dynamic changes in actin cytoskeleton, although the exact mechanism by which the TCR and B cell receptor (BCR) initiates actin polymerization is incompletely understood. The Vav family of guanine nucleotide exchange factors (GEF) is implicated in generation of TCR and BCR signals and immune synapse formation. Using live cell imaging, we show that Vav participation in signaling microclusters is critical for initiation of actin polymerization. Surprisingly, the intrinsic GEF activity is largely dispensable for Vav function in antigen receptor signaling in lymphocytes indicating a linker role.

Recently, ITAM signaling by DAP12 and FcRγ chains has also been implicated in outside-in signaling by integrin receptors in myeloid cells. For example, integrin engagement can induce rapid generation and release of reactive oxygen species (ROS) by phagocyte NADPH oxidase (phox/NOX2/gp91), also termed oxidative burst, a critical antimicrobial mechanism of neutrophils. Genetic mutations in the NOX2 complex are associated with chronic granulomatous disease characterized by recurrent and life-threatening microbial infections. We provide genetic and biochemical evidence that the VavGEFs, and phospholipase C-γ2 are critical mediators of adhesion-dependent ROS production in neutrophils critically required for neutrophil-dependent host defense against systemic infection by *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

We also identified integrin-mediated activation of DAP12 and FcRγITAM signaling as a central regulatory mechanism for antigen presentation in dendritic cells (DCs). We demonstrate the requirement for this pathway in helper T cell priming and induction of experimental autoimmune encephalomyelitis (EAE). Although the cellular mechanisms of antigen processing and presentation have been studied extensively, the precise signaling pathways governing antigen presentation are incompletely understood. In this context, immature DCs specialize in antigen capture and maintain a highly dynamic pool of intracellular major histocompatibility complex class II (MHCII) that continuously recycles from peptide loading compartments to the plasma membrane and back again, a process that facilitates sampling of environmental antigens for presentation to T helper cells. We show that the signaling pathway mediated by DAP12 and FcRγ and Vav family GEFs controls the half-life of surface peptide-MHCII (pMHCII) complexes and is critical for CD4 T-cell triggering. Strikingly, mice with disrupted DC ITAMs show defective T helper cell priming in vivo and are completely protected from EAE

induction. Mechanistically, we show that deficiency in ITAM signaling results in increased pMHCII internalization, impaired recycling, and an accumulation of ubiquitinated MHCII species that are prematurely degraded in lysosomes. We propose a novel mechanism for control of T helper cell priming.

T-Cell Progenitors and T-Cell Leukemia

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The elucidation of physiological pathways of T-cell progenitors migrating from bone marrow to thymus remains challenging. The prevailing model postulated a common lymphoid progenitor as the upstream progenitor that gives rise to T-cell progenitors in the thymus. However, in vitro, intrathymic T-cell progenitors have abundant myeloid potential, casting doubt on the original dichotomous (lymphoid vs. myeloid) model and supporting a new myeloid-based model of hematopoiesis. Recent fate mapping experiments showed: (1) that most T-cell progenitors in the thymus arise from an interleukin 7 receptor (IL-7R)-expressing progenitor, consistent with a lymphoid-primed route from bone marrow to thymus, and (2) that majority of thymic myeloid cells are progeny of IL-7R negative cells. Therefore, T-cell progenitors in the thymus (and common lymphoid progenitors in the bone marrow) can be driven in vitro towards myeloid fates but they have little, if any myeloid potential in vivo. This is different when Notch1 is deleted, using a T cell-biased Cre-deleter, in T-cell progenitors. Such Notch1-deficient T-cell progenitors are blocked in their T-cell development, and give rise to conventional and plasmacytoid dendritic cells in the thymus. Finally, a widely accepted hallmark of T-cell progenitors is their lack of self-renewal, which would render thymus function absolutely dependent on continuous supply of new progenitors from the bone marrow. We have now found experimental conditions that reveal an ability of the thymus for autochthonous T-cell development in the absence of de novo progenitor colonization. I will consider how fit progenitors from the bone marrow prevail over exhausted progenitors in the thymus, and present implications for an origin of T-cell leukemia in the thymus.

Generating Self-Antigen Diversity for Central Tolerance

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In the course of central self-tolerance induction a highly diverse repertoire of T cell receptors (TCRs) is probed against a matching array of their ligands, namely self-peptide/MHC complexes. While much has been learnt about the molecular mechanisms underlying the generation of TCR diversity, the cellular and molecular strategies responsible for intra-thymic expression and presentation of self-antigen repertoires are less well understood. The diversity of self-peptide display is on the one hand afforded by the remarkable heterogeneity of thymic antigen-presenting cells and on the other hand by their unconventional molecular pathways of antigen expression, processing and presentation (Derbinski and Kyewski 2010; Klein et al. 2009). Here we will focus on one mechanism, namely promiscuous gene expression (pGE). pGE denotes the property of a specific subset of thymic stromal cells—medullary thymic epithelial cells (mTECs)—to express a highly diverse set of tissue-restricted antigens (TRAs) representing essentially all tissues of the body. This allows self-antigens, which otherwise are expressed in a spatially or temporally restricted manner to become continuously accessible to developing T cells. While the pool of promiscuously expressed genes encompasses more than 1,000 TRAs, each particular self-antigen is expressed by only 1–3% of mTECs in a mosaic fashion. How gene expression at the single cell level faithfully adds up to the full complement of pGE is not clear. We addressed this issue by single cell RT-PCR analysis of ex vivo isolated selected subsets of mouse and human mTECs. Our studies reveal both stochastic and deterministic expression patterns. As a result single mTECs express partly redundant and partly complementary sets of self-antigens. In this way it is ensured that (1) each antigen will be expressed by a critical number of mTECs and (2) that antigen diversity will be generated.

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Ontogeny and TCR Diversity of Regulatory Foxp3$^+$ T Cells

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Regulatory T cells that express the Foxp3 transcription factor (Tregs) are critical for the maintenance of peripheral tolerance, and lack of functional Tregs results in a severe autoimmunity in humans and in mice. Majority of Tregs differentiate in the thymus (natural nTregs). It is also postulated that these cell lineage commitment occurs as a result of high affinity interactions between antigen receptors (TCRs) expressed by Tregs precursors and self MHC/peptide complexes expressed on thymic stromal cells. However, the diversity of TCRs expressed on Tregs is very high and can match or possibly exceed the diversity of TCRs found on majority of Foxp3$^-$CD4$^+$ T cells. In addition, Tregs can specifically recognize non-self antigens bound to MHC or foreign MHC molecules, so the postulated inherent bias of these cells TCRs to self-antigens remains a contentious issue.

It has also been shown that upon suboptimal conditions of antigen presentation, Foxp3 expression is induced outside the thymus, in vivo or ex vivo, in naïve CD4$^+$ T cells. This "conversion" of naïve CD4$^+$ T cells often requires TGF-β, and can be further augmented by retinoic acid. Some organs like the intestine may also represent a unique milieu where recruitment of naïve CD4$^+$ T cells to Tregs lineage occurs more frequently than in other organs. Treg clones that differentiated outside the thymus are called adaptive or induced Tregs. Whereas the key role of nTregs in peripheral tolerance is established, the exact role of adaptive Treg's maintenance of homeostasis requires further research.

This presentation will provide the current overview of the key topics in contemporary Tregs research that have to be further investigated to understand these cells ontogeny and antigen specificities.

Autoimmunity Caused by T Cells Escaping Negative Selection

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More then 15 years ago it was shown that a considerable number of conventional human peripheral T cells express two T cell receptor (TCR) α chains (Padovan et al. 1993). Based on this it was postulated that such T cells could escape negative selection and might induce autoimmunity. However, up to now very little experimental evidence supporting this hypothesis is available.

We have now created a double transgenic mouse model in which T cells escaping negative selection in the thymus by expressing two TCR α chains induce autoimmunity. Thus, mice expressing a transgenic TCR specific for influenza hemagglutinin (HA) and expressing HA under control of the CD11c promoter show early in life anemia, B-cell hypoplasia and myeloid hyperplasia and develop later in life severe arthritis and high titers of IgG anti-nuclear autoantibodies. Negative selection of transgenic TCR expressing T cells in the thymus of these double transgenic mice is practically complete. However, activated T cells expressing the transgenic TCR can be found in the periphery and a large part of these express a second TCR α chain. The fact that TCR-HA $\times$ CD11c-HA mice on a RAG deficient background have no T cells and do not show any signs of autoimmunity strongly suggest that the TCR-HA expressing T cells in the double transgenic mice require a second TCR α chain in order to get out of the thymus. Thus, the TCR-HA/CD11c-HA mouse model indeed shows that autoreactive T cells can escape negative selection by expressing a second TCR α chain and can cause autoimmunity.

In order to test whether Tregs can prevent and/or cure the above-described diseases, newborn double transgenic mice were injected with HA specific Tregs or polyclonal Tregs. Preliminary findings indicate that HA specific Tregs can prevent the early form of disease (anemia, B-cell hypoplasia and myeloid hyperplasia) but not the late form of disease. In marked contrast no improvement of the health status of double transgenic mice was observed after transfer of polyclonal Tregs.

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The Pro-Inflammatory Role of CD8 Effector T Cells

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Inflammatory reactions are complex biological responses believed to be triggered by pathogens or "danger signals" that have a major protective role by recruiting innate immunity cells, favoring lymphocyte activation and differentiation, and thus contributing to the sequestration and eventually eliminating the injurious stimuli. Although certain T lymphocyte types such as TH17 cells may co-participate in inflammatory reactions, the generation of such cells from a naïve T cell pool requires previous differentiation steps within a pre-existing inflammatory milieu. In this context inflammation is regarded as beginning with an innate immune response mediated by tissue resident cells that may eventually be perpetuated and amplified by the recruitment and/or differentiation of certain T cell types.

We will show that besides γ-interferon and cytotoxic activities, CD8 responses also generate effectors with a potent pro-inflammatory capacity. The generation of these effectors does not require the presence of "danger signals" and does not follow the general rules believed to govern CD8 differentiation, since they are generated early in the response when antigen is abundant and extensive division did not occur. These effectors induce the immediate recruitment of lymphocytes and different accessory cell types to the place where the immune response takes place, what should favor the control of the exogenous aggression. Moreover, CD8 inflammatory effectors also up-regulate local S1P concentrations, preventing circulating cells' egress. These events will have a fundamental role in the recruitment of antigen-specific naïve cells that are dispersed through the body to the site of the immune reaction. These results demonstrate that CD8 differentiation into effector functions is not necessarily preceded by an expansion phase. They show that CD8 cognate interactions have a major role in inducing the early recruitment of multiple cell types to the place where the antigen is present. They demonstrate for the first time that local inflammatory reactions can modify S1P concentrations in tissues, thus modulating local S1P gradients. Conceptually, they also show that although inflammation does modulates cognate responses, CD8 T cells cognate responses also have a fundamental role in initiating and modulating inflammatory reactions.

Prevention of Type 1 Diabetes

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Immunologists have not been very good in inducing specific tolerance in the adult immune system, and hence the pharmaceutical industry has developed generally immune suppressive drugs that however, are of limited use because they can cause severe infections and lymphomas that threaten the life of patients who are supposed to be helped by these drugs. We have focused our attention on preventing type 1 diabetes by inducing regulatory T cells that interfere with the autoimmune response to insulin which is essential in the spontaneous development of type 1 diabetes in NOD mice, representing a valid model of human disease. Experiments from a variety of labs have indicated that T cell clones which cause disease recognize autoantigens with low affinity or of low agonist activity, perhaps due to the fact that such T cells escape negative selection in the thymus. On the other hand we have found that conversion of naïve T cells into Treg requires the sub-immunogenic delivery of TCR ligands with high agonist activity. We thus have obtained a mimetope of the natural insulin epitope believed to be the most important peptide in inducing the disease. We have found that the sub-immunogenic delivery of this mimetope in young NOD mice protects the mice from disease by an increased number of $Foxp3^+$ cells that suppress the insulin specific response. Thus, the antigen-specific induction of Treg can replace general immunosuppression to yield specific tolerance that is not associated with the bad side effects of general immunosuppression. One might argue that there is no need to prevent type 1 diabetes since it can be treated by exogenous insulin. Unfortunately this is a bad argument. Problems with delivering the right dose of exogenous insulin have resulted in severe complications such as kidney failure and the need to amputate severely infected legs. Thus, the described simple way of preventing type 1 diabetes in the absence of general immunosuppression could help to solve the present day problems of managing the disease.