Instituto Juan March
de Estudios e Investigaciones

86

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Transcription Factors in Lymphocyte Development and Function

Organized by

J. M. Redondo, P. Matthias and S. Pettersson

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Introduction

J. M. Redondo, S. Pettersson and P. Matthias

During the last 6-8 years there has been a rapid expansion in our understanding of the molecular mechanisms underlying the regulation of gene transcription, signal transduction cascades and developmental programs in mammals. In particular, the hematopoietic system typified by the lymphoid system has established itself as the foremost paradigm for the study of cell-specific transcription and signal transduction in a physiological setting. In addition, many of the findings have led to new insights into the developmental cues that guide the development as well as execute the effector functions of the immune system. The two arms of this system, the T and B lymphocytes, share many common regulatory mechanisms and also have some notable differences. To properly function, the immune system requires an intimate interplay between these two cell types. Therefore, the idea to organize a workshop that would bring together scientists working on transcription control and signal transduction in B and T lymphocytes made perfect sense, as was evidenced during the course of the meeting.

The contributions presented in the workshop highlighted the major `role that transcription factors play in controlling the development and homeostasis of the immune system. Thus, many of the specific functions of transcription factors as regulators of hematopoietic development at early and late stages, in lymphocyte differentiation processes, or as integrators coupling extracellular stimuli to gene expression programs (lymphoid cell maturation, differentiation, activation etc.) were presented and discussed. Many of the experimental approaches were based on the generation of knockout mice lacking transcription factor genes or transgenic mice overexpressing them. The results presented evidenced the critical involvement of different transcription factors in T and B lineage development, lymphoid-cell differentiation as well as the multiple biological functions needed to maintain the regulatory and effector functions of the immune system. Similar strategies using transgenic and knockout mice expressing or lacking signal transduction components allowed the identification of signalling cascades and kinases regulating T cell development. Also, experiments using mice harbouring miniloci transgenes comprising the enhancers of B or T cell-specific genes revealed the role of these cis-acting elements in the tight temporal and spatial regulation of recombination and cell-type specific gene expression.

These results also introduced chromatin as an important component in the regulation of complex developmental pathways. Besides these analyses using mice models, a number of talks of the workshop established functional connections between transcription factor activity and the regulation of differentiation and function of lymphocytes using in vitro models. Thus, several contributions were concerned with the analysis of the molecular mechanisms by which transcription factors may regulate cell cycle control, proliferation or apoptosis in lymphocytes. Similarly, work on the role of signalling cascades connecting extracellular stimuli with transcription factor activation (or deactivation) and the subsequent effects on lymphoid gene expression was also presented.

This workshop gathered scientists working on transcription in the B and T cell fields, and provided a unique and exciting forum to exchange, discuss, and integrate the most recent information on lymphocyte development and function, critical issues for a better understanding of the function and regulation of the immune system.

J. M. Redondo

P. Matthias

S. Pettersson

Session 1: Molecular basis of hematopoietic gene loci regulation

Chair: Harinder Singh

The interacting transcription factors, PU.1 and Pip, analysis of their roles in lymphocyte development and activation.

Harinder Singh, Howard Hughes Medical Institute, The University of Chicago, Chicago, IL USA

PU.1 is a unique regulatory protein required for the generation of both the innate and adaptive immune system. It functions exclusively in a cell-intrinsic manner to control the development of granulocytes, macrophages and B and T lymphocytes. Multipotential lymphoid-myeloid progenitors ($AA4.1^+$, Lin^-) are significantly reduced in PU.1 -/- embryos and fail to generate pro B cells in response to stromal contact and IL-7. PU.1 -/- progenitors are defective in the expression of IL-7Rα as well as the B lineage transcription factors EBF and Pax-5. Retroviral transduction of PU.1 cDNA into PU.1 -/- progenitors induces expression of IL-7Rα and the generation of proB cells in response to IL-7. These results will be discussed in the context of a regulatory network of transcription factors specifying B lineage development.

Pip is a lymphoid-specific IRF transcription factor that is essential for B and T cell activation. In B lymphocytes, Pip is recruited by PU.1 to composite sites which are essential for the activity of immunoglobulin light chain gene enhancers. Pip is recruited to its binding site on DNA by phosphorylated PU.1. PU.1/Pip interaction is shown to be template-directed and involves two distinct protein-protein interaction surfaces: (1) the ets and IRF DNA-binding domains and (2) the phosphorylated PEST region of PU.1 and a lysine-requiring putative α-helix in Pip. Thus, a coordinated set of protein-protein and protein-DNA contacts are essential for PU.1/Pip ternary complex assembly. To inhibit gene activation by these factors *in vivo*, we engineered chimeric repressors containing the ets and IRF DNA-binding domains connected by a flexible POU domain linker. When stably expressed, the wild-type fused dimer strongly represses the expression of a rearranged immunoglobulin lambda gene, thereby demonstrating the functional importance of Ets/IRF complexes in B cell gene expression. The degree of repression exerted by the fused dimers correlates with their DNA-binding specificity and stability. These results establish the utility of DNA-binding domain fusions as gene-specific repressors.

Developmental regulation of V(D)J recombination at the T cell receptor alpha/delta locus.

Michael S. Krangel, Cristina Hernandez-Munain, Michelle McMurry, Xiao-Ping Zhong, Juan Carabana, Joseph Roberts, Pilar Lauzurica, Barry Sleckman, Fred Alt. Duke University Medical Center, Durham, NC, USA and Harvard Medical School, Boston, MA, USA.

The process of V(D)J recombination assembles T-cell receptor genes from variable (V), diversity (D) and joining (J) gene segments during thymic differentiation of T cells, leading to the production of alpha/beta and gamma/delta T lymphocytes. We are interested in understanding the molecular basis for the developmentally regulated rearrangement and expression of TCR genes. We have focussed our studies on the TCR alpha/delta locus, because it contains two different sets of TCR gene segments that are differentially regulated during thymocyte development. Rearrangement and expression of the centrally located TCR delta gene segments initiates at the double negative (DN) stage of thymocyte development, whereas rearrangement and expression of the distally located TCR alpha gene segments initiates at the subsequent double positive (DP) stage. TCR alpha rearrangement deletes TCR delta gene segments. An unrelated and distinctly regulated antiapoptosis gene, Dad1, is located immediately downstream of TCR alpha. The tight linkage of TCR delta, TCR alpha and Dad1 raises questions concerning not only how the individual genes are activated, but as well, how their distinct patterns of regulation are maintained. Our goal is to understand how cis-acting elements effect the developmental program at this locus.

The two best characterized cis-regulatory elements within the locus are the TCR delta and TCR alpha enhancers. The former is located within the Jdelta-Cdelta intron, and the latter, downstream of Calpha. We have evaluated the roles of these enhancers as developmental regulators of V(D)J recombination using a human TCR delta gene minilocus, composed of V, D, J and C gene segments, as a V(D)J recombination reporter substrate in transgenic mice. Our analysis of V(D)J recombination in versions of the minilocus that carry wild-type or mutant versions of the TCR delta enhancer, wild-type or mutant versions of the TCR alpha enhancer, or no enhancer, indicates that the two enhancers provide region-specific developmental control to the process of V(D)J recombination at the endogenous TCR alpha/delta locus. Our analysis suggests that these enhancers regulate V(D)J recombination by modulating the accessibility of recombination signal sequences to the V(D)J recombinase.

We would like to understand the mechanisms by which the TCR delta and TCR alpha enhancers are differentially activated (and perhaps inactivated) during T cell development. To do so, we have assessed the

effects of mutations in individual transcription factor binding sites on the ability of these enhancers to activate V(D)J recombination and transcription within the context of the transgenic minilocus. To better understand how multiprotein complexes are assembled on these enhancers, we have used genomic footprinting to assess the consequences of enhancer mutations on enhancer occupancy in vivo. Armed with this information, we are currently using genomic footprinting to analyze the occupancy of these enhancers within the endogenous locus to evaluate the extent to which changes in the activities of the two enhancers across the DN to DP transition can be explained by changes in enhancer occupancy.

As our studies impicate the TCR delta and TCR alpha enhancers as region-specific developmental regulators of V(D)J recombination within the TCR alpha/delta locus, they raise the question of how the regulatory effects of these enhancers are limited to discrete regions of the locus. This led us to consider the possibility that there may be boundary or enhancer-blocking elements within the locus that delimit the regulatory domains influenced by the two enhancers. We have used stable transfection approaches to identify enhancer-blocking activities between TCR delta and TCR alpha gene segments, and between TCR alpha and Dad1. A gene targeting approach is currently being used to evaluate the in vivo relevance of the putative blocking element between TCR alpha and TCR delta gene segments.

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The dynamics of ß-globin gene switching

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The most important level of the regulation of the ß-globin genes is by activation of all of the genes by the Locus Control Region (LCR) and repression of the early genes by as yet unknown factors acting on sequences flanking the genes. Superimposed on this is a mechanism in which the early genes (ε and γ) suppress the late genes (δ and ß) by competition for the interaction with the LCR. Although this extra level of gene regulation is quantitatively of less importance than the direct repression mechanism, it has important implications and has provided an excellent assay system to probe the regulation of transcription at the single cell level.

These studies indicate that the LCR interacts with individual globin genes and that LCR/gene interactions are dynamic with complexes forming and dissociating continually. The levels of expression of each of the genes appear to depend on: (1) the frequency of interaction which is itself dependent on the distance of the gene to the LCR, (2) the affinity of the LCR for the gene and (3) the stability of the LCR/gene complex. The latter two are dependent on the balance of transcription factors. The role of individual transcription factors and the effects of chromatin structure on this process will be discussed.

INTEGRATION OF MULTIPLE ELEMENTS IN THE CONTROL OF CD4 GENE EXPRESSION DURING T CELL DEVELOPMENT. Gerald Siu, Department of Microbiology, Columbia University College of Physicians and Surgeons, 701 West 168th Street, New York, NY 10032.

Our lab studies the control of CD4 gene expression as a model system for characterizing the molecular signaling pathways that mediate T cell development and selection. We and other have previously identified four elements that are critical in controlling CD4 expression: two enhancers that function only in mature T cells, a promoter, and a transcriptional silencer. We have utilized mouse genetic approaches to identify new transcriptional control elements in the CD4 locus. Using a transgenic approach, we have identified a novel thymocyte enhancer over 70kb downstream of the CD4 promoter that is important for mediating CD4 transcription in $CD4^+CD8^+$ thymocytes; in addition, we have determined that one of the previously-identified enhancers is not responsible for controlling CD4 transcription *in vivo*. Thus, appropriate CD4 expression requires the coordination of four control elements, each of which functions at different stages of T cell development. In addition, we have utilized a molecular approach to characterize the factors that bind to the functional sites in each of these transcriptional control elements. We have determined that c-Myb and the Notch pathway intermediate HES-1 bind to two of the three sites required for silencer function. We have also cloned a gene that encodes a novel protein which we refer to as Silencer Associated Factor, or SAF, that binds to the third functional site of the CD4 silencer. We are currently conducting both genetic and molecular studies to determine the role of all of these factors in CD4 gene expression and in overall T cell development.

The role of Ikaros and Aiolos in lymphocyte differentiation and homeostasis.

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Ikaros and Aiolos encode a family of transcription factors which are essential for lymphocyte differentiation and homeostasis. Lack of Ikaros proteins from the hemopoietic system causes an early and complete arrest in the production of fetal and postnatal B lymphocytes and natural killer cells. T cell differentiation is also blocked at the fetal stage; however, after birth a small number of T cell precursors is detected in the thymus which display aberrant differentiation along the CD4 lineage. Ikaros also plays a critical role in the regulation of T-cell proliferation.

Lack of or a substantial reduction in the levels of Ikaros in immature thymocytes and mature T cells causes augmentation of their proliferative response and rapid transformation to a neoplastic state. The increase in the proliferative response displayed by Ikaros mutant T cells correlates with the reduction in Ikaros activity indicating that it sets thresholds for T cell activation.
In contrast to Ikaros, Aiolos is not essential during the specification of B and T cell precursors.

However, in a similar fashion to Ikaros in T cells, Aiolos regulates later stages of B cell differentiation and proliferation. Aiolos sets thresholds of receptor signaling which control B cell activation, maturation to germinal centers and isotype switching.

The physiological effects of lack of Aiolos from the B cell lineage are a breakdown in B cell tolerance and the development of B cell lymphomas. Taken together, Ikaros and Aiolos proteins are essential in lymphocyte differentiation and act as potent tumor suppressors in the T and B lineage respectively. These factors which are thought to control the potential for gene expression in cells of the lymphoid lineage participate in a higher order protein complex.

The composition of this complex and the dynamic changes it undergoes during the cell cycle suggests a mechanism for their function.

Session 2: Factors important for early hematopoietic development

Chair: Michael S. Neuberger

Fritz Melchers, Basel Institute for Immunology, Basel, Switzerland

B cell development - decisions between life and death

B lineage cells develop in fetal liver during embryogenesis and in bone marrow during adult life from pluripotent stem cells. This process includes a commitment to become B-lineage cells, the activation of the Ig gene rearrangement machinery, the stepwise rearrangements of IgH and L chain gene segments, the expression of Ig H chains first in pre-B cell receptors together with surrogate L chains, and later with L chains in B cell receptors. Cells expressing these receptor are subject to selections which involve the pre-B cells and B cell receptors, selections which can be positive or negative. The presentation will summarize the cellular stages, the kinetics of proliferation, maturation and apoptosis in interactions with the environment during this development of B lineage cells to mature B cells and in B cell responses.

HMG BOX TRANSCRIPTION FACTORS IN DEVELOPMENT AND CANCER

Hans Clevers. Department of Immunology University Hospital Utrecht, The Netherlands

The identification of the mammalian sex-determining gene Sry has led to the discovery of a large family of related ("HMG box") transcription factors that control development in yeast, C. elegans, Drosophila and vertebrates. In lymphocyte differentiation, several HMG box factors play a decisive role, i.e. the Sry homolog Sox-4 and two members of the related Tcf subfamily Tcf-1 and Lef-1. By gene knockout and the construction of radiation chimearas, we have shown that Sox-4 is essential for the expansion of the earliest B cell progenitors.

Similarly, gene knockout revealed that Tcf-is essential for expansion of the earliest double-negative thymocytes. While Lef-1 knockout mice reportedly have no lymphoid phenotype, double knockout Tcf-1/Lef-1 mice have an even more severe thymocyte phenotype than the single Tcf-1 knockout mice, implying some redundancy between Tcf-1 and Lef-1. Tcf/Lef proteins have recently been found to constitute downstream components of the Wingless/Wnt signal transduction pathway. In flies, this pathway controls a.o. segment polarity; in Xenopus it controls the formation of e body axis. Deregulation of the pathway appears to be the primary event in intestinal and various other tumors. Based on these observations, it is implied that Wnt signaling plays a decisive role in early lymphoid development and possibly in leukemogenes.

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HELIX-LOOP-HELIX PROTEINS IN B CELL DEVELOPMENT AND B CELL LEUKEMIAS.

Kees Murre

The activity of HLH proteins, including the E2A proteins, is required for proper B lymphocyte development. The E2A gene products bind to sequence motifs, termed E box sites, that are present in the regulatory regions of many B lineage specific genes. The activity of these proteins in other organisms has been shown to be regulated by antagonists which have the ability to dimerize but lack the ability to bind DNA. In vertebrates, four antagonists are present, designated as Id1, Id2, Id3 and Id4. Both Id2 and Id3 are expressed in the B cell lineage at all stages. To examine their role in B lymphocyte development we have generated null mutant mice for Id2 and Id3. Id3 null mutant do not show signicant B cell abnormalities.

However, mice lacking Id2 show decreased numbers of pro-B and pre-B lymphocytes. Interestingly, pro-B cells in Id-2 null mutant mice show increased numbers cells expressing cytoplasmic Ig Kappa. The data suggest that Id-2 functions, at least in part, to control the activity of E2A. E2A activity is also upregulated upon activation of primary B splenocytes. We have examined the role of E2A during B cell activation by overexpression of Id-2 using retroviral-mediated gene transfer. The results indicate that the E2A gene products are required for Ig isotype switching. In summary, the data indicate that the E2A proteins are essential during various stages of B lymphocyte differentiation and during early B lymphocyte development are regulated by the antagonist, Id-2.

Lineage commitment by Pax5 (BSAP) in the lymphohematopoietic system

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BSAP, the product of the *Pax5* gene, is a DNA-binding protein expressed throughout B-cell development with the exception of terminally differentiated plasma cells. We have previously demonstrated, by gene targeting in the mouse, that BSAP is essential for B-cell development to proceed beyond the early pro-B cell stage in adult bone marrow (1,2). Interestingly, BSAP is required early at the onset of B-cell development in the fetal liver, as B-cell progenitors could not be detected during embryogenesis (2).

We now present data to demonstrate that these early pro-B cells in the *Pax5* (-/-) mice are in fact non-committed lymphoid progenitors. This is despite the observation that they transcribe a large number of genes commonly associated with lineage committed B-lymphocytes (3). In vitro cultured *Pax5* (-/-) pro-B cells can, upon injection into *Rag2* (-/-) hosts, reconstitute T-lymphopoiesis in the thymus, producing $>10^7$ T-cell precursors which proceed to the single positive CD4 or CD8 stage, express a functional TCR and migrate to the spleen. Moreover, *Pax5* (-/-) pro-B cells can, upon IL-7 withdrawal, in vitro differentiate into a variety of morphologically distinct cell types, which display phenotypic and functional characteristics of other lymphoid derived lineages. For example, upon IL-2 addition, *Pax5* (-/-) lymphoid progenitors can be induced to lyse YAC target cells, which is a characteristic of natural killer cells. Conversely upon GM-CSF stimulation, the same cells become functional dendritic cells as determined by cell surface phenotype and antigen presentation in a mixed lymphocyte reaction. B-cells in vitro have long been known to be capable of differentiation into myeloid lineages. Indeed *Pax5* (-/-) pro-B cells can, after IL-7 withdrawal, differentiate into the monocyte lineage, resulting in terminally differentiated phagocytic macrophages and multi-nucleated TRAP-positive osteoclasts.

One model for lineage determination of hematopoietic cells is the concept of "lineage priming" (4). According to this model, non-committed cells express low levels of a number of different lineage determinants and progression along a particular lineage occurs only after a combination of transcriptional regulators are co-expressed. In this regard it is significant to note that we have recently demonstrated that the *Pax5* gene is transcribed monoallelically at the onset and during late stages of B-cell development (5). One possible function of this allele-specific regulation could be that stochastic *Pax5* expression may facilitate the bifurcation of, for example, B- and T-lymphocytes early in their cellular development.

Together, these data provide strong evidence that the Pax5-deficient pro-B cell represent an early hematopoietic progenitor which is blocked in B-cell development, but retains the capacity to differentiate into other lymphoid and myeloid cell types. Thus, the definitive lineage restriction of B-lymphocytes requires Pax5 and occurs much later in development than has been previously anticipated. These Pax5-deficient pro-B cells will, in the future, provide a valuable tool as they enable the inducible specification of cell fate and are amenable to genetic manipulation in vitro.

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Cooperative DNA binding by Pax and Ets proteins requires highly conserved sequences within the novel β-turn motif of the paired domain.

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The binding of Pax-5 (BSAP) recruits proteins of the Ets proto-oncogene family to bind a composite site that is essential for efficient transcription of the early B cell-specific *mb-1* promoter in vivo. Cooperative DNA binding by these proteins requires only their respective DNA-binding domains (paired and ETS domains). Here, we show that binding of the promoter requires both the amino- and carboxyl-terminal subdomains of the Pax-5 paired domain. Using an assay that replaces the function of the carboxyl-terminal subdomain by tethering Pax-5 to a heterologous DNA-binding domain, we show that 73 amino acids of the intact amino-terminal subdomain are necessary and sufficient for cooperative DNA binding with Ets-1. Furthermore, amino acids within the highly conserved β-turn motif of this domain are essential for DNA binding and efficient recruitment of Ets-1. Recruitment of Ets proteins is a shared property of Pax proteins, as demonstrated by cooperative DNA binding of Ets-1 with Pax-2, Pax-3, or Pax-6. Together, these data suggest Pax:Ets interactions are important for regulating transcription in diverse tissues during cellular differentiation.

Session 3: Factors important for late B cell development and function

Chair: Meinrad Busslinger

Fine tuning of IgH gene expression by the transcription factors NF-kB and PU.1. Sven Pettersson, Center For Genomcis Research, Karolinska Institute, Stockholm, Sweden.

Enhancers in the 3´end of the IgH locus have been suggested to regulate IgH-gene expression and perhaps class switch recombination in B lineage cells. Previous experiments have indicated that this region may act as a Locus Control Region (LCR) in cell lines. To assess adress this issue further, we generated transgenic mice harbouring a construct in which known 3´cis-elements were linked to a Vh promoter-driven human β-globin gene reporter. Subsequent analysis of several different founder strains suggest that i) expression of the transgene is confined to lymphoid cells ii) Transgene expression appears to be restricted to B lineage cells iii) Transgene expression appears to be copy dependent although two founder lines exhibits high copy numbers and yet low levels of expression. The activation of one of these elements, the IgH 3´enhancer (HS1,2) is guided by multiple signalling pathways that converge to induce key factors required for enhancer activity. Moreover, it has been postulated that the 3´enhancer in part may be regulated by the transcription factor BSAP encoded by the Pax-5 gene. Downregulation of Pax-5 expression in cell lines appears to be concomittant with increased IgH-gene expression and the recruitment of binding of a protein complex, NF-αP, to the 3´enhancer. By screening a λGT11 expression library, we identified the NFαP-binding protein to be the ets protein PU.1. Here we demonstrate that PU.1 in combination with NF-κB, can potentiat the 3´enhancer dependent transcription of a heterologous reporter gene in both plasmacytomas and in reconstituted COS cells. Moreover, NF-κB and PU.1 can form complex in solution. In addition, the insertion of a Pax-5 binding site 4 helix turns upstream of a PU.1/NF-κB promoter construct, indicate that BSAP can suppress PU.1/NF-κB dependent reporter gene expression in a position dependent manner. Our data will be discussed in the context of mechanisms guiding Ig- gene expression in developing B lineage cells.

SOMATIC HYPERMUTATION OF IMMUNOGLOBULIN GENES

Michael S. Neuberger. Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH.

Following antigen encounter, the productively rearranged immunoglobulin V genes in germinal centre B cells are subjected to localised hypermutation.

Nucleotide substitutions are introduced into a domain that extends from shortly downstream of the V gene promoter through into the enhancer/matrix attachment regions in the major J-C intron. The mutations are introduced over several generations and in a stepwise manner.

The detailed mechanism of the hypermutation is not known although information has been obtained by analysis of mutation patterns of modified immunoglobulin transgenes, by analysis of the effects of gene disruptions affecting DNA repair and by use of transformed cell lines. Thus, evidence from transgenics points to mutation recruitment being a transcription-linked process with the location of the mutation domain being defined by the position of the V gene promoter. The V gene itself is not needed for mutation recruitment .

The precise targeting of nucleotide substitutions within the mutation is not random and is biased by local DNA sequence. Indeed, germline V gene sequences appear to have evolved to facilitate strategic targeting of somatic mutations so as to aid affinity maturation.

Clues to possible mechanisms of mutation fixation obtained from analysis of mutation pattterns in cell-lines and in repair-deficient mice will be discussed.

THE B CELL-SPECIFIC COACTIVATOR OBF-1 (aka OCA-B, BOB1) IS REQUIRED FOR IMMUNE RESPONSE AND B CELL DEVELOPMENT

P Matthias

The highly conserved octamer motif -ATGCAAAT- has been associated with the B cell-specific transcription of several genes, in particular the immunoglobulin (Ig) genes. B lymphocytes contain two transcription factors of the POU homeodomain family that bind specifically to this motif: Oct-1, a ubiquitous protein, and Oct-2, a mostly B cell-restricted protein. Oct-1 and Oct-2 both can, through their POU domain, recruit the transcriptional coactivator OBF-1 (aka OCA-B, Bob-1) to a subset of octamer sites.

This recruitment of OBF-1 is thought to be essential for mediating high level transcription through the octamer site in B cells. Expression of OBF-1 is highly cell-restricted and is observed constitutively in B cells of all differentiation stages, as well as transiently in activated T cells. In mice that have been made OBF-1 deficient by gene targeting, early B cell development and Ig gene transcription are surprisingly largely normal (Schubart et al., Nature 383, 538-542). Strikingly, however, these mice show a dramatically impaired immune response to several antigens, accompanied by the complete absence of germinal center formation.

The impaired immune response reflects an intrinsic B cell defect as T cell function appears to be normal. We have crossed OBF-1 deficient mice with Xid mice that are deficient in the cytoplasmic tyrosine kinase BTK; from these animals data will be presented that identify a novel and crucial role for OBF-1 in B cell development. In addition, experiments aimed at defining the specific relative roles of OBF-1 and Oct-2 will be presented.

Ananda L. Roy

Multifunctional transcription factor TFII-I: a target of Bruton's tyrosine kinase

Bruton's tyrosine kinase (Btk) is required for normal B cell development as defects in Btk lead to X-linked immunodeficiency (xid) in mice and X-linked agammaglobulinemia (XLA) in humans. Although Btk has been shown to interact with several proteins, no functional targets have yet been clearly identified. Btk has been shown to constitutively interact with one such protein, BAP-135, *in vivo* and tyrosine phosphorylate it *in vitro*. BAP-135 is identical to the previously characterized initiator element binding transcription initiation factor TFII-I. Here we demonstrate a functional interaction between TFII-I and Btk as ectopic expression of wild type Btk enhances transcriptional activation of ectopically expressed TFII-I *in vivo*. Mutation of Btk in either the PH domain (R28C, as in the murine xid mutation) or the kinase domain (K430E) compromises its ability to enhance TFII-I transcriptional activity. Coimmunoprecipitation studies revealed that TFII-I associates *in vivo* with wild type and kinase mutant Btk but not xid mutant Btk. Finally, we show that the subcellular localization of TFII-I in wild type and xid derived primary B cells is markedly different. Taken together, these data lead us to hypothesize that mutations impairing the physical and/or functional association between TFII-I and Btk lead to diminished TFII-I dependent transcription and defective B cell development and/or function.

GENETIC RECONSTITUTION IN VITRO OF PRIMARY B CELL TERMINAL DIFFERENTIATION.

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Cell cycle arrest and apoptosis are tightly coupled to cellular differentiation during terminal differentiation of mature B cells to antibody-secreting plasma cells in the humoral immune response. The mechanisms that underlie this intricately orchestrated process, however, are not well understood and the characteristics of normal plasma cells remain largely to be determined. To address these questions, we have reconstituted B cell terminal differentiation in vitro in which resting primary mouse B lymphocytes undergo orderly activation and differentiation, cell cycle arrest and apoptosis with high efficiency in response to CD40 ligand and cytokine stimulation.

Using B lymphocytes isolated from various genetically altered mice, we have investigated the roles of cyclin-dependent kinase (CDK) inhibitors in the activation and arrest of the cell cycle during B cell terminal differentiation in vitro. p18INK4C, but not other CDK inhibitors, is found to be essential for both negative control of CD40-signaled cell cycle activation in B lymphocytes and the subsequent exit from the cell cycle during final maturation to plasma cells. These results corroborate our previous finding that the expression of p18INK4C is specifically activated and sufficient to reconstitute cell cycle arrest during terminal differentiation of human lymphoblastoid cells induced by IL-6. The physiologic role of p18 in antigen-specific T-dependent humoral immune response has been investigated in the newly created p18INK4C-deficient mice and will be discussed.

Plamacytomas, the mouse counterparts of multiple myelomas in humans, are the major plasma cell cancers characterized by massive accumulation of plasmacytoid cells, presumably due to uncontrolled cell proliferation. Investigation of the time course of plasmacytoma development in IL-6 transgenic mice, however, reveals that plasmacytoma cells are arrested in the G1 phase of the cell cycle and their accumulation is preceded by enhanced expansion of activated B cells. Thus, plamascytomagenesis has its molecular basis in both dysregulation of B cell activation and inappropriate survival of the non-replicating plasmacytoma cells Dysregulation of B cell activation resulting from overexpression of IL-6 has been recapitulated in B cell terminal differentiation in vitro, indicating that sustained cell cycling driven by IL-6 is B cell autonomous.

Accumulation of IL-6 transgenic plasmacytoma cells, however, does not occur in vitro without the help of marrow stromal cells. The biochemical mechanisms that mediate CD40 and IL-6 signals for cell cycle control and apoptosis during primary B cell terminal differentiation in vitro will also be discussed.

Session 4: Signal transduction and transcription in lymphoid cells

Chair: Anjana Rao

JAKs and STATs in Cytokine Signal Transduction Pathways

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A variety of functions are regulated through the interaction of cytokines with members of the cytokine receptor superfamily. This family of receptors shares in common the requirement to interact and activate members of the Janus family of cytoplasmic protein tyrosine kinases (Jaks). The role of Jak3 and Jak2 have been established through the derivation of mice in which the genes are disrupted. Mice lacking Jak3 suffer from severe combined immunodeficiency (SCIDs) and the functions of all the receptors that utilize the common (chain component are lost. Mice lacking Jak2 die during embryogenesis due to lack of definitive erythropoiesis and the responses to a variety of cytokines that had previously been shown to activate Jak2 are lost. Together the results demonstrate the essential, non-redundant role that Jaks play in the function of specific cytokine receptors. A common feature of cytokine signaling is the ability to recruit and activate members of the signal transducers and activators of transcription (Stat) transcription factors. There are currently seven mammalian Stats whose function has been examined through gene deletions. Mice lacking Stat6 have specific defects in their immune functions that are primarily regulated by IL-4 and/or IL-13 while mice lacking Stat4 have specific defects in their immune responses that are normally associated with functions regulated by IL-12. The two related Stat5 genes (a and b) are activated by multiple cytokines and consequently might be suggested to play more general functions in cellular regulation. Mice lacking Stat5a have a deficiency of homozygous females to lactate. Mice lacking Stat5b have phenotypes consistent with deficiencies in growth hormone signaling. However, mice lacking both genes have a more severe phenotype consist with the loss of all prolactin and growth hormone responses. Surprisingly hematopoiesis is normal in these mice although a number of cytokines that affect lymphoid and myeloid functions activate the Stat5 proteins. Lastly, and most strikingly, mature T cells from mice lacking both Stat5 genes fail to respond to activation stimuli although the differentiation of the cells occurs normally. Together the knockouts support the view that individual Stat proteins have evolved to fulfill specific functions for specific cytokines.

T cell development and signalling plasticity in the absence of Csk

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Engagement of preTCR and αβTCR leads to multiple changes in cellular metabolism which underlie the differentiation, proliferative expansion and selection of developing T cells. It is thought that both the strength and the pattern of TCR-induced signalling are controlled by src-family protein kinases (src-PTKs) Lck and Fyn. To study the role of src-PTKs in T cell development and activation we attempted to generate an experimental sytem in which the activation of src-PTKs is disengaged from preTCR or αβTCR. In T lineage cells as well as in cells of other types the activity of src-PTKs is negatively regulated by carboxy-terminal Src kinase (Csk). Accordingly, deficiency in Csk leads to ligand-independent activation of src-PTKs. Conditional csk gene inactivation has been employed to generate csk-deficient T lineage cells *in vivo*. Using mice in which the inactivation of *csk* occurs at stages preceeding the TCR expression, we have addressed the question, to which extent the differentiation of αβT lineage cells is controlled by src-PTKs. We found that inactivation of *csk* in immature thymocytes abrogates the requirement for preTCR, αβTCR and MHC class II for the development of $CD4^+8^+$ double-posititve (DP) and CD4 single positive (SP) thymocytes as well as peripheral CD4 αβT lineage cells. Unless Csk-deficiency affects the activity of proteins other than src-PTKs, these data show that src- PTKs govern the entire process of αβT cell development.

Inactivation of *csk* in $CD4^+8^+$ double positive and CD4 single positive thymocytes allows the MHC class II independent development of CD4 T cells or the development of CD4 T cells with TCR specificities normally selected by MHC class I. These data demonstrate that the negative control that Csk exerts overs Src-PTKs is a prerequisite for normal selection of αβT cells.

Assuming that activation of src-PTKs is proportional to the strength of the αβTCR-mediated signal, we have used Csk-deficient T lineage cells to address the molecular mechanism of T cell responses to signals of increased strength. We found that inactivation of *csk* in thymocytes or peripheral T cell leads to down-regulation of TCR-CD3 and CD4/CD8 co-receptors. Moreover, the Csk deficiency in peripheral T cells is asssociated with reduced expression levels of Fyn and Lck and dramatic reduction of the basal and anti-CD3ε induced protein tyrosine phosphorylation. Surprisingly, neither the

anti-CD3ε induced Ca^{++} mobilization nor the T cell proliferation are negatively affected by the observed changes in signalling molecules. These data suggest that activation of src-PTKs might induce a negative feedback mechanism allowing adaptation of T cells to sustained hyperactivation of src-PTKs. We speculate that the adaptive capacities of T cells may define their fate during development and immune responses.

THE IKK SIGNALSOME: A MULTI-PROTEIN SIGNALING COMPLEX REGULATING NF-κB ACTIVATION

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NF-κB is a key regulator of immune and inflammatory gene expression such as TNFα, interleukins, T-cell receptor β chain, GM-CSF, beta interferon and major histocompatibility complex class I. Studies of NF-kB in lymphocytes reveal potent effects in preventing apoptosis, activation of the immune response, and T and B cell differentiation. Targeting the NF-κB pathway therefore offers the potential for the development of novel anti-inflammatory and immunosuppressive therapeutics. To this end, we purified a large multi-protein complex, the IκB kinase (IKK) signalsome, that contains a regulated IκB kinase activity that phosphorylates IκBα and IκBβ. Two components of the IKK signalsome, IKK-1 and IKK-2, were identified that are closely related protein serine kinases containing leucine-zipper and helix-loop-helix protein interaction motifs. We are presently engaged in sequencing and molecularly cloning additional components of the IKK signalsome. Identification and analysis of all the IKK signalsome components will provide invaluable insights into the mechanism by which extracellular stimuli regulate the function of this elaborate protein complex, and how it in turn mediates the activation of NF-κB.

Regulation of NF-kB by IkBepsilon

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In most unstimulated lymphoid cells, transcription factors of the NF-kB family are sequestered in the cytoplasm by inhibitor proteins called IkBs. Several different IkBs have been cloned, and they share a number of properties. Each 1) contains multiple ankyrin repeats, 2) retains dimers in the cytoplasm, 3) inhibits DNA binding by dimers in vitro, and 4) undergoes signal-induced phosphorylation at two serine residues in the N-terminal region of the protein (Ser 18 and Ser 22 in IkBepsilon). Each is present in all cells we have examined to date (including various lymphoid cell lines and normal peripheral blood T cells), although the relative amounts of RelA and c-Rel bound by each varies widely from cell line to cell line.

The IkBs also differ from one another in several ways. In contrast to IkBalpha, which turns over very rapidly, we have shown that IkBepsilon has a very long half-life in cells. IkBepsilon also differs structurally from IkBalpha in having a much longer N-terminal region that is rich in serine residues. To investigate whether residues other than Ser 18 and Ser 22 play a role in signal-induced phosphorylation and degradation, we have created stable transfectants with a battery of IkBepsilon mutants. The resulting cells were stimulated with LPS and monitored for degradation of the transfected IkBepsilon. We found that degradation was governed solely by Ser 18 and Ser 22: mutation of either of these residues abolished degradation, while all other mutations were without effect. Thus, signal-induced degradation of IkBepsilon in response to LPS appears to be governed by the same two conserved serines residues as found in IkBalpha.

While the serine requirement is comparable, IkBalpha and IkBepsilon are degraded at quite different rates following stimulation. We have observed significantly slower degradation of IkBepsilon in response to LPS, and much much slower degradation in response to TNF. In THP-1 cells in the presence of TNF, for example, IkBalpha was completely degraded within 10 min, whereas the level of IkBepsilon was little changed after an hour. This suggests that the two IkBs differ in their affinity for the signal-induced kinases, and the basis for this difference is the subject of our current studies.

Dual role of NF-κB activation for apoptosis protection and cell cycle progression

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NF-κB and IκB proteins control a number of immune and stress responses and play a role in proliferative processes in the immune system. The nuclear IκB homologue and oncoprotein Bcl-3 interacts with the NF-κB subunits p50 and p52. We have identified a set of nuclear co-factors for Bcl-3, which link Bcl-3 to other transcription factor families involved in transformation. Some of these factors stimulate the transcriptional activation potential of p50-Bcl-3 complexes. The amount of cellular Bcl-3-p50 complexes is augmented by diverse cytokines and other stimuli known to activate NF-κB. Like NF-κB-p65, Bcl-3 has anti-apoptotic properties and its overexpression leads to strongly reduced apoptosis in TNF-a treated or serum starved cells. The anti-apoptotic effects of NF-κB/Rel are further discussed in the context of Hodgkin's lymphoma.

Persistent activation of NF-κB p50-RelA is a characteristic common property of Hodgkin/Reed-Sternberg cells, which discriminates these tumor cells from most other cell types studied so far. Constitutive nuclear NF-κB has an anti-apoptotic effect in these cells. Furthermore, NF-κB drives cell growth in these tumor cells by stimulating cell-cycle progression. This dual function of NF-κB in vitro is consistent with its role for tumor growth in xenotransplanted mice. We have analyzed how NF-κB might generally control progression during the G1 phase of the cell cycle.

Session 5: Factors regulating T lymphocyte function and differentiation

Chair: Hans Clevers

ROLE OF p38 MAPK PATHWAY ON T CELL DEVELOPMENT IN THE THYMUS

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Upon antigenic stimulation, precursor CD4+ helper T cells differentiate into two subsets of effector cells, Th1 and Th2. These two subpopulations are defined by the pattern of cytokine expression that distinguishes these differentiated cells from their precursors. We have used reporter transgenic mice here to show that, during differentiation of precursor T cells into effector Th1 or Th2 cells, high levels of preformed AP-1 complexes are accumulated. However, upon stimulation, the preformed AP-1 complexes in effector Th2 cells, but not in Th1 cells are able to induce high levels of AP-1 transcriptional activity. Furthermore, in contrast to precursor T cells, the induction of AP-1 transcriptional activity is independent of calcium and costimulatory signals in effector Th2 cells. This AP-1 transcriptional activity appears to be mediated by JunB complexes which accumulate differentially in effector Th2 cells, but not in precursor $CD4^+$ T cells or effector Th1 cells. Unlike precursor cells, the activation of AP-1 does not appear to be mediated by c-Jun NH_2-terminal kinase (JNK) in effector Th2 cells. Like AP-1, only the NFAT complexes present in eTh2 cells are able to mediate transcription, and no NFAT transcriptional activity was induced in eTh1 cells, although NFAT DNA binding is similarly induced in both eTh1 and eTh2 cells upon antigen stimulation. These results indicate that during differentiation of T cells, and probably other cell-types, the signal requirements for the AP-1 and NFAT transcription machinery are reprogrammed to enable the differentiated cells to perform their specialized functions.

p38 MAP kinase can be activated by multiple stimuli, such as proinflammatory cytokines and environmental stress. We have shown that pyridinyl imidazole compounds (specific inhibitors of p38 MAP kinase) block the production of interferon-γ (IFNγ) by Th1 cells without affecting IL-4 production by Th2 cells. In transgenic mice, inhibition of the p38 MAP kinase pathway by the expression of dominant-negative p38 MAP kinase results in selective impairment of Th1 responses. In contrast, activation of the p38 MAP kinase pathway by the expression of constitutively activated MKK6 in transgenic mice caused increased production of IFNγ during the differentiation and activation of Th1 cells. Together, these data demonstrate that the p38 MAP kinase is relevant for Th1, not Th2 cells, and that inhibition of p38 MAP kinase represents a possible site of therapeutic intervention in diseases where a predominant Th1 immune response leads to a pathological outcome.

Factors that Control Th2 Differentiation

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We have recently shown that the provision of three distinct proteins, the c-maf protooncogene, the transcription factor Nuclear Factor of Activated T cells (NFAT), and a novel nuclear antigen NIP45, confers on a non-producer cell the ability to produce IL-4. The phenotypes of c-maf and NFAT genetic mutant mice recently produced in our laboratory provide compelling evidence for a critical role of the c-maf and NFAT proteins in controlling the Th2 differentiative program. We have overexpressed c-maf in vivo using both transgenesis and retroviral gene transduction. C-maf transgenic mice have an increased Th2 immune response in vivo and in vitro which can be ablated by backcrossing onto an IL-4 deficient background (1). C-maf retroviral gene transduction of the Jurkat Th1 cell line or normal, primary Th1 cells confers on them the ability to produce IL-4. Finally, we have targeted the c-maf locus and find that c-maf knockout mice have severely impaired IL-4 production. These data provide incontrovertible evidence that c-maf regulates the production of IL-4 in vivo.

NFAT was first identified as a transcriptional regulatory complex important for the expression of the T cell cytokine. There are currently four members of the NFAT family: NFATc, NFATp, NFAT4 and NFAT3 that are highly homologous within a region distantly related to the Rel domain. Mice lacking NFATc in the lymphoid system (as evaluated by RAG-2 blastocyst complementation) have mildly impaired proliferation and a selective decrease in IL-4 production (2,3) consistent with a function of NFATc as a positive regulator of the immune system and as a direct transcriptional activator of the IL-4 gene. Conversely, some hints that NFATp and NFAT4 might negatively control proliferative responses, Th2 cell formation and lymphocyte activation were obtained from the characterization of NFATp and NFAT4 deficient animals (4-6). We and others (4,5) demonstrated that mice lacking NFATp display modest splenomegaly, T and B cell hyperproliferation and cytokine dysregulation during the course of an immune response with a moderate increase in Th2-type cytokines. Mice lacking NFAT4 have normal peripheral T cell proliferation and cytokine production although an increased percentage of T and B cells display a phenotype characteristic of memory/activated cells (6). Mice lacking both NFATp and NFAT4 (7) develop a profound lymphoproliferative disorder likely due to a lowered threshold for TCR signaling coupled with increased resistance to apoptosis secondary to defective FasL expression. NFAT mutant mice also have allergic blepharitis, interstitial pneumonitis, and a 10^3 to 10^4 fold increase in serum IgG1 and IgE levels, secondary to a dramatic and selective increase in Th2 cytokines. This phenotype may be ascribed to unopposed occupancy of the IL-4 promoter by NFATc. Our data demonstrate that lymphoid homeostasis and Th2 activation require a critical balance among NFAT family members.

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MAPKs AND SUBCELLULAR LOCALIZATION OF NFAT

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The transmission of signals derived from T cell stimulation leads to the activation of the Ca^{2+}-calcineurin pathway which in turn trigger the translocation of NFAT. NFAT is family of transcription factors composed by at least four structurally related members NFATp/NFAT1, NFATc/NFAT2, NFAT3 and NFAT4 that are expressed in cells of the immune system as well as in cells and tissues outside the immune system. Upon calcium signaling, the Ca^{2+}-activated calcineurin dephosphorylates NFAT proteins leading to the translocation of NFATs from the cytoplasm to the nucleus. NFATs remain in the nucleus while Ca^{2+} is elevated, and are rapidly phosphorylated and exported to the cytoplasm upon termination of calcium signaling or by calcineurin inhibition with Cyclosporin A or FK-506. Although the role of NFAT proteins in the regulation of the inducible expression of a number of genes implicated in the regulation and function of the immune system has been previously established, the molecular mechanisms involved in the regulation of NFAT activation and subcellular localization are not completely understood.

Among the signaling pathways identified in mammalian cells those involving MAP kinases (MAPKs) have been shown to be major mediators in the response to extracellular stimuli by coupling signal transduction and activation of transcription factors and thus triggering specific gene expression programs. Three major members of MAPKs have been described in mammalian cells, which includes the extracellular signal regulated kinases (ERKs), the stress-activated protein kinases/c-jun terminal kinases (SAPKs/JNKs), and the p38/HOG kinases. Although the implication of signaling cascades mediated by these MAPKs in the activation of transcription factors have been extensively analyzed, little is known on the signals that counteract and switch off such activation. Recently, the analysis of the regulation of the nuclear shuttling of NFAT has evidenced the role of different kinases in the attenuation of activation signals.

We have analyzed the role of the different MAPKs the T cell activation and found that p38 MAP is activated in a sustained fashion during T cell activation. The activation of p38 signal transduction pathway but not that of JNK or ERK pathways promotes the nuclear export of NFATp. Conversely, blockade of this pathway results in inhibition of the NFATp nuclear export and the subsequent retention of the transcription factor in the nucleus of activated T cells. These results indicate that p38 signal transduction pathway plays a role in the homeostasis of the immune system by counteracting calcium signals during T cell activation.

GENE REGULATION IN THE IMMUNE RESPONSE

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The expression of inducible genes by antigen-stimulated T cells occurs in two distinguishable stages. The first stage occurs in naive T cells that are differentiating into mature effector cells as a result of initial encounter with antigen, and involves chromatin remodelling and active CpG demethylation of genes whose expression is important for the differentiated effector phenotype. Thus T cells stimulated with antigen and IL-4 show chromatin remodelling of the linked IL-4 and IL-13 genes, while T cells stimulated with antigen and IL-12 show remodelling of the interferon-γ gene. We are studying the nuclear factors and mechanisms that regulate these remodelling events.

When reexposed to antigen, the differentiated effector T cells acutely transcribe a variety of inducible genes. This acute phase of gene transcription is regulated by inducible transcription factors including NFAT, AP-1 (Fos/Jun) and NFκB. The DNA-binding domains of NFAT and NFκB/Rel proteins show a striking structural similarity. NFAT proteins form strong cooperative complexes with AP-1 at composite NFAT:AP-1 sites, which are present in the regulatory regions of most NFAT-dependent genes. The nuclear translocation of NFκB/Rel proteins is controlled by specific IκB kinases (IKK-1 and IKK-2), while nuclear translocation of NFAT proteins is controlled by the calmodulin-dependent phosphatase calcineurin. The activity of calcineurin is countered by one or more NFAT kinases. The N-terminal transactivation domains of NFAT proteins bind histone acetyltransferases in the nucleus, an interaction that is essential for transactivation by NFAT.

CONTROL OF T LYMPHOCYTE TRANCRIPTION BY CYCLOOXYGENASE 2

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Cyclooxygenase (Cox), known to exist in two isoforms, is a key enzyme in prostaglandin synthesis and the target for most of the non-steroidal antiinflammatory drugs (NSAIDs). We have found that human T lymphocytes express both Cox enzymes. However, while Cox-1 was constitutive, Cox-2 mRNA and protein were induced by activation through the TcR/CD3 or PMA in both Jurkat and purified T cells. Cox-2 mRNA was induced very early after activation and superinduced by protein synthesis inhibitors, whereas it was inhibited by the immunosuppressive drug cyclosporin A (CsA), identifying it as an immediate early gene. Cox-2 promoter dependent transcription required both protein kinase C and calcium dependent signals. Induction of Cox-2 promoter in T cells by TcR mediated signals was controlled by NFAT and AP-1. Ectopic expression of NFAT cooperated with PMA to activate the promoter whereas overexpression of a dominant negative NFAT inhibited its transcription. Expression of a dominant negative c-jun also completely abolished Cox-2 transcription

Interestingly, blockade of Cox-2, but not Cox-1, activity with specific inhibitors severely diminished early and late events of T cell activation including, CD25 and CD71 cell surface expression, IL-2, TNF-α and IFN-γ production and cell proliferation but not the expression of CD69, an immediate early gene. Moreover, treatment of T cells with Cox-2 inhibitors abolished transcription of reporter genes driven by IL-2 and TNF-α promoters and inhibited the activation of transcription factors, nuclear factor kappa-B (NF-κB) and nuclear factor of activated T cells (NFAT) in activated T cells. Interestingly, ectopic expression of COX-2 in Jurkat T cells was sufficient to activate NF-κB but not NFAT. This induction was reversed Cox-2 inhibitors. These results may have important implications in antiinflammatory therapy and open a new field on Cox-2 selective NSAIDs as modulators of the immune activation and transcription.

POSTERS

REQUIREMENTS FOR PRE-B CELL RECEPTOR EXPRESSION, ASSEMBLY, AND FUNCTION

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Previously our laboratory identified two pre-B cell specific DNaseI hypersensitivity sites in the human (*hu*) lambda (λ) 5 locus. To further define the roles of cis-regulatory regions *in vivo*, we generated *huλ5* transgenic mouse lines. We have used these mice not only to identify the tissue- and stage-specific transcriptional regulatory regions but also the protein requirements for a functional pre-B cell receptor (BCR). The *huλ5* transgenic mice express high levels of *huλ5* mRNA and protein restricted to the pro-B and pre-B stages, indicating that the 28 kb XhoI transgene fragment encompassing the *huλ5* gene contains the regulatory regions necessary for the tissue- and stage-specific expression.

When crossed with *mλ5* nullizygous (-/-) mice, the *huλ5* transgene does not rescue the *mλ5* (-/-) phenotype to restore normal B cell development. Abelson derived cell lines from *huλ5* (+/-)/mλ5 (-/-) bone marrow show by immunoprecipitation and fluorescent staining that a chimeric huλ5, mVpreB, mμ, and pre-BCR is assembled and expressed on the cell surface. Fluorescent staining of *huλ5* transgenic bone marrow also shows that the huλ5 protein is synthesized in pro-B and pre-B, but not B cells. Our results suggest that assembly and surface expression of the pre-BCR is not sufficient for pre-BCR signaling and subsequent B lineage differentiation. This system provides a model to elucidate the molecular requirements of the human and mouse pre-B cell receptor, critical for B lymphocyte differentiation.

IDENTIFICATION AND CHARACTERIZATION OF ACII, A TRANSCRIPTION FACTOR THAT IS REQUIRED FOR MHC CLASS II EXPRESSION

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Expression of Major histocompatibility complex (MHC) class II molecules is strongly regulated and is controlled by many *cis* and *trans* acting elements. MHC class II molecules are constitutively expressed only in dendritic and B cells and are induced by interferon γ (IFNγ) in macrophages and fibroblast.

ACII (activator of class II) has been identified as a transcription factor that binds to the X box of the promotor of the mouse MHC class II IAβ gene. The binding site corresponds to the X1 (5') site of the X box. Human cDNA for ACII was also cloned showing a 76 % homology with mouse cDNA.

The ACII gene codes for a protein of about 33.5 kD. By western blot analyssis of total and nuclear extracts four bands are detected, two major bands of about 36 kD and two minor bands of about 33 kD. In mouse, ACII mRNA and protein are present in B cells, but are undetectable in T cells and fibroblasts. IFNγ induces ACII mRNA and protein expression in macrophages and fibroblast cells. Thus, the pattern of expression and regulation of ACII gene is corralated to that of the MHC class II genes. Furthermore, IFNγ induction of ACII mRNA is rapid, independent of protein synthesis and precedes expression of class II transactivator (CIITA) and MHC class II genes.

Antisense oligonucleotides of ACII gene were shown to inhibit the protein expression of the MHC class II IAα gene, suggesting that ACII is important for the expression of MHC class II genes.

In conclusion, ACII is an important transcription factor for the expression of MHC class II genes. Interestingly, ACII shows a cell specific expression and is induced by IFNγ.

A locus control region that regulates early B lineage specific expression of the mouse λ5/V$_{preB1}$ locus

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We have used studies in transgenic mice to identify a locus control region (LCR) in the λ5/V$_{preB1}$ locus. The λ5 and VpreB genes are expressed in pro-B and pre-B cells and are silenced in mature B cells. Their protein products complex together to form the surrogate light-chain. In pre-B cells, the surrogate light-chain forms part of the pre-B cell receptor which is thought to signal proliferation of cells that have a productive μ rearrangement (Melchers 1995). A set of DNase I hypersensitive sites (HS) located 3' of λ5 is required for position independence and correct levels of expression of both genes at single copy. Transgene expression is fully tissue and stage-specific. The strongest of the LCR HS is also present in mature B cells where the genes are silenced and contains multiple overlapping binding sites for EBF, E47 and Ikaros. Ectopic expression of EBF and E47 has previously been shown to induce expression of the genes in an early pro-B cell line (Sigvardsson et al., 1996). The role of Ikaros as a candidate repressor of the genes is being investigated by mutagenesis of Ikaros binding sites.

A novel PCR assay has been used to identify a transgene integration into centromeric γ satellite DNA. We find that the λ5/V$_{preB1}$ LCR gives full copy dependent expression even when integrated into centromeric heterochromatin. This result implies that the spectrum of positively acting factors that act on the genes in pro and pre-B cells is dominant over the silencing effect of heterochromatin and has implications for models for chromatin-mediated silencing of the genes.

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Activation and Differentiation of JNK1-deficient T Cells

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c-Jun N-terminal kinase (JNK) is a novel MAP kinase that phosphorylates c-Jun component of AP1 transcription factor. JNK and AP1 activation is suggested to be involved in T lymphocyte development, activation, tolerance and apoptosis, and cytokine production by effector helper T cells. To understand the functional role of JNK, our laboratory has generated mice deficient in JNK1 gene. T cells develop normally in these mice with normal T to B and CD4 to CD8 cell ratio in the spleen. To analyze whether the JNK1 deficient T cells are competent in immune response, I stimulate these cells with various stimuli and measure their proliferation and IL-2 production. I am also differentiating the CD4 T cells to effector helper T cells and analyze if they are capable of producing cytokines. The results from these experiments will be presented in the meeting.

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CONTROL OF TRANSCRIPTION AND V(D)J RECOMBINATION AT THE T-CELL RECEPTOR α/δ LOCUS BY THE TCR α AND δ ENHANCERS

The T-cell receptor (TCR) α/δ locus represents a fascinating model for the analysis of V(D)J recombination and transcription within the immune system. The locus spans at least 1000 kb of DNA, within which are included both the TCR α and TCR δ genes, with TCR δ gene segments nested between TCR α gene segments. The organization of the TCR α/δ locus dictates that the entire TCR δ gene is deleted upon TCR α gene rearrangement. The developmental stage- and lineage-specificity of TCR gene segment rearrangement and expression at the TCR α/δ locus implies the presence of precise and localized regulation that is critical for the maturation of both αβ and γδ T lymphocytes.

Using a transgenic mouse system carrying an unrearranged human TCR δ gene minilocus, our laboratory has demonstrated that the TCR α and δ enhancers, Eα and Eδ, direct both developmental stage- and lineage-appropiate activation of V(D)J recombination *in vivo* within the TCR α/δ locus. Eδ function is controlled by juxtaposed binding of CBF/PEBP2 and c-Myb, whereas Eα function is controlled by binding of ATF/CREB, TCF/LEF, CBF/PEBP2 and Ets family members. We are analyzing loading of transcription factors to these two enhancers *in vivo*. In the case of Eα, we have found that formation of a multiprotein complex on this enhancer is highly cooperative and that no single factor can access chromatin in vivo to play a unique initiating role in its assembly. Rather the simultaneous availability of multiple binding proteins is required for chromatin disruption and stable binding site occupancy as well as activation of transcription and V(D)J recombination. In contrast with Eα, *in vivo* studies of Eδ occupancy revealed that transcription factor loading to this enhancer is hierarchical rather than all-or-none. Studies about occupancy of these enhancers during thymic development are in progress and will be discussed.

Transcription Factors for Interleukin-4

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Interleukin-4 (IL-4) plays a central role in the pathogenesis of allergic inflammation by inducing Ig class switch to IgE. IL-4 is also the most potent factor that drives naive T helper (Th) cells to differentiate to the Th2 phenotype. Based on cytokine production patterns Th cells can be grouped into two distinct subsets: Th1 and Th2 cells. The Th1 cells produce interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor (TNF) and lymphotoxin and are associated with cell-mediated immune functions. The Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and assist the humoral immune response. Recently, efforts have been made to explore the molecular basis of the Th2-specific IL-4 expression in CD4 T cells. Transcription factors, such as GATA-3, NF-IL6 and c-Maf, were found to be preferentially expressed in Th2 cells and to play an important role in regulation of the IL-4 promoter activity. We have recently shown that Th2-specific DNA/protein interactions at two regulatory regions of the human IL-4 promoter. These findings provide evidence that tissue-specific expression of the IL-4 gene may involve interactions of Th2-specific transcription factors with the promoter/enhancer elements of the IL-4 promoter. Yet, other transcription factors may be indirectly involved in Th2-specific expression of IL-4 even though they are present in both Th1 and Th2 cells. Recently, CD28, a costimulatory cell surface molecule in T cell activation, has been shown to promote the production of Th2 cytokine including IL-4 and to play a role in differentiation of T helper cells into either Th1 or Th2 type. Two important IL-4 enhancer elements are found to respond to CD28-stimulation-induced transactivation. In correlation with the CD28 induced transcriptional activation, proteins of AP-1 and NF-kB/Rel family members are found to bind to these two regulatory elements upon CD28 stimulation. In conclusion, Th2-specific expression of the IL-4 gene appears to be controlled by a multi-factor-system.

TRANSCRIPTIONAL UPREGULATION OF eNOS EXPRESSION: AP-1, NF-AT AND REDOX STATE.

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Cyclosporine A (CsA) and Tacrolimus (FK506) are claimed to be very effective drugs, specially in the field of transplantation. Among their side effects, one of the most well known is damage to the vascular endothelium associated in some cases to the presence of hypertension and/or microangiopathic hemolytic anemia. Hypertension seems to be related to perturbations in the balance of vasoconstrictors/vasodilators within the vascular wall. However, little is known about the molecular changes in signal transduction and gene expression induced by CsA or FK506 within the endothelium. CsA and FK506 increase eNOS mRNA expression (2-2,5 fold) in cultured bovine aortic endothelial cells (BAEC). Since preincubation with an inhibitor of the RNA polymerase II (dichlorbenzimidazol riboside) prevents this upregulation, CsA appears to increase eNOS mRNA levels mainly by increasing the rate of transcription. Results of transient transfections using a 1.9 Kb construct of the eNOS promoter supported this contention: a 2-fold increase in the promoter activity with CsA. CsA and FK506 induced an increase of ROS synthesis with the fluorescent probe used, DHR123. The ROS generating system glucose oxidase (GO) increased the expression of eNOS mRNA in BAEC. This upregulation of eNOS mRNA by CsA or GO was abrogated by catalase, but potentiated by PDTC, an antioxidant which promotes AP-1 activation. As AP-1 is a redox-sensitive transcription factor and the bovine eNOS promoter has an AP-1 cis-regulatory element, a role for this factor in the upregulation of eNOS mRNA was studied. Electrophoretic mobility shift assays were consistent with an increase in AP-1 DNA-binding activity in BAEC treated with CsA or glucose oxidase. In preliminary transient transfection assays in BAEC using an AP-1-driven reporter anchored to luciferase (-73Col-Luc), CsA appears to increase its activity. The potential participation of ROS and the transcription factor AP-1 in the regulation of eNOS gene expression is suggested. In the human eNOS promoter a consensus NF-AT binding site is present and overlaps with an AP-1 binding site (-1532: AGGAAAATGAGTCA). The functional significance of these sites or their implication in the upregulation of eNOS mRNA by CsA have not been described. EMSAs, using an oligonucleotide that includes this overlapping sequence, show a single band which behaves as an AP-1 regulatory site. Preliminary results show an increase in the NF-AT binding activity in BAEC treated with superoxide dismutase, suggesting a possible redox regulation of the NF-AT signalling pathway in this cell type.

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Dual Functions of p300/CPB integrate Raf/Rac-signalling pathways to induce NF-ATc Activity in T cells

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NF-ATc is a prominent member of family of NF-AT transcription factors and inducibly expressed in peripheral T lymphocytes where NF-ATc appears to control the induction of numerous genes upon activation. We show here that the nuclear co-activators p300/CBP bind to and enhance the activity of a strong inducible N-terminal transcactivation domain of NF-ATc, TAD-A. Stimulation of both Raf/Erk- and Rac-involved signalling pathways enhance, and inhibition of MEK protein kinase activity impairs the p300/CBP mediated increase of TAD-A and NF-ATc activity. Although stimulation of Raf/Erk-involved pathways results in an inducibly phosphorylation of TAD-A, direct phosphorylation of TAD-A appears to play a minor, if any role for the binding and activation of TAD-A by p300/CBP. Instead, constitutive active versions of c-Raf and Rac are able to act synergistically in the p300/CBP-mediated stimulation of TAD-A activity. Mutations of CBP which abolish HAT activity diminuish but do not abolish CBP-mediated NF-ATc activation indicating an important, but not sole role for histone acetylation in NF-ATc activity. Since, in addition, MAP kinase signals do not appear to affect histone acetylation in Jurkat T cells one may conclude that functions of p300/CBP others than histone acetylation play an important role in the induction of NF-ATc activity in T cells

Functional modulation of E47 activity by Early B cell Factor (EBF).

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The development of a mature B lymphocyte from a bone marrow precursor cell is a highly ordered event where linage and stage specific transcriptional control is a key issue. One transcription factor suggested to have a key function in B cell development is the basic helix-loop-helix (bHLH) transcription factor E47. Basic-helix-loop-helix (bh-l-h) proteins are a large evulotionary conserved family of transcriptionfactors with key roles in such diverse events as sex determination and formation of the periphiral nervous system in drosophila and development of B lymphocytes and myogenisis in mice. The function of the bh-l-h proteins are regulated by interactions with other proteins such as calmodulin and the specific bh-l-h inhibitors of the Id family. The role of h-l-h proteins in B cell development has been extensively studied and several B cell restricted genes has been suggested as genetic targets for the bh-l-h protein E47. These targets does however represent a broad range of genes that are expressed at different stages of B cell development. The finding that the same trascription factor appears to regulate genes with such diverse expression patterns as the surrogate light chain λ5 and the joining chain (J-chain) with expression restricted to the pre-B cell and plasma cell stage respectivly, suggests that E47 participates in a complex network to control stage specific transcription.

We have investigated the interplay between E47 and a h-l-h related transcription factor Early B cell factor (EBF) normally not found in plasma cells, and found that expression of EBF in a plasma cell repress the functional activity of several reporter genes under the control of Ig enhancer elements. This repression is present in the absence of EBF binding sites in the control elements. Further more, coexpression of E47 and EBF act in synergy to activate a λ5 reporter gene in HeLa cells while EBF repress the E47 induced activation of an Ig heavy chain enhancer in the same cells. The mechanism of inhibition appear to differ from that of Id proteins since functional repression can be observed also using a E47 forced homodimer that should be resistant Id proteins. We also demonstrate that E47 interacts fysically with EBF both *in vitro* and in cells.

Based on these findings we suggest that EBF acts as a modulator of E47 activity in B cell development.

PJA-BP EXPRESSION AND T CELL RECEPTOR-δ DELETION DURING HUMAN T CELL DIFFERENTIATION

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Recombination of δRec to ψJα deletes the T cell receptor (TCR)δ gene and is assumed to precede Vα to Jα gene rearrangements during human T cell differentiation. The TCRδ gene deletion is thought to play an important role in the bifurcation of the TCRαß versus TCRγδ differentiation lineages. We recently detected a DNA-binding protein in human thymocytes, the so-called PJA-BP, which recognizes the ψJα gene segment and might be one of the factors involved in the regulation of preferential δRec-ψJα rearrangements. Now we investigated PJA-BP expression and its correlation with TCRδ gene deletion in thymocytes.

Our electrophoretic mobility shift assay experiments showed that the PJA-BP is evolutionary conserved in human, murine, and simian thymocytes. Using a large series of human hematopoietic malignancies (n=30), we conclude that PJA-BP expression is thymocyte specific, and seems to be restricted to thymocytes committed to the TCRαß lineage.

Analysis of seven well-defined human thymocyte subpopulations showed that preferential δRec-ψJα rearrangements as well as PJA-BP expression can be detected from the immature CD34$^+$/CD1$^+$/CD3$^-$/CD4$^+$/CD8α$^+$ß$^-$ thymocyte differentiation stage onwards. These experiments indicate that expression of PJA-BP in human thymocytes starts simultaneously with preferential δRec-ψJα rearrangements, which supports our hypothesis that the PJA-BP is one of the factors involved in the preferential recombination of δRec to ψJα. Furthermore, our RT-PCR studies on Vα–Cα mRNA show that transcription of the TCRα gene, and therefore TCRα gene rearrangements, occurs after TCRδ gene deletions have started.

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