Instituto Juan March
de Estudios e Investigaciones

130 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Basis of Human Congenital Lymphocyte Disorders

Organized by

H. D. Ochs and J. R. Regueiro

J.-L. Casanova
V. Cerundolo
T. Chatila
M. E. Conley
M. D. Cooper
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The lectures summarized in this publication were presented by their authors at a workshop held on the 3rd through the 5th of December, 2001, at the Instituto Juan March.

Depósito legal: M-54.854/2001
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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Introduction
H. D. Ochs and J. R. Regueiro

A meeting on "Immunodeficiencies of genetic origin" was organized by Drs A Arnaiz and A Fischer on March 1995 with the support of the Juan March Foundation (booklet nº 38). Since then, an enormous progress has taken place in the field: first, the genes responsible for most congenital immunodeficiencies and for several lymphoproliferative disorders have been identified; second, a wealth of new information has accumulated on the biological role of the proteins involved, and on their particular localization within a limited number of biochemical pathways; and third, the first successful gene therapy trial in humans has been reported in immunodeficient children, raising hope for those suffering other genetic diseases including cancer.

The present meeting has reviewed this progress, concentrating on human congenital lymphocyte disorders caused by defects in the biochemical pathways involved in DNA rearrangement, regulation and repair (including Ig isotype switch and somatic hypermutation), cytokine recognition, antigen presentation and recognition, cytolysis, apoptosis, and cytoskeleton control (see table below). The defects may cripple signal transduction, DNA methylation or transcription, vesicle sorting or secretion. The molecules dedicated to those biochemical pathways (mostly proteins, but also RNA in one instance) are thus crucial either for producing immunocytes, or for initiating, maintaining or terminating immune responses, and sometimes for apparently unrelated biological functions in other cell types, such as melanocytes. These "experiments of nature", no matter how rare, have opened unexpected and exciting avenues for future research which will further increase our understanding of the immune system and, in turn, should give way for improved diagnostic and therapeutic procedures.

H. D. Ochs and J. R. Regueiro

HUMAN CONGENITAL LYMPHOCYTE DISORDERS

PROTEIN	SYNDROME
• Defects of DNA modification and repair pathways	
RAG, Artemis	T^-B^- SCID, Omenn
RNAse MRP	Cartilage-hair hypoplasia
DNA methyltransferase DNMT3B	ICF (Immune/Centromeric/Facial)
CD40L/CD40/ NEMO (IKKγ) /AID	Hyper IgM
ATM, hMRE 11, Nibrin	Ataxia Telangiectasia and AT-Like
• Defects of cytokine recognition pathways	
γc / IL-7Rα / Jak 3	Severe Combined ID (SCID)
IL-2R α	SCID
IL-12 / IFN-γ / STAT-1 network	Mycobacterial susceptibility

• Defects of antigen receptor recognition and signalling pathways	
λ5 / μ/ Igα/ BLNK (non X-linked)	Agammaglobulinemia
Btk (X-linked)	Agammaglobulinemia
CD3γ, ε, ζ, CD8α	CID (Combined immunodeficiency)
CD45	SCID
Zap 70, Lck	SCID
• Defects of antigen presentation pathways	
MHC-class I (TAP1,TAP2)	Granulomatosis, CID
MHC-class II (RFX5,AP,B, CIITA)	SCID
• Defects of cytolysis pathways	
Perforin	Hemophagocytic lymphohistiocytosis
Lyst	Chediak-Higashi
Myosin-5a / RAB27A	Griscelli
SAP or SH2D1A (X-linked)	Lymphoproliferative disease, Duncan
• Defects of apoptosis pathways	
TNFR1, cryopyrin, melvalonate kinase	Autoinflammatory/Recurrent fevers
CD95L/CD95/Caspase 10/Foxp3	Autoimmune lymphoproliferation
• Defects of cell mobility and adhesion	
WASP	Wiskott Aldrich / XLT / neutropenia
CD18/GDP-FUCOSE TRANSPORTER	Leukocyte adhesion deficiency

Session 1: Defects of DNA regulation and repair
Chair: Hans D. Ochs

SCID due to Rag defects

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Central to the differentiation of both B and T lymphocytes is their ability to rearrange genes coding for receptors specific for antigens. In the genomes of both mouse and man, there are several genes encoding for lymphoid specific rearranging genes, which are composed of one or a few regions coding for the constant C portion of these receptors and a huge number of genomic fragments which must be selected in order to codify for their variable domain. Functional receptors are then the result of a sophisticated process in which two (V-J) or three (V-D-J) DNA fragments, called Variable, Diversity and Joining segments, are assembled together in a single exon, in a process restricted to immature lymphoid cells that has been called V(D)J recombination (Tonegawa et al., 1983).

Rearrangement is directed by recombination signal sequences (RSS) that flank each antigen receptor gene segment. These recombination signal sequences consist of a heptamer sequence directly adjacent to the coding element, and a nonamer element separated from the heptamer by a spacer of either 12 or 23 base pairs. Efficient recombination occurs between a pair of gene elements with recombination signal sequences that have different spacer lengths, the so- called 12/23 rule. Recombination activating genes (Rag1-1 and Rag-2) play a fundamental role by initiating the 'cut-and-paste' process leading to the assembling of the V, (D) and J segments, which together form the variable portion of the receptors. Disruption of Rag-1 and Rag2 function blocks initiation of VDJ recombination and leads to the absence of mature B and T cells in mice and human (Shinkai et al. 1992; Mombaerts et al. 1992; Schwarz et al.1996), whereas natural killer cells, that do not rearrange antigen receptor genes, do not show any defects. Patients with Severe Combined Immunodeficiency secondary to Rag mutations may have Omenn syndrome (OS), a rare combined immunodeficiency characterized by the presence of a substantial number of oligoclonal, activated T cells, lack of B lymphocytes and peculiar clinical features such as : generalized erytrodermia, lymphoadhenopathy, hepato-splenomegaly and increased occurrence of life threating infections (Omenn 1965). On the basis of our findings, we have hypothesized that in humans the persistence of partial Rag activity could be responsible for OS, whereas mutations abolishing Rag function could give rise to T-B-SCID (Villa et al, 1998; Villa et al., 2001; Corneo et al., 2001).

Here we report the analysis of Rag genes in a large series of immunodeficient patients, evaluating the role of specific domains during the VDJ recombination process and suggesting that Rag dependent immunodeficiency covers a spectrum broader than previously thought. We have identified Rag defects in a cohort of patients with some, but not all, the clinical and immunological feautures of OS, a condition we called 'atypical SCID/OS'. Interestingly, missense mutations are over-represented in patients with atypical SCID/OS, as they are in Omenn syndrome.

We have analyzed the biochemical consequences of Rag mutants through the evaluation of their ability to bind Dna , to introduce nick and produce coding and signal joints. In addition, we have highlighted the role of the N-terminus domain of Rag1,

considered to be dispensable for recombination, but playing an important role in nuclear localization and in enhancing the recombination activity of Rag1 (Santagata et al., 2000; Noordzij et al., 2000).

In addition, we have investigated the activity of Rag2 in the VDJ reaction. Recently a variety of sequence analysis tools has revealed that the Rag2 active core contains six internal repeats of approximately 50 amino acids which were first identified in the Drosophila kelch repeat (Callebaut et al.,1998). The observation that Rag2 contains such repeats has allowed to better understanding the role of this protein in the VDJ reaction (Gomez et al., 2000; Corneo et al., 2000). The analysis of Rag2 mutants found in T-B- SCID and Omenn patients, combined with the analysis of an extensive panel of site-directed mutations within each of the six kelch motifs, support the sequence analysis predictions that Rag2 may form a β-propeller structure composed of six kelch repeats.

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Villa A, Sobacchi C, Notarangelo LD *et al.* V(D)J recombination defects in lymphocytes due to RAG mutations: a severe immunodeficiency with a spectrum of clinical presentations. Blood 2001; 97:81--88.

Lymphocyte disorders resulting from mutations in the *ATM*, *NBS1* and *hMRE11* genes

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Ataxia-telangiectasia (A-T) is classified as a primary immunodeficiency disorder. Both humoral and cellular immune responses are affected in A-T patients including absence or abnormal development of the thymus and impaired function of both B and T cells, a low number of alpha/beta receptor carrying T lymphocytes and antibody deficiencies particularly IgA deficiency which might be an indicator of a defect in switch recombination. Patients also show a reduced number of lymphocytes, IgG2 subclass deficiency (1) and poorer T cell responses to PHA. In the past few years knowledge of the *ATM* gene, the range of mutations in patients, variations in clinical phenotype and the interactions and functions of the ATM protein, have increased enormously (2) but details of its role in immune system development and function have not emerged at the same rate. A major feature of A-T indicating the role of ATM in lymphoid cells is the very high frequency of lymphoid tumours among these patients (3).Cells from A-T patients are unusually sensitive to the killing effects of ionising radiation. The *ATM* gene is involved in a variety of cellular responses to DNA double strand breaks including cell cycle arrest, apoptosis and DNA repair (2). The ATM protein interacts with many other cellular proteins mainly through its role as a protein kinase. Two target proteins that ATM phosphorylates are Nbs1 and hMre11 that form the hMre11/Nbs1/hRad50 repair complex involved in the cellular response to DNA double strand breaks (4-7). Mutation of the *hMRE11* gene also gives rise to an ataxia-telangiectasia-like disorder (ATLD) (8) and mutation of *NBS1* to Nijmegen Breakage Syndrome (NBS) (9). Both of these disorders also show immunodeficiency and NBS is associated with a high frequency of lymphoid tumours.

The defect in both B and T cells at least in A-T and NBS is manifested, therefore, as both a relatively mild immunodeficiency but also in a very high risk of lymphoid tumours. With regard to possible defects underlying the immunodeficiency, there is no major defect in DNA double strand break repair in A-T, NBS or ATLD cells, although a small proportion of breaks may be refractory to repair. V(D)J recombination is normal in both A-T and NBS cells (10-14). There is no evidence for any major defect in switch recombination or somatic hypermutation. Despite the absence of a defect in V(D)J recombination, an important feature common to all three groups of patients is the occurrence of a high frequency of chromosome translocations in T cells involving breakage of the T cell receptor genes (3). The consequences of these translocations may be different in each disorder. For example in A-T, it is known that translocations involving a TCR gene with either TCL1 (T cell leukemia gene) or MTCP1 (mature T cell proliferation gene) can develop into a T cell leukaemia (T-PLL) although not in Nijmegen Breakage Syndrome where tumours are predominantly of B cell origin. Loss of ATM is also associated with a defective p53 response to DNA double strand breaks. Failure to stabilise p53 will result in failure of apoptosis. Therefore, damaged and unrepaired cells may survive and contribute to a pool of potentially malignant cells.

The atm null mouse also has immune defects and develops thymomas (15). These tumours can be be suppressed by the simultaneous inactivation of the RAG machinery, demonstrating that breaks generated by V(D)J recombination are required for their development (16). The ATM, NBS1 and hMre11 proteins clearly have important roles in both B and T lymphocyte functions although the nature of these roles is presently not known.

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The ICF syndrome, a genetic disease involving a DNA methylation defect

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The ICF syndrome is a rare autosomal recessive disorder characterized by a combined Immunodeficiency, Centromeric instabilities and Facial abnormalities (Hultén 1978 ; Maraschio et al 1988) . ICF is the only example of genetic transmission of a constitutional DNA methylation defect affecting different DNA sequences and particularly the classical satellites, one of the major components of human heterochromatin (Miniou et al 1994; Miniou et al 1997a and b, Bourch'his et al 1999, Kando et al 2000). The disease was casually related to mutations in the catalytical domain of the gene encoding DNMT3B, a *de novo* methyltransferase involved in the establishment of methylation profiles during early development (Xu et al 1999). The ponctual mutations observed in the patients are accompanied by a residual expression of the fonctional protein (Xu et al 1999, Hansen et al 1999). DNMT3B knock-out mice (Okano et al 1999) containing complete deletion of the catalytic domain present a hypomethylation of several DNA sequences but the phenotypical abnormalities are more severe than those observed in ICF patients.

The lack of mutations in the catalytical domain of DNMT3B observed in some patients and the heterogeneity of the methylation defect of several DNA sequences led us to propose the existence of two types of ICF patients having definite genetic and epigenetic defects. This distinction underlines the specificity of the methylation process and prone either to the existence of mutations in other regions of the DNMT3B gene or to the involvement of another locus in this syndrome. ICF syndrome remains an intriguing pathology and may offer an opportunity to understand the contribution of DNA methylation in the induction of phenotypic abnormalities.

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The immunodeficiency in cartilage-hair hypoplasia, a genetic disorder of the skeleton, caused by mutations in *RMRP* gene

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We have recently demonstrated that mutations in the *RMRP*, an untranslated gene located to 9p13, are the genetic cause of cartilage-hair hypoplasia (CHH), an autosomal recessive multi-organ condition (Ridanpää et al., 2001). The clinical phenotype of CHH consists of short stature due to metaphyseal chondrodysplasia, hair hypoplasia, defective erythrogenesis, defective cell-mediated, and often, humoral immunity, defective spermatogenesis, variable Hirschsprung disease, and risk of malignancies. CHH is common among the Old Order Amish in the USA and the Finns, and rare in other populations.

Reviewing the clinical presentation of the immunodeficiency, McKusick and his co-workers observed a propensity to infections in their original description of CHH among the Amish patients, and, in particular, they reported varicella as a severe, occasionally lethal condition. They also reported on lymphopenia and reduced response of lymphocytes to several mitogens (McKusick et al., 1965). Since then a number of patients with severe, chronic, and even fatal infections due to both viral and bacterial etiologies have been reported (Polmar and Pierce, 1986; Castigli et al., 1995; Berthet et al., 1996). Recently, we reported on increased mortality in a cohort of 120 Finnish patients with CHH attributable to defective immunity, especially in children (Mäkitie et al., 2001). The standardized incidence ratio of malignancies was significantly increased, and that of lymphomas was about 90 in CHH patients (Mäkitie et al., 1999).

Laboratory studies have repeatedly confirmed a marked, constant, but variable decrease in the total count of T-lymphocytes, and reductions in the proliferative response and in the IL-2 and IFN-gamma synthesis of the CHH lymphocytes to mitogens (PHA, ConA, PWM, MLR, etc.) (Virolainen et al., 1978; Polmar and Pierce, 1986; Kooijman et al., 1997; Mäkitie et al., 1998). The reductions could not be restored by addition of rIL-2 (Kooijman et al. 1997). In lymphocyte subpopulation studies, a reduction of 50 % in CD4+ cell count, and a reduction of 30 % in CD4+/CD8+ cell ratio have been reported. The B-lymphocyte count was usually normal, whereas the NK cell count often was elevated (Mäkitie et al., 1998). A third of the Finnish CHH patients also have defective humoral immunity presenting as isolated IgA and IgG subclass deficiencies, whereas severe combined immunodeficiency (SCID) is rare (Mäkitie et al., 2000). It has been suggested that reduction in lymphocyte count is due to intrinsic defect in cell proliferation (Polmar and Pierce, 1986). The levels of mRNA encoding c-myc, IL-2R alpha, IL-2, and IFN-gamma were decreased in the stimulated CHH lymphocytes, whereas those of other early activation gene products, such as c-fos and c-jun, were not impaired. The authors suggested that in CHH there may be an abnormality in a component of intracellular signaling pathways or in an expression regulating factor (Castigli et al, 1995). Bone marrow transplantation completely restored the cellular and humoral immunity in a child with SCID (Berthet et al., 1996).

The primary transcript of *RMRP* gene consists of 267 nucleotides, only, and encodes the RNA component of the RNase MRP complex formed by the RNA molecule and several protein subunits. The biological functions of the complex, an endoribonuclease, are only partially understood in humans. It has at least two functions required for cell proliferation, namely involvement in nuclear the processing of precursor rRNA and in the sequence-specific cleavage of RNA in mitochondrial DNA synthesis. Other functions involved in cell division, differentiation, migration or apoptosis probably exist (see, Eenennaam et al., 2000).

The disease causing mutations in the *RMRP* are localized to either the promoter or transcribed region of the gene. Mutations in the promoter region block transcription from that allele, and if located there in both alleles, most probably result in a lethal condition. The mutations in the transcribed region are located at sites that are conserved in human, cattle, rat, mouse, and even in *Xenopus*, *Arabidopsis*, and *Saccharomyces*. However, the mutations have not been observed to interfere with the association of the RNA component and the protein subunits of the RNase MRP complex. Screening the Finnish and Amish CHH patients, and patients from a number populations for mutations in the *RMRP* gene, a variety has been detected. In the Finnish patients, the major mutation is nt70A->G substitution which is the most common in patients from other populations, as well. In addition, there is a minor mutation, nt262G->T, special for the Finnish patients. Haplotype analyses to determine the age of the major mutation and genotype-phenotype correlation studies are in progress.

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ARTEMIS, a novel DNA double-strand break repair/V(D)J recombination protein is mutated in Human Severe Combined Immune Deficiency with increased radiosensitivity (RS-SCID)

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Human T- B- Severe Combined Immunodeficiency (T-B-SCID) is characterized by a virtually complete absence of both circulating T and B lymphocytes, while natural killer (NK) cells are normally present and functional. This autosomal recessive condition is due to a defect in the V(D)J recombination leading to an early arrest of both T and B cell maturation, caused by mutations in either the Rag1 or Rag2 gene in a subset of patients. In some patients (RS-SCID), the Rag1 and Rag2 genes are found normal and the immunodeficiency is accompanied by an increased radiosensitivity to ionizing radiations of both bone marrow cells and primary skin fibroblasts and by an impaired coding joint formation in V(D)J recombination.

Genetic linkage analysis in several consanguineous families enabled us to clearly demonstrate that none of the known genes involved in V(D)J recombination/DNA repair is at the origin of RS-SCID, suggesting that a yet undescribed factor accounts for this disorder.

We recently mapped the disease related locus to the short arm of human chromosome 10 in a 6.5cM region delimited by two polymorphic markers, D10S1664 and D10S674. Here we report the identification and cloning of Artemis, a gene encoding a novel protein involved in V(D)J recombination and DNA repair, whose mutation causes human RS-SCID. Artemis belongs to a large family of molecules that adopted the metallo-beta-lactamase fold as part of their putative catalytic site.

Session 2: Defects of cytokine recognition pathways
Chair: Max D. Cooper

Autosomal Dominant Recurrent Fevers

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Hereditary periodic fevers (HPFs) comprise a group of rare disorders characterised by intermittent self-limited inflammatory episodes with fever, synovial/serosal inflammation, skin rashes, and conjunctivitis [reviewed 1, 2]. Despite clinical similarities between all conditions in this category there is a clearcut distinction in the mode of inheritance between the two main autosomal recessive conditions, familial Mediterranean fever (FMF, MIM249100), and hyperimmunoglobulinemia D (HIDS, MIM260920 –MIM refers to the Mendelian Inheritance in Man classification number), and the less well classified autosomal dominant recurrent fever (ADRF) syndromes. There are at least three recognised Mendelian dominant periodic fever syndromes and the most common and extensively studied is familial Hibernian fever (FHF, MIM142680), first described as a separate clinical entity in a Scottish-Irish family in 1982 [3]. The less common ADRFs include Muckle-Wells syndrome (MWS, MIM:191900) [4], and familial cold urticaria (FCU, MIM:120100) [5]. Autoantibodies are not a general feature of TRAPS or the recessively inherited FMF, and for this reason the term *autoinflammatory* was proposed rather than *autoimmune* to define these disorders [6]. Autosomal dominant cyclic hematopoiesis (ADCH, MIM162800), also known as cyclic neutropenia [7] is also sometimes included in this category.

The discovery of the underlying genetic basis of all the main periodic fevers (FMF, FHF, MWS/FCU, cyclic neutropenia and HIDS) in the past 4 years has led to a major expansion of this field, both in terms of investigation and treatment. Identification of missense mutations in the extra-cellular domains of the 55 kDa tumor necrosis factor receptor (TNFRSF1A) led to the adoption of the more descriptive acronym TRAPS (Tumor necrosis factor Receptor-Associated Periodic Syndrome) for FHF [6]. Mutations in the *ELA2* gene, encoding neutrophil elastase on chromosome 19 underlie susceptibility to cyclic neutropenia [8], and the Muckle Wells (MWS)/familial cold urticaria (FCU) syndrome is caused by mutations in the NACHT domain of the *CIAS1/NALP3* gene [9, and unpublished].

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Defective cytokine signaling in severe combined immunodeficiency: SCID associated with mutations in the common cytokine receptor γ chain, Jak3, and the IL-7 receptor α chain

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Severe combined immunodeficiency (SCID) represents a syndrome comprising the most severe forms of inherited immunodeficiencies. The most common form, X-linked SCID, in which development of T cells and NK cells is profoundly reduced, results from mutations in the common cytokine receptor γ chain, γ_c, which is shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. γ_c-dependent signaling requires Jak3, the Janus family tyrosine kinase that associates with and is immediately downstream of γ_c. Mutations in Jak3 result in an autosomal recessive form of SCID clinically and immunologically indistinguishable from XSCID. In these diseases, the basis for defective T-cell development can be attributed to defective IL-7 receptor-dependent signaling, as humans with some forms of $T^-B^+NK^+$ SCID have been shown to have mutations in the *IL7R* gene. In contrast, defective NK-cell development can be attributed to defective IL-15 signaling. Whereas Jak1 and Jak3 are both required for T-cell and NK-cell development, Stat5a and Stat5b are STAT proteins that are downstream of these Jaks and activated by IL-7 and IL-15. Based on murine knockout models, Stat5a and Stat5b are together essential for NK-cell development and also contribute to T-cell development. Whether mutations in either the *STAT5A* or *STAT5B* gene can result in a clinically important immunodeficiency in humans is not yet known; however, based on the murine knockout models some degree of compromise can be anticipated. Stat5a and Stat5b are essential for IL-2-induced upregulation of the IL-2 receptor α chain gene, which in turn is required for expansion of T-cells in response to antigen. This is based on the presence of two Stat5-dependent IL-2 response elements in the gene, one of which is located 5' to the promoter whereas the other is in the first intron. Thus, in addition to the requirement of Stat5a and Stat5b for normal NK-cell and T-cell numbers, these STAT proteins are critical for IL-2 responsiveness following antigen-induced expansion of T-cells, an essential function that is cooperatively served by two different IL-2 response elements. Moreover, analysis of Stat5 knockout and transgenic mice has revealed a role for Stat5 proteins in CD8 T-cell homeostasis. This like explains, at least in part, the greatly diminished numbers of $CD8^+$ T cells in humans and mice lacking γ_c or Jak3.

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SCID from gene identification to gene therapy

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Severe combined imunodeficiencies (SCID) consist in rare inherited conditions characterized by defective development of the T cell lineage variably associated with other developmental defects. Molecular mechanisms of several SCID conditions have been identified, including impaired γc-cytokine receptor signaling (gc, JAK-3, IL7Ra), impaired V(D)J recombination (Rag-1, Rag-2, Artemis) impaired TCR mediated signaling (CD3, ZAP-70, CD45) or defective protection from apoptotic death (ADA,PNP). These investigations bring insight into many aspects of T-cell development as examplified by the identification of Artemis, a gene product involved in the formation of coding joints during the V(D)J recombination process as well as in DNA repair through the non homologous end joining process.

SCID also represent optimal models for gene therapy as being lethal conditions with hitherto partially unsatisfactory treatment while it is expected that transgene expression into lymphoïd progenitors should provide survival/growth selective advantage leading to a flurry of long-lived T cells. This is what has been demonstrated in the SCID-X1 condition (gc deficiency). By using a retroviral vector ex vivo gc gene transfer into hematopoïetic progenitors restores both in the gc (-) mouse and SCID patients, T cell development. The effect was found to be sustained so far up to 2.5 years in patients. Antigen-specific T /B cell responses and a clinical response were documented. These results validate the selective advantage concept which could be the rationale to the treatment by gene transfer of other T cell deficiencies.

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Inherited disorders of the IL-12-IFNγ axis

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Individuals with inherited disorders of interferon-γ (IFNγ-mediated immunity appear to be specifically vulnerable to mycobacterial infections. The severity of clinical features of affected individuals varies from case to case. Some patients die of mycobacterial infection in early childhood, whereas others have long asymptomatic periods in childhood and adulthood. This syndrome shows high allelic and non-allelic genetic heterogeneity. Mutations in *IL12B*, encoding the IL-12 p40 subunit, and in *IL12RB1*, encoding the β1 chain of the IL-12 receptor, result in impaired IFNγ production. Mutations in *IFNGR1*, *IFNGR2*, and *STAT1*, encoding the two IFNγ-receptor chains and a key signaling molecule, result in impaired cellular responses to IFNγ. Different types of mutation define two types of complete and two types of partial IFNγR1 deficiency. Complete and partial IFNγR2 deficiency have also been described.

Healthy individuals and patients with the seven known genetic disorders impairing cellular responses to IFNγ may be classified into four broad groups based on genotype, cellular phenotype, and clinical phenotype (normal individuals and patients with mild, intermediate, or severe disease). There is virtually no variation in the clinical phenotype of unrelated patients sharing a given *IFNGR1*, *IFNGR2* or *STAT1* genotype. This correlation suggests that IFNγ-mediated cell activation is a genetically-controlled quantitative trait and that it determines the outcome of mycobacterial invasion in man.

In contrast, we identified two siblings with complete IL-12Rβ1 deficiency but markedly different clinical phenotypes. Both are homozygous for an *IL12RB1* missense mutation that prevents receptor expression and abolishes cellular responses to IL-12. One had the expected phenotype of disseminated Bacille Calmette-Guérin (BCG) vaccine infection in early childhood, whereas the other did not develop even local BCG infection despite three inoculations with live BCG at various ages. Abdominal tuberculosis was diagnosed in this second child at 18 years of age. These observations show unexpected interfamilial and intrafamilial heterogeneity of the clinical phenotype associated with complete IL-12Rβ1 deficiency. They further suggest that severe forms of tuberculosis may be favored by IL-12Rβ1 deficiency.

Altogether, these studies suggest that IFN-γ plays a non redundant (in each individual) and conserved (between individuals) role in protective immunity to mycobacteria. Whereas, IL-12 seems to play an important but partly redundant and variable role, and differences in clinical phenotype between patients with the same *IL12RB1* genotype are probably accounted for by variations in IL-12-independent IFNγ-mediated immunity. These observations are consistent with the idea that within the human IL-12-IFNγ axis, IFNγ is the single effector, and IL-12 one of several helper cytokines.

Session 3: Defects of antigen receptor recognition and signalling pathways
Chair: Alain Fischer

Autosomal recessive agammaglobulinemia

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There are at least four autosomal recessive disorders that can result in a clinical and laboratory picture that is very similar to that seen in patients with X-linked agammaglobulinemia (XLA). Of the 15% of patients with an XLA phenotype who do not have mutations in Btk, approximately one third have defects in μ heavy chain. Seven different mutations in μ heavy chain have been identified in 15 patients. Three mutations are large deletions that remove all of the exons of the μ constant region as well as the J region and D region genes. One mutation is a single base pair insertion in exon 1, one is a two base pair deletion in exon 2, one is a single base pair substitution resulting in the substitution of glycine for the wild type cysteine in the CH4 domain of μ heavy chain. The last mutation, which is a single base pair substitution at the alternative splice site that is used to produce the membrane form of μ heavy chain, has occurred in six unrelated families on several different immunoglobulin haplotypes, indicating that this is a hot spot for mutations in this gene.

Patients with defects in μ heavy chain tend to be a little sicker than the patients with XLA. They are recognized to have immunodeficiency at an earlier age and they are more likely to have enteroviral infections and life threatening infections. However, many of these patients do very well if they are treated with gammaglobulin replacement.

The μ heavy chain is first expressed on the surface of the pre-B cell along with the surrogate light chain and the two transmembrane signal transduction molecules, Igα and Igβ.The surrogate light chain consists of two proteins, VpreB, which has strong homology to a light chain variable gene segment, and λ5, which has homology to the lambda constant region. The surrogate light chain tests the ability of the μ heavy chain to fold properly and bind to a conventional light chain. One of the patients with a clinical disorder indistinguishable from that seen in patients with XLA had two different mutations in λ5. The maternal allele had a premature stop codon in the first exon and the paternal allele had a proline to leucine substitution in the third exon, the exon that encodes the segment with homology to the lambda constant region. Mice with defects in λ5 have a block in B cell differentiation at the pro-B cell to pre-B cell transition but the block is leaky such that 4 month old mice have 20% of the normal number of B cells. By contrast, the 10 year old patient with λ5 deficiency has less than 1% of the normal number of B cells in the peripheral circulation. Of interest, λ5 is a highly polymorphic gene. At least 9 different variants with amino acid substitutions have been identified. These different alleles may contribute to the variation in severity seen in patients with XLA.

We have looked for defects in Igα and Igβ, the signal transduction molecules that escort μ heavy chain to the cell surface. Although we have not found any defects in Igβ, we have identified one patient, a Turkish girl, with a homozygous splice defect in Igα. The defect, an A to G substitution at the –2 position of the splice acceptor site for intron 2, results in faulty splicing of the Igα mRNA.

The majority of transcripts delete exon 3 and a small number of transcripts use a cryptic splice site within exon 3 that causes the deletion of 13 bases of coding sequence. Both of these altered types of transcripts cause frameshift mutations. Like the patients with μ heavy chain deficiency, the Igα deficient patient has a complete block at the pro-B cell to pre-B cell transition. In her peripheral circulation, less than 0.01% of her lymphocytes express CD19.

Like Btk, the adaptor protein BLNK (B cell linker protein) is phosphorylated early in the signal transduction pathway that is activated by crosslinking of the pre B cell or B cell receptor complex. In addition, like Btk deficient B cell lines, BLNK deficient cell lines are unable to flux calcium appropriately in response to cross-linking of the antigen receptor. These two findings suggested that defects in BLNK might cause a clinical syndrome similar to XLA. A 20 year old male who was thought to have XLA, but who did not have a mutation in Btk, was found to have a homozygous splice defect at the splice donor site for intron 1 of BLNK. Analysis of cDNA from this patient's bone marrow showed the complete absence of BLNK transcripts.

All but two of the 18 patients that we have identified with rare autosomal defects in B cell differentiation have been homozygous for their gene defect. Although only 5 of the patients were from families with known consanguinity, the majority of the remaining patients had grandparents or great grandparents who came from the same small town.

A variety of defects can be associated with markedly decreased numbers of B cells in the peripheral circulation. These include disorders that cause myelodysplasia, like monosomy 7, trisomy 8 and dyskeratosis congenita. Some patients with XLP have hypogammaglobulinemia and reduced numbers of B cells. There are also patients with CVID who have late onset of disease, hypogammaglobulinemia and reduced numbers of B cells. We have not found mutations in any of the gene that we have screened in most of these CVID patients.

It is likely that the patients with early onset infections, panhypogammaglobulinemia and marked decreased numbers of B cells who do not have defects in Btk or any of the genes known to cause autosomal recessive are a diverse group. Some of these patients may have mutations in the known genes (Btk, μ heavy chain etc.) that are unusually difficult to detect. Others may have abnormalities in genes that have not yet been described or genes that have been described but not yet associated with immunodeficiency. Still others may have disorders that are not due to a single gene defect. A better understanding of the etiology of the B cell defects improves our understanding of normal B cell development.

X-linked agammaglobulinemia: subcellular location of Btk and gene expression profiling

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X-linked agammaglobulinemia (XLA) is a hereditary disease caused by mutations in the *BTK* gene resulting in a block in B lymphocyte development. The gene (Brutons agammaglobulinemia Tyrosine Kinase) was identified in 1993[1,2]. The *BTK* gene encodes a cytoplasmic protein-tyrosine kinase found in hematopoietic cells. It is localized in the 21.3-22 region of the long arm of the X chromosome (Xq). Several genes have been found to cluster in this area, but there is no evidence for any co-regulated expression or functional interaction among these genes or their products. The Btk locus encompasses about 38 kb in humans and 44 kb in the mouse, mainly due to different lengths of two of the intronic regions. The Btk gene is composed of 19 exons including a 5' untranslated exon[3]. More than 400 unique mutations have been identified and are collected in the BTKbase mutation database[4]. Btk is expressed in hematopoietic cells and is selectively downregulated in T cells and plasma cells. The promoter region is TATA-less and contains multiple binding sites for Sp1/Sp3 factors, two of which seem to be of functional importance, as well as a site for the Ets-family members PU.1 and Spi-B.

The Btk protein belongs to the Src superfamily of cytoplasmic protein-tyrosine kinases, but forms its own subfamily[5]. Src and eight related molecules form the largest family of cytoplasmic PTKs. The Tec family is the second largest, and is composed of five mammalian members: Btk, Bmx, Itk, Tec and Txk. Btk can be subdivided into five functional domains. The C-terminus contains the catalytic region and is also referred to as the Src homology 1 (SH1) region. This 280 amino acid moiety has tyrosine kinase activity, i.e. phosphorylates a substrate on tyrosine residues. In the very N-terminus the pleckstrin homology (PH) domain is found, followed by the Tec homology (TH), SH3 and SH2 regions. The PH domain is believed to have a membrane tethering function. Btk is involved in signal transduction and is believed to transfer signals from the B cell receptor via phospholipase C gamma 2 and protein kinase C activation[6]. We have previously demonstrated the phosphatidylinositol-3 kinase-dependent membrane translocation of Btk[7], as well as the nucleocytoplasmic shuttling of this molecule[8]. We have now identified a novel binding partner for Btk residing in the cytoplasmic membrane. This interaction seems to have a negative regulatory function for Btk. Using filter-based microarrays as well as Affymetrix oligonucleotide arrays we have studied more than 12,500 genes in order to get a global view of expressed genes in human Epstein-Barr virus-transformed B lymphocytes. Data from both filter and oligonucleotide arrays were compared to the gene clusters of a previously published lymphoma expression profile by linking to the UNIgene transcript database clusters. Our findings demonstrate for the first time the use of microarray technology to study the influence of mutations in the *BTK* gene and the use of functional annotation and validation of expression data by comparison of microarray analyses.

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Learning from human CD3 deficiencies

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TCR/CD3 complexes contain variable antigen-binding αβ heterodimers non-covalently associated to invariant CD3 proteins (γ, δ, ε and ζ) , which participate in TCR/CD3 assembly, expression and signal transduction both in the thymus and in the peripheral lymphoid tissues (1). The lack of individual CD3 chains (to date CD3γ, ε or ζ causes a group of T lymphocyte immunodeficiencies characterized by a prominent TCR/CD3 expression defect without overt lymphopenia, associated to respiratory tract infections which may be lethal (2, 3 and A. Fischer, personal communication). From these experiments of nature we learned that CD3 chains improve TCR expression, intrathymic selection events and antigen recognition, but their absence does not block them completely in humans, illustrating the high redundancy of the immune system. Their adaptative value is nevertheless dramatically illustrated by the patients, whose survival can be reduced without treatment. Gene transfer of the normal CD3 chains has thus been considered to treat these disorders (4).

From the detailed study of CD3γ-deficient mature T lymphocytes we learned that the lack of CD3γ impairs, but does not block, the surface expression of the rest of the TCR/CD3 complex (5) or its ligand-induced downregulation and concomitant re-expression (6). In contrast, phorbol ester-induced downregulation is completely abrogated (7). In addition, the lack of CD3γ revealed the existence of hitherto unrecognized biochemical differences between mature $CD4^+$ and $CD8^+$ T lymphocytes in the intracellular control of αβTCR/CD3 assembly, maturation, or transport (8). We also learned that CD3γ contributes essential specialized signaling functions to certain mature T cell responses such as (sustained) protein tyrosine phosphorylation, cell adhesion and polarization (6), or IL-2 induction (7). In contrast, it seems redundant for other responses such as proliferation, cytotoxicity or TNFα induction (7).

Recently,. we learned that the observed TCR/CD3 expression and downregulation defects are selectively corrected by retroviral gene transfer of CD3γ, but severe functional adverse effects cripple the transduced cells, which become constitutively IL-2 positive and apparently self-reactive (9). These results indicate that post-thymic gene supplementation disrupts the intrathymic calibration of CD3-deficient T cells. This could be harmful to the host and thus precludes the future use of peripheral blood T lymphocytes as targets for gene therapy in CD3 deficiencies.

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ZAP-70 severe combined immune deficiency

Naomi Taylor

Severe combined immune deficiency (SCID) is caused by a variety of mutations that interfere with the differentiation or function of T and/or B lymphocytes. Patients with ZAP-70-deficiency have an unusual SCID phenotype characterized by a selective inability to produce CD8 single positive T cells and a signal transduction defect in peripheral CD4+ cells[1-3]. In order to study signaling and gene transfer in ZAP-70 deficient cells, we performed initial experiments in HTLV-I transformed thymocyte and CD4+ T cell lines which were established from patients deficient in ZAP-70 and normal individuals. To determine whether signaling could be reconstituted by ZAP-70 gene transfer, wild-type ZAP-70 was introduced into the HTLV-I-transformed deficient cell lines with a ZAP-70-expressing retroviral vector (G1ZAP70SvNa). Upon TCR stimulation, ZAP-70 was phosphorylated on tyrosine in reconstituted cells and was associated with the z chain of the TCR and the Lck PTK. Kinase activity of ZAP-70 in the reconstituted cells was appropriately upregulated by receptor aggregation and calcium flux, a measure of appropriate downstream TCR signaling, was restored[4]. Nevertheless, although transformation was useful in circumventing problems associated with the maintenance of ZAP-70-deficient T cells and low gene transfer levels, the presence of HTLV-I precluded any biological studies.

In order to continue this work in a system which is more "physiologic" than HTLVI-transformed T cell lines, we have recently modified a system developed by Hans Yssel and colleagues to grow primary T cells in culture using irradiated PBLs and an irradiated EBV transformed cell line in media containing human A/B serum, transferrin, fatty acids, ethanolamine and bovine serum albumin[5]. We have successfully expanded T cells from several new ZAP-70-deficient patients (obtained from Klaus Schwarz, Ulm, Germany) using this method. In two of these patients, we identified a novel mutation in the kinase domain resulting in an alanine to valine substitution at residue 507[6]. Interestingly, we found that the related protein tyrosine kinase Syk was expressed at 5-10 fold higher levels in all thymocyte and T cell lines lacking ZAP-70. Syk and ZAP-70 are both expressed at high levels in thymocytes but Syk expression is normally decreased 3-4 fold in peripheral T cells[7]. Despite the similarities between ZAP-70 and Syk, the presence of high levels of Syk was not sufficient to result in appropriate signaling in ZAP-70-deficient T cells following induction of the T cell receptor. Indeed, phosphorylation of Syk in primary ZAP-70-deficient T cells results in a differential activation of downstream signaling molecules with higher levels of calcium mobilization than that observed in control ($ZAP\text{-}70^{+}$) T cells, and a profound decrease in CD3-mediated IL-2 secretion and proliferation relative to control T cells[6]. The differences in the ensemble of signaling molecules stimulated by Syk and ZAP-70 suggest that their respective activation may modulate thymocyte differentiation and T cell activation.

The many advances in gene transfer technology were paramount in allowing us to successfully transduce these primary T cells derived from ZAP-70-deficient patients. Primary CD4+ T lymphocytes derived from normal individuals and ZAP-70-deficient patients were transduced with ZAP-70-retroviral vectors using optimized protocols. The levels of ectopic ZAP-70 in transduced patient T cells were approximately 2-3 fold higher than endogenous ZAP-70 levels in control T cells. Cells were expanded and assessed for EGFP expression at days 3, 40 and 60 post-transduction.

Importantly, we always observed a marked augmentation in the percentage of ZAP-70/EGFP-transduced patient T cells upon ex vivo expansion. In one representative gene transfer experiment, the percentages of Pt. 1 and Pt. 2 T cells transduced with ZAP-70/EGFP increased from 31 to 67% and 36 to 74%, respectively, following 2 months of in vitro culture. In contrast, the percentage of patient T cells expressing EGFP remained essentially unchanged following transduction with the control EGFP vector. Collectively, these results indicate that ZAP-70-transduced patient T cells have a strong selective growth advantage over their non-transduced counterparts. Importantly, this selective growth advantage of gene-corrected cells was associated with a gain-of-function of TCR-induced MAPK activation, IL-2 secretion and proliferation[8].

The feasibility of stem cell gene correction for human ZAP-70 deficiency was assessed using a ZAP-70 knock out model and a SCID/hu mouse model. ZAP-70-deficient murine bone marrow progenitor cells were transduced with a retroviral vector expressing the human ZAP-70 gene. Engraftment of these cells in irradiated ZAP-70-deficient animals resulted in the development of mature CD4+ and CD8+ T cells. In marked contrast, both populations were absent in ZAP-70-/- mice transplanted with BM progenitor cells harboring a control vector. Importantly, the reconstituted cells proliferated in response to T cell receptor stimulation. These ZAP-70+ T cells expressed Fas and CD25 activation markers at levels similar to those observed in control mice. Nevertheless, the ZAP-70-reconstituted mice differed from control mice in that there were significantly higher percentages of memory T cells in the former. Further work will be necessary to understand the basis for this difference. The presence of ZAP-70 in newly formed B cells did not modify lipopolysaccharide- and IL-4-mediated immunoglobulin isotype switching. In separate experiments, the effect of retrovirally-expressed ZAP-70 on intrathymic human T cell development was studied using SCID/hu (thymus/liver) mice. T cell differentiation proceeded normally when human CD34+ progenitor cells transduced with the ZAP-70-vector were engrafted into recipient mice. Altogether, these data indicate that retroviral-mediated gene transfer of the ZAP-70 gene may prove to have a therapeutic benefit for patients with ZAP-70-SCID.

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Genetic and molecular analysis of the mutant CD8 alpha molecule responsible for familial CD8 deficiency

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CD8 glycoproteins play an important role in both the maturation and function of MHC class I-restricted T lymphocytes. We have described an immunodeficient patient without any CD8+ lymphocytes. A 25-year-old man, from a consanguineous family, with recurrent bacterial infections and total absence of CD8+ cells, was studied. Antibody deficiencies and others immunodeficiencies affecting CD8 T cells as ZAP-70 and TAP defects were ruled out. Since the parents were consanguineous, the members of his family were analysed. Two sisters were also found to have a complete absence of CD8 cells. Conversely, high percentages of CD4-CD8-TCRalpha-beta T cells were found in the three CD8 negative siblings. The patient, its parents, the CD8-deficient siblings, and other brothers and sisters were studied further. Membrane and intracellular expression of CD8 molecules in T and NK lymphocytes and serum sCD8 concentration were below detectable limits in the three affected siblings. Mean fluorescence intensity of CD8 expression on cell surface and sCD8 were decreased in the parents and two brothers, but normal in the remaining siblings. Weak expression of CD8 was detected by Western blot analysis in Pumps of the patient and two CD8-deficient sisters; however, CD8beta membrane expression was absent. Messenger Ranks for CD8 alpha and beta were detected by RT-PCR at similar levels in the CD8-negative individuals, their relatives, and normal controls. Genetic studies of CD8 molecule were conducted in view of these results. A missense mutation (gly90 ser) in both alleles of the immunoglobulin domain of the *CD8 alpha* gene was shown to correlate with the absence of CD8 expression found in the patient and two sisters. The glycine at position 90 (gly90) is located in the immunoglobulin domain of the CD8 alpha chain and is conserved in all reported species, and also in the corresponding region of the closely related molecules CD8 beta and CD7. Site-directed mutagenesis and transfection studies were performed to establish a direct correlation between the mutation found in the CD8 immunoglobulin domain and the absence of any detectable CD8 antigen in the homozygous members of the family. The results demonstrate that the presence of serine at position 90 precludes CD8 expression. Similar experiments were performed with the murine CD8 alpha molecule (Lyt-2) with identical results. Chimeric molecules have been derived in order to analyse the transit and cellular localization of the mutant CD8 molecule. Functional characteristics and binding to HLA class I molecules will be examined. A novel autosomal recessive immunologic defect characterized by absence of CD8+ cells is described. These findings may help to further understanding of the role of CD8 molecules in human immune response.

SCID due to CD45 deficiency

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The hematopoietic-specific transmembrane protein tyrosine phosphatase, CD45 plays a pivotal role in signaling via lymphocyte antigen and cytokine receptors by regulating the activity of receptor-associated tyrosine kinases [1]. These include T- and B-cell antigen receptor-associated src kinases and cytokine receptor-associated Jak kinases [2, 3]. In experimental animals, CD45 deficiency is associated with severe T cell depletion due to impaired thymocyte expansion and positive selection. While B cell development is spared, antibody responses are compromised. In man, SCID due to loss of function mutations in the CD45 gene exhibits many of the features of experimental CD45 deficiency[4, 5]. It presents soon after birth with recurrent infections and failure to thrive associated with T cell depletion and failure of the residual T cells to respond to mitogens. The NK cell are also decreased in number while the B cell, whose development is spared, fail to develop germinal centers or sustain normal immunoglobulin production. These findings emphasize the pivotal role of CD45 in regulating lymphocyte development and function and identify CD45 deficiency as a novel cause of SCID in man.

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Linking antigen receptor signaling with B cell differentiation

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Early B lineage differentiation is dependent upon the expression of pre-B cell receptors (preBCR) composed of μ heavy chains, surrogate light chains (SLC) and Igα/Igβ transmembrane signaling elements. Since preBCR are expressed at remarkable low levels *in vivo*, we employed an enhanced immunofluorescence assay that can detect < 100 cell surface molecules per cell to determine precisely when SLC are expressed during B lineage differentiation. We found that preBCR were expressed only by a limited subpopulation of relatively large pre-B cells. The receptor-positive pre-B cells had downregulated expression of the recombinase activating genes, *RAG-1* and *RAG-2*. In contrast, their receptor-negative pre-B cell progeny were relatively small, expressed *RAG-1* and *RAG-2*, and exhibited selective downregulation of *VpreB* and *λ5*. Comparative analysis of the two pre-B cell subpopulations indicated that SLC gene silencing results in the coordinate loss of the preBCR expression, exit from the cell cycle and the light chain gene rearrangement required for B cell differentiation. Differential SLC expression thus governs pre-B cell differentiation, first through ligand interaction to promote clonal expansion. Subsequent extinction of preBCR expression removes the proliferative stimulus to allow the development of BCR-bearing B cells. Antigen-mediated triggering of the B cells can be enhanced or inhibited by a variety of immunoglobulin-like receptors. For example, five recently-identified Fc receptor homologs are differentially expressed by human B cells. The cytoplasmic domains of these new receptors possess activating motifs or inhibitory motifs or both, thereby inferring their participation in the fine tuning of B cell responses. Given that the signaling competencies of a variety of cell surface receptors can govern the progression of normal B lineage differentiation, deficiencies in these receptors or their downstream signaling elements may result in aberrant B cell development and either immunodeficiency or autoimmunity.

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Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development

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Developing B cells must pass a series of checkpoints that are regulated by membrane bound Ig-mu through the Ig-alpha/Ig-beta signal transducers. To determine how Ig-mu expression affects B cell development and antibody selection in humans we analyzed Ig gene rearrangements in pro-B cells from two patients that are unable to produce Ig-mu proteins. These patients suffer from primary immunodeficiencies and present a B cell blockage in the bone marrow at the pro-B cell stage. For one we have detected a complete deletion of the Ig-mu chain gene and for the other a nucleotide insertion in the CH1 domain of the Ig-mu gene, leading to the presence of a stop codon.

We found that lack of Ig-mu expression does not alter VH, D or JH segment usage and is not required for human Ig-kappa and Ig-lambda gene recombination or expression. However, IgH and IgL chains found in Ig-mu deficient pro-B cells differed from those in normal B cells in that they showed unusually long CDR3s. In addition, we demonstrated that the Ig-kappa repertoire in Ig-mu-deficient pro-B cells was skewed to downstream J-kappas and upstream V-kappas consistent with persistent secondary V(D)J rearrangements. Thus, Ig-mu expression is not required for secondary V(D)J recombination in pro-B cells. However, B cell receptor expression shapes the antibody repertoire in humans and is essential for selection against antibodies with long CDR3s.

Session 4: Defects of antigen presentation pathways
Chair: Luigi D. Notarangelo

TAP deficiency syndrome

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Seven adults with necrotizing granulomatous lesions in the upper respiratory tract and skin associated with recurrent bacterial respiratory infections and skin vasculitis were identified (1,2). A diagnosis of Wegener's granulomatosis was considered in all of them, but abandoned because of an incompatible disease course, and resistance to immunosuppressive treatments. As all seven patients were homozygous for the HLA locus, we looked for genetic defects located within the Major Histocompatibility Complex (MHC).

A severe reduction of HLA class I molecule surface expression was observed in all patients. In all patients we showed that defective expression of the Peptide Transporter (TAP) genes was responsible for the HLA class I down-regulation, and in two patients we identified a mutation in the TAP 2 gene responsible for the defective expression of the TAP complex.

Analysis of the patients' lymphocyte repertoire in the peripheral blood revealed an expansion of NK and γδ T cells in most patients (1). We demonstrated the presence of autoreactive Natural Killer (NK) cells and gamma-delta (γδμ T lymphocytes in two patients' peripheral blood cells. Correction of the genetic defect *in vitro* restored normal expression of HLA class I molecules and prevented self-reactivity of patients' cells. Histology of granulomatous lesions revealed the presence of a large proportion of activated NK cells.

Our findings define the aetiology and pathogenesis of a novel syndrome which affects patients with a defective expression of HLA class I molecules. The primary objective of therapy for patients with *TAP deficiency syndrome* is tight control of infections in order to prevent the development or progression of bronchiectasis and ultimately respiratory failure. The syndrome resembles Wegener's granulomatosis both clinically and histologically. Patients suffer from chronic necrotizing granulomatous lesions in the upper respiratory tract and skin, recurrent infections of the respiratory tract and skin vasculitis. The presence of a predominant NK population within the granulomatous lesions suggests that the pathophysiology of skin lesions may relate to the failure of HLA class I molecules to turn off NK responses. Our findings illustrate the general principle that an accurate genetic analysis of a defined syndrome can provide a better understanding of the aetiology and pathogenesis of a disease.

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Molecular basis of MHC class II deficiency

Walter Reith

Major Histocompatibility Complex class II (MHCII) molecules direct the development, activation and homeostasis of the CD4+ T helper cell population. They thus play a central role in key processes of the adaptive immune system, such as the generation of T cell mediated immune responses, the regulation of antibody production, and the development and maintenance of tolerance. Given these key functions it is not surprising that the absence of MHCII expression results in a severe primary immunodeficiency disease called MHCII deficiency, frequently also referred to as the Bare Lymphocyte Syndrome (BLS) (reviewed in Klein et al 1993).

The disease is genetically heterogeneous. Four genetic complementation groups corresponding to defects in four distinct genes have been defined. The genetic defects do not lie within the MHCII locus itself, but in four genes encoding transcription factors required for MHCII expression (reviewed in Reith and Mach 2001). These genes encode RFXANK, RFX5, RFXAP and CIITA (Steimle et al 1993, Steimle et al 1995, Durand et al 1997, Masternak et al 1998). The first three are subunits of RFX, a ubiquitously expressed factor that binds to the promoters of all MHCII genes. RFX binds cooperatively with other factors to form a highly stable macromolecular nucleoprotein complex referred to as the MHCII enhanceosome (Masternak et al 2000). This enhanceosome serves as a landing pad for the coactivator CIITA, which is recruited via protein-protein interactions (Masternak et al 2000). The enhanceosome complex and CIITA collaborate in activating transcription by promoting histone H3 and H4 hyperacetylation and by recruiting components of the general transcription machinery, such as TFIID and RNA polymerase II (unpublished data; reviewed in Harton and Ting 2000, Reith and Mach 2001).

CIITA is the master control factor for MHCII expression. Three independent promoters exhibiting differential cell type specificity and induction by interferon-γ control transcription of the CIITA gene. The highly regulated expression pattern of these three promoters ultimately dictates the cell type specificity, induction and level of MHCII expression (see for example Landmann et al 2001, Waldburger et al 2001; reviewed in Harton and Ting 2000, Reith and Mach 2001). The specificity of CIITA for MHCII genes, and its highly regulated pattern of expression, set CIITA apart from other known transcriptional co-activators. CIITA thus represents a paradigm for a novel type of gene specific and highly regulated transcriptional cofactor.

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Session 5: Defects of apoptosis pathways and other lymphocyte disorders
Chair: C.I. Edvard Smith

Primary defects in lymphocyte apoptotic pathways

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Lymphocyte cell death is a key event in the homeostasis of the immune system. Lymphocytes can be induced to die because of exposure to toxic agents, of cytokines withdrawal or because specific cell surface receptors are engaged by their ligands. A number of such receptors belonging to the TNF receptor family have been described in the recent past. Among them, the role of the Fas/ Fas ligand / receptor interaction in the induction of lymphocyte cell death has been enlightened by the study of natural mutants, firstly described in murine strains, then in humans.

Autoimmune Lymphoproliferative Syndrome (ALPS)

More than 30 years ago, Canale and Smith as well as others reported a condition characterised by non malignant lymphadenopathies associated with autoimmune features in children (1) It turned out that these patients and a number of newly described ones have a genetic disorder caused by mutations of the Fas encoding gene (2-4) The syndrome was also named autoimmune lymphoproliferative syndrome (ALPS) (5). Cases of more than sixty patients have now been reported in the literature (6-14).

ALPS is not unique as at least five types can be described, i-e.

a) ALPS caused by autosomal recessive Fas mutations (ALPS 0),
b) ALPS caused by autosomal dominant Fas mutations (ALPS Ia),
c) ALPS caused by autosomal dominant FasL mutations (ALPS Ib),
d) ALPS associated with defective lymphocyte Fas – mediated apoptosis, without Fas mutation (ALPS II).
e) ALPS associated with neither detectable lymphocyte Fas nor FasL- mediated apoptosis defect (ALPS III).

ALPS 0 and ALPS 1a

The ALPS 0 form of the disease is characterized by the pre- or neo- natal onset. So far, only a few patients have been described. In contrast, more than 60 patients carrying an heterozygous Fas mutation have been reported in the litterature (6-14). In these cases, onset of symptoms occurs in early childhood (0 to 5 years, around 6 to 12 months in most cases). Lymphoproliferation involved CD4 (-), CD8 (-), CD45 RA (+), HLA class II(+), CD57 (+) T cells. This subset accounts for 1 to 60% of the blood T cell counts. Polyclonal hyperimmunoglobulinemia G and A is a very frequent finding while level of serum IgM is usually reduced. Lymphocytes are insensitive (ALPS 0) or poorly sensitive (ALPS 1a) to the pro-apoptotic effect uf anti-Fas antibody (12). The magnitude of the defect (varies) from one patient to another.

Autoimmune manifestations are the second most frequent event in patients with ALPS Age at onset varies considerably (in contrast to the lymphoproliferative syndrome) (8, 9, 13, 15).It is therefore likely, that among reported patients, some who have not yet developed such manifestations, will do it later on. In the most case the autoimmunity involves the hematological lineage leading to peripheral cytopenia. In some of the patients autoimmunity tends to be severe, requiring aggressive immunosuppressive treatments including Steroids and Cyclophosphamide in order to be controlled. Splenectomy often performed because of discomfort and hypersplenism, was also, in some cases required because of protracted autoimmunity directed toward blood cells. In addition, Fas mutations represent a significant risk factor for lymphomas (16).

Genetic and molecular basis :

A variety of Fas gene mutations have been found associated with ALPS including mutations leading to truncated products or modified sequences (8, 9, 13). A low number of mutations affects the extracellular domain of Fas. It should be noted however that some relatives carrying the same heterozygous Fas mutation are no symptomatic despite in vitro abnormal Fas-mediated apoptosis. This strongly suggests that a second event (see below) should be associated to induce an overt ALPS 1a syndrome. It is likely that genetic rather than environemental factors, influence the ALPS expression, and account for the variable penetrance of some of the mutations.

ALPS Ib

Among a series of patients with systemic lupus, one, who also presented with chronic lymphoproliferation was shown to carry an heterozygous mutation in the Fas ligand gene (17). No other fasL deficient patient with ALPS has been detected so far.

ALPS II

A number of patients present with all typical clinical and immunological features of ALPS including abnormal lymphocyte Fas – mediated apoptosis. However, the Fas molecule expression and sequence are normal in these patients (18) (7)]. Wang et al have reported the occurrence in 2 families in whom caspase 10 mutations were found associated with ALPS (19). One of the mutation exerts a transdominant negative effect impairing Fas mediated cell apoptosis, while the second requires expression on both alleles to induce a full blown clinical picture.

ALPS III

We have investigated a number of patients (over 30) who present with a clinical condition close to mild ALPS, associated with hypergammaglobulinemia and excess of blood CD4-CD8- T cells (unpublished observation). Patients' lymphocytes exhibit a normal activation of the FasL / Fas apoptotic pathway. No molecular defect has been found so far. Although not demonstrated, it is plausible that in these patients, another lymphocyte apoptotic pathway is impaired. If this hypothesis turns out to be correct, this would lead to the conclusion that Fas-independent apoptotic pathway(s) is (are) important mediators of the control of lymphocyte homeostasis in humans. It would thus be important to determine which it is (they are).

Conclusion :

The recent unravelling of the molecular events leading to the ALPS syndromes in humans has brought a significant insight into the understanding of the function of the FasL / Fas pathway in peripheral tolerance. More questions are now raised about the physiological roles of the other lymphocyte apoptotic pathways and how other gene polymorphisms can combine to promote auto-immunity.

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Abnormal NK cell function in X-linked lymphoproliferative disease

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X-linked lymphoproliferative disease (XLP, MIM 308240) is a primary immune deficiency (PID) that is characterized by a unique proclivity to severe complications (including fulminant infectious mononucleosis or B-cell lymphomas) following infection by the Epstein-Barr virus (EBV). The gene that is defective in XLP maps on human chromosome X at q25 and encodes a 128 amino acid long intracytoplasmic polypeptide (*SH2D1A*, *SAP* or *DSHP*) characterized by a Src homology 2-containing (SH2) domain (1). We show that in normal NK cells SH2D1A binds to 2B4 (CD244) (2) and to a novel surface molecule termed NTB-A (3). NTB-A is a novel 60 kD surface molecule encoded by a gene located on human chromosome 1 close to that encoding CD84 (4). It is expressed on resting and activated lymphoid populations including NK, T and B lymphocytes. Functional analysis revealed that NTB-A, similar to 2B4, function as a co-receptor in the induction of NK cell-mediated cytotoxicity. Moreover, mAb-mediated masking experiments suggested that NTB-A may recognize cell surface ligand(s) expressed on EBV-infected B lymphocytes. Biochemical analysis reveals that NTB-A associates with SHP-1 and, upon tyrosine phosphorylation, with SH2D1A. As in the case of 2B4, recruitment of SH2D1A appears to displace SHP-1 from the NTB-A cytoplasmic tail that is characterized by two TxYxxV/I motifs. At variance with 2B4, the NTB-A cytoplasmic portion also contains a classical Immune Tyrosine-based Inhibitory Motif (ITIM) and, upon tyrosine phosphorylation associates with SHP-2. Analysis of NK cells from XLP patients reveals a dramatically altered function of both 2B4 and NTB-A. Indeed, due to the lack of SH2D1A, these molecules associate with SHP-1 and transduce inhibitory rather than activating signals that down-regulate cytotoxicity induced via the main triggering NK receptors (3). These are represented by the NKp46, NKp30 and NKp44 molecules collectively termed Natural Cytotoxicity Receptors (NCR) (5). As a consequence, XLP-NK cells are unable to kill EBV+ targets. Thus, in addition to the 2B4/CD48 interaction, another inhibitory signal is generated by the interaction between NTB-A and yet unknown ligand(s) expressed on EBV^+ cells. Indeed, a virtually complete restoration of XLP-NK cell cytotoxicity against EBV^+ cells is detected by the simultaneous mAb-mediated masking of both 2B4 and NTB-A molecules.

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The spectrum of immunodeficiency with hyper-IgM: defects in CD40L, CD40, AID and NEMO

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Development and maturation of antibody responses are regulated by both changes in the genetic programme of cells belonging to the B-lymphocyte lineage and interaction of B cells with other cell types and with soluble factors. While the first stages of B lymphocyte differentiation (up to generation of mature B cells) occur primarily in the bone marrow and are antigen-independent, terminal maturation of B lymphocytes is dependent on antigenic challenge and occurs in secondary lymphoid organs and in mucosa-associated lymphoid tissue (MALT).

Three events characterize terminal B cell differentiation: immunoglobulin isotype switching, somatic hypermutation, and memory B cell generation. These are all finalized to mount high-affinity and diversified antibody responses that can be sustained upon re-encounter with the antigen.

Over the past ten years, the molecular basis involved in these processes have been extensively investigated. In particular, cross-talk between B and T cells in the germinal center plays a major role in eliciting terminal B cell differentation. Interaction of CD40 (constitutively expressed by B cells) and CD40 ligand (CD40L), expressed mostly by activated CD4+ T cells, together with cytokine-mediated B cell activation, is a key factor in promoting immunoglobulin isotype switching, as indicated by analysis of patients with X-linked hyper-IgM (HIGM1). These patients are able to synthesize low-affinity IgM, but do not produce other isotypes. In addition, upon antigen re-challenge, they mount IgM-restricted antibody responses of low affinity, and also show a marked defect in memory B cell generation. We and others have shown in 1993 that HIGM1 results from mutations in the *CD40L* gene, that maps on the long arm of the X chromosome. Importantly, HIGM1 also suffer from opportunistic infections and neutropenia, suggesting that CD40L-CD40 interaction is also essential to T-cell immunity and myeloid cells homeostasis.

While most patients with hyper-IgM syndrome do have mutations in CD40L (and in many cases an obvious X-linked inheritance in apparent), it was well known that hyper-IgM syndrome can also occur in females, and that in some families it is inherited as an autosomal recessive or even as an autosomal dominant trait. Importantly, the clinical and immunological evaluation of most cases of autosomal recessive hyper-IgM syndrome (HIGM2) had shown some peculiarities that distringuish it from X-linked HIGM1. In particular, HIGM2 patients show a milder clinical course, mostly characterized by recurrent upper and lower respiratory tract bacterial infections, and markedly enlarged tonsils and adenoids. Immunohistochemical evaluation of these tonsils had shown markedly enlarged germinal centers, a striking difference vs. what seen in HIGM1, where no germinal centers are present. Through an International collaborative work, Durandy et al. have identified a novel gene, termed Activation-Induced cytidine Deaminase (*AID*), expressed in germinal centers B cells, that is mutated in HIGM2. This gene appears to be an essential component of the poorly characterized switch recombinase. In addition, it is also involved in somatic hypermutation, as indicated by the fact that HIGM2 patients and *AID*-targeted mice have a profound defect in somatic hypermutation.

More recently, we have identified a further subgroup of patients with autosomal recessive hyper-IgM (designed HIGM3), whose clinical features were more similar to HIGM1 than to HIGM2: these patients, in fact, present with early onset opportunistic infections and/or neutropenia. A detailed immunological analysis of these patients has shown that they present a defect of immunoglobulin isotype switching, somatic hypermutation, and memory B cell generation. We have shown that HIGM3 is due to mutations of the *CD40* gene, thus explaining the phenotypic similarities with HIGM1. Some of the clinical manifestations of HIGM3 may be explained on the basis of defective function of dendritic cells (that constitutively express CD40). Indeed, we have shown that dendritic cells from these subjects fail to undergo terminal maturation, and are inefficient in the stimulation of antigen-primed T-cell activation.

The spectrum of hyper-IgM syndrome has been enriched by yet another form, inherited as an X-linked trait, in which immunological disturbances associate with hypohydrotic ectodermal dysplasia. The disease is due to mutations of the *NEMO/IKK gamma* gene, that encodes for a regulatory component of the IKK complex, which is involved in activation and translocation of the NF-κB transcription factor. Importantly, this pathway is located downstream to CD40L/CD40 interaction and signaling. Thus, three forms of hyper-IgM syndrome are strictly inter-related. It remains to be seen whether activation of this pathway plays a role also in *AID* gene transcription.

From a practical point of view, following the identification of the molecular and cellular basis of the hyper-IgM syndrome, we are now able to provide adequate diagnosis (including prenatal diagnosis), prospect prognosis, and design patient-specific therapeutic strategies in this highly heterogeneous group of disorders.

The Wiskott-Aldrich syndrome protein (WASP): Its function and its relevance for the clinical phenotype

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The Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT) are caused by mutations of the WAS protein (WASP) gene. The clinical manifestations of the classic WAS phenotype consists of immunodeficiency, eczema and thrombocytopenia. The phenotype of WAS/XLT correlates with the effect of the WASP mutations on the function of the protein. As a rule, missense mutations within exons 1, 2 and 3 result in expression of mutated WASP, often at a reduced amount, and in a clinical phenotype designated as XLT (Scores 1 and 2). Nonsense mutations, deletions and insertions (resulting in frameshift and premature termination), splice site mutations and complex mutations result in lack of WASP or in truncated protein, causing the classic WAS phenotype (scores 3, 4 and 5). Autoimmune manifestations and malignancies (usually lymphoma) are rare in XLT and frequent in WAS.

The unique domains of WASP are responsible for its complex biological function. The PH domain of WASP interacts with membrane phospholipids, such as PIP2, and may facilitate the transfer of WASP from the cytoplasm to the cell membrane. Due to its reversible auto inhibitory contact between the GTPase-binding domain and the verprolin/cofilin homology domain and acid region, WASP can undergo functionally important conformational changes that are controlled by the activation/phosphorylation of Cdc42. The release of the carboxy terminal region facilitates the interaction of WASP with the Arp2/3 complex, a group of proteins that directly regulates the dynamic function of the acting cytoskeleton by inducing acting polymerization; this affects cell survival by contributing to cell cycle progression, cell division, filopodia formation and podosome assembly and disassembly. The interaction of WASP with WIP is important for signal transduction and acting polymerization in hematopoietic cells. In addition, WASP plays an important role in intracellular signaling of lymphocytes and platelets by interacting with the SH3 domains of selected cytoplasmic proteins.

Finally, lack of WASP is associated with increased expression of caspase-3 and Fas, and decreased expression of Bcl-2, resulting in accelerated cell death. To date, platelets are the only primary cells in which inducible tyrosine phosphorylation of WASP has been consistently demonstrated. Thus, WASP plays a central role, not only in acting polymerization and direct regulation of the cytoskeleton, but also in intra-cellular signaling and cell survival. The differential diagnosis of WASP includes IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked, a recently defined syndrome caused by mutation of FOXP3, a DNA binding protein; and GATA-1 which, if mutated, results in X-linked thrombocytopenia associated with a thalassemia phenotype; all three genes are closely linked on the X chromosome.

Identifying the proteins involved in secretory pathway by studying genetic diseases

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Secretory cells of the hemopoieitic lineage use an unusual secretory organelle which is a modified lysosome. Cells possessing this organelle use specialised machinery to both sort and secrete proteins. In order to identify the machinery involved in this specialised secretory pathway we are studying a series of genetic diseases in which secretory lysosome function is selectively disrupted. We use the cytotoxic T lymphocyte (CTL) as a model system and derive CTL from patients with Hermansky-Pudlak, Chediak-Higashi and Griscelli syndrome. In addition a number of mouse models of these diseases have also been examined. These studies reveal critical roles for Rab27a, Rab modifying enzymes and the adapter protein AP3 involved in protein sorting.

POSTERS

Fourteen years follow-up of an autoimmune patient lacking the CD3(subunit of the T-lymphocyte receptor)

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A familial defect of T cell receptor (TCR)/CD3 complex expression by T lymphocytes, due to a selective deficiency of the CD3gamma subunit, was reported in 1986. Two brothers (V and D) had inherited mutations in both the paternal and maternal CD3gamma genes that severely truncated the protein. V had very low counts of peripheral blood CD3+ cells, a severe combined immunodeficiency and autoimmune enteropathy with gut epithelial cell autoantibodies. He died at 32 months of age after severe infections and autoimmunity. Still living sibling D shared the same genotype regarding CD3gamma, but had milder laboratory immunological and clinical parameters.

D was born in 1981 and started with clinical symptoms at 12 months of age, including infrequent bacterial infections, vitiligo, atopic eczema and a mild dilated cardiomyopathy. Otherwise, D has had a normal development and life and is currently 19 years old.

In this study, the most relevant immunological parameters are summarised: the mean CD3+ cells percentage remains approximately 20% lower in D (ranging from 44% to 57%) than in normal controls (ranging from 60% to 80%) and the surface density of CD3 molecules per cell is four times lower in D than in normal controls. Serum autoantibodies were never found before June 1999, when anti-tyroglobulin (185 UI/ml; normal range=0-89 UI/ml) and anti-thyroid peroxidase autoantibodies (251 UI/ml; normal range=0-41 UI/ml) appeared, they were positive in January 2000 and have been maintained so until present. Several D alterations (vitiligo, DCM, specific thyroid autoantibodies) may certainly have an organ-specific autoimmune etiopathogeny. These autoimmune anomalies may be directly related to the CD8 cytotoxic/suppressor -CD4 lymphocyte disbalance and to the persistent high numbers of memory T cell clones (CD4+CD45R0+).

The redundant and overlapping functions among proteins of the immune system permit the existence of healthy but immunodeficient individuals, the best known being congenital C2-, IgA-, HLA class I- and adenosine deaminase-deficiencies. These results will be discussed within the context of the recently described CD3gamma-deficient mice.

Immune function is apparently sufficient for the survival of the CD3gamma lacking D patient, but continuing a close follow up will enable us to determine the real importance of adaptive vs non-adaptive immune mechanisms along individual's life and to study the mechanisms that relate autoimmunity and immunodeficiency.

WIP (WASP Interacting Protein) deficiency reveals a differential role for WIP and the actin cytoskeleton in T and B cell activation

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WIP stabilizes actin filaments and is important for filopodium formation. To define the role of WIP in immunity, we generated WIP-deficient mice. WIP-/- mice have normal lymphocyte development but their T cells fail to proliferate, secrete IL-2, increase their F-actin content, polarize and extend protrusions following T cell receptor ligation. In contrast, WIP-deficient B lymphocytes have enhanced proliferation and CD69 expression following B cell receptor ligation and mount normal antibody responses to T-independent antigens. Both WIP-deficient T and B cells show a profound defect in their subcortical actin filament networks. These results suggest that WIP is important for T cell activation and also regulates B cell activation

Characterization of *Herpesvirus saimiri*-transformed T lymphocytes from Common Variable Immunodeficiency patients

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INTRODUCTION. The term CVID is used to describe an incompletely defined syndrome characterized by defective antibody formation. Intrinsic MHC, monocyte, B and T cells defects have been reported. The description of X-linked hyper IgM syndrome as a T cell (CD154) defect, has rekindled the discussion about the role of these cells in CVID.

METHODS. We have used *Herpesvirus saimiri* (HVS) for the targeted transformation of CVID $CD4^+$ and $CD8^+$ T cells and subsequent functional evaluation by flow cytometry of their capacity to generate cell surface (CD154, CD69) or soluble (IL-2, TNF-α, IFN-γ) help after CD3 engagement.

RESULTS. Unexpectedly, the results showed that 40 different CVID blood samples exposed to HVS gave rise with a significantly increased frequency to transformed $CD4^+$ T cell lines, as compared to 40 age-matched controls (27% *vs* 3%, $p < 0.00002$) suggesting the existence of a CVID-specific signaling difference which affects $CD4^+$ cell transformation efficiency. The functional analysis of 10 $CD4^+$ and 15 $CD8^+$ pure transformed T cell lines from CVID patients did not reveal any statistically significant difference as compared to controls. However, half of the $CD4^+$ transformed cell lines showed CD154 (but not CD69) induction (mean value of 46.8%) under the lower limit of the normal controls (mean value of 82.4%, $p<0.0001$). Exactly the same five cell lines showed, in addition, a significantly low induction of IL-2 ($p<0.04$), but not of TNF-α or IFN-γ. None of this differences were observed in the remaining $CD4^+$ cell lines or in any of the transformed $CD8^+$ cell lines.

CONCLUSION. We conclude that certain CVID patients show selective and intrinsic impairments for the generation of cell surface and soluble help by $CD4^+$ T cells, which may be relevant for B lymphocyte function. The transformed T cell lines will be useful to establish the biochemical mechanisms responsible for the described impairments.

Activation-induced inmmunodeficiency: a CD4+ T lymphocyte defect of HIV-infected individuals

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Observation of the changes of immune cellular activation that occur after infection with HIV could provide information of the pathogenesis of immunodeficiency and could be useful in the understanding of the causes of some inherited immune disorders. We performed a prospective follow-up lasting 54 months in a cohort of 85 HIV-IDUs to assess which of the lymphocyte activation antigen changes in CD4 T cells can provide additional prognostic value for the development of AIDS. CD4+ activation was associated with subsequent decline of CD4+ cell levels. Using Cox multivariate regression analysis adjusted for both CD4+ cells and plasma HIV-1 RNA levels, we found that the increase in the percentage of CD4+CD7- and CD4+CD38+DR+ T cells retained additional predictive value (RH 3.81, 95% CI 1.19-12.17, $p = 0.02$ and RH 1.07, CI 1.04–1.11, $p < 0.0001$, respectively). Spearman correlation between levels of CD4+CD7- and CD4+CD38+DR+ T cells, CD4+CD7- T cells and CD4+ T cell count were 0.51 ($p<0.0001$) and –0.42 ($p<0.0001$), respectively. Congenital deficiency of CD7 expression is reported to be associated with a clinically severe combined immunodeficiency (1). It has been suggested that cellular immune activation itself is an additional contributing factor to subsequent disease progression (2). Increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression (3).

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Phenotypic correction of T cells from fanconi anemia patients using retroviral vectors

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Fanconia Anemia (FA) is a genetic recessive chromosomal instability syndrome (CIS) with at least 7 distinct genes implicated in the disease. Cells of FA patients show marked defects in apoptosis and DNA repair to crosslinking agents such as Mytomicine C (MMC) and diepoxybutane (DEB). Aiming of subtyping the population of spanish FA patients, peripheral blood T cells were transduced with retroviral vectors encoding the *EGFP* (as a marker), *FANCA, FANCC* and *FANCG* cDNAs (accounting for about 90% of all FA patients) and then exposed to increasing concentrations of MMC. So far, we have analyzed T cell samples from 32 patients. In 27 cases (84.4%), samples showed the characteristic hypersensitivity to MMC and were evaluated for retroviral complementation. Current data show that *FANCA* retroviral vectors corrected the MMC-hypersensitivity in 22 out of the 27 MMC-sensitive samples (81.5%). In one patient (3.7%), *FANCG* vectors reversed the MMC sensitivity, while in 4 patients (14.8%) no correction was observed. Samples from two of these patients were then transduced with a retroviral *FANCF* vector, but neither this vector was able to reverse their MMC-hypersensitivity, being these patients provisionally classified as non-*FANCA/C/F/G* FA patients. In a number of FANCA patients, the chromosomal stability of untransduced and *FANCA*-transduced LCLs was studied. As deduced from the cytogenetic analyses of samples exposed to DEB, a significant improvement in the chromosomal stability of these samples was obtained as a result of the insertion of the *FANCA* cDNA sequence. Further patients are being studied, aiming to characterize the involvement of the different *FANC* genes within the Spanish population of FA patients.

Identification of a myeloid intrathymic pathway of dendritic cell development marked by expression of the GM-CSF receptor

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In this study, the finding that a significant proportion of all dendritic cells (DCs) resident *in vivo* in the human postnatal thymus displayed a myeloid-related phenotype prompted us to re-examine the developmental origin of thymic DCs, a cell type hitherto considered to represent a homogeneous lymphoid-derived population. We show here that these novel intrathymic DCs are truly myeloid, as they arise from $CD34^+$ early thymic progenitors through $CD34^{lo}$ intermediates which have lost the capacity to generate T cells, but display myelomonocytic differentiation potential. We also demonstrate that phenotypically and functionally equivalent myeloid precursors devoid of T cell potential do exist *in vivo* in the postnatal thymus. Moreover, although IL-7 supports the generation of such myeloid intermediates, we show that their developmental branching from the main intrathymic T cell pathway is linked to the upregulation of the myelomonocytic GM-CSF receptor, to the downregulation of the IL-7 receptor and to the lack of pre-TCRα (pTα) gene transcriptional activation. Taken together, these data challenge the current view that the thymus is colonized by a lymphoid-restricted progenitor and provide evidence that a more immature precursor population with lymphoid and myelomonocytic potential is actually seeding the human postnatal thymus.

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A role for CD3g in thymocyte selection

Edgar Fernández

T cell development in CD3gd double deficient mice is completely arrested at the CD4-CD8-CD44-CD25+ stage. Introduction of a transgenic human CD3d in these mice (hereafter referred to as g-/- mice) restored the generation of CD4+CD8+ double positive cells and T cell receptor (TCR) expression, thus allowing by the first time the analysis of the specific role of CD3g in thymocyte positive and negative selection. Numbers of mature SP thymocytes were consistently reduced in g-/- mice compared with wild-type animals, suggestive of an alteration in thymic selection events. To test this in more detail, transgenic TCRs were introduced onto the CD3g deficient background. A severe defect in negative selection was observed in HY-TCR transgenic males lacking CD3g, that correlated with a markedly decreased sensitivity of non-TCR transgenic g-/- thymocytes to apoptosis induction by anti-TCR antibodies both in vitro and in vivo. Thymocytes expressing either class I- or class II-restricted TCRs were positively selected in mice lacking CD3g, albeit with severely diminished efficiency compared with g+/+ mice. Taken together, these data indicate that CD3g plays a critical role in coupling TCR signalling to positive and negative selection of thymocytes.

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Lentiviral-mediated gene transfer into umbilical cord and adult CD4+ T cells is differentially regulated by the IL-7 cytokine

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Gene therapy protocols involving the transfer of genes that are required for T cell development and activation have potential relevance for patients with genetic and acquired immunodeficiencies. While we and others have achieved high levels of retroviral-mediated gene transfer in T lymphocytes, transduction is almost always performed after T cell receptor (TCR) stimulation. The ability to transfer genes into resting T cells stimulated solely with cytokines is vital in situations where TCR activation is defective, as in many genetic severe combined immunodeficiencies. Furthermore, TCR stimulation results in a loss of the naïve subset; all cells acquire markers associated with the memory T cell subset. Importantly, gene transfer into the naive T cell subset is necessary for any protocol targeting neontatal or umbilical cord (UC) T cells as the vast majority of these lymphocytes are naive. We have therefore compared gene transfer into naive and memory T cells following stimulation with IL-7, a cytokine which promotes T cell survival without inducing either the expression of activation markers or a change in the phenotype of naive T cells. Here we show that IL-7 has distinct regulatory effects on adult peripheral blood (APB) and UC CD4+ lymphocytes. Neither naive nor memory APB CD4+ cells proliferated in response to IL-7 while naive UC CD4+ lymphocytes underwent multiple divisions. Nevertheless, both naive and memory IL-7-treated APB T cells progressed into the G_{1b} phase of the cell cycle, albeit at higher levels in the latter subset. Progression through the G_{1a}/G_{1b} checkpoint of the cell cycle has been shown to be necessary for completion of HIV-1 reverse transcription in T cells stimulated via the TCR. Intriguingly though, the IL-7-treated memory CD4+ lymphocyte population was significantly more susceptible to infection with an HIV-1-derived vector than dividing CD4+ UC lymphocytes. However, activation through the TCR rendered UC lymphocytes fully susceptible to HIV-1-based vector infection. These data demonstrate that cell cycle progression is not an absolute gauge of the susceptibility of a cell to lentiviral transduction. Rather, the global activation state appears to regulate HIV-1-based gene transfer in T cells. These results have significant implications for the development of T cell-based gene therapy protocols in the absence of ex-vivo TCR stimulation. Moreover, these data support a view of naive UC and APB lymphocytes as functionally disparate. It is of crucial importance to further elucidate the unique characteristics of neonatal T cells.

Adverse effects of post-thymic gene transfer revealed by correction of human CD3γ-deficient mature T lymphocytes

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The CD3 subunits of the T-cell receptor/CD3 complex (TCR/CD3) help to regulate surface TCR/CD3 expression, and participate in signal transduction leading to intrathymic selection and peripheral antigen recognition by T lymphocytes. Humans who lack individual CD3 chains show impairments in the expression and activation-induced downregulation of TCR/CD3, and the defective immune responses that result may be lethal. We have investigated delivery of a normal CD3 chain to treat disorders of this type. Retroviral transduction of CD3γ into CD3γ-deficient peripheral blood T lymphocytes from two unrelated patients selectively corrected the observed TCR/CD3 expression and downregulation defects, but unexpectedly also disrupted IL-2 (but not IFN-γ) synthesis, which became constitutive. In addition, the reconstituted T cells specifically caused cell death of untransduced autologous T lymphocytes, most likely by an autoreactive recognition mechanism. These data suggest that gene transfer into post-thymic lymphocytes carrying mutations on T-cell recognition or activation pathways may disrupt their intrathymic calibration and become harmful to the host.

IPEX is caused by mutations of the human *scurfy* (FOXP3) gene

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Immune deficiency/dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX), a fatal congenital disorder has been mapped to chromosome Xp11.23-Xq13.3. Murine scurfy shares phenotypic features with IPEX and maps to a region of conserved synteny on the mouse X-chromosome. The *scurfy* (*Foxp3*) gene and the human orthologue (*FOXP3*) have been cloned, revealing a novel forkhead DNA binding protein. Murine scurfy due to a 2 base pair insertion upstream of the forkhead domain, is a congenital X-linked lethal disorder characterized by wasting, infections, scaly skin, diarrhea, anemia and thrombocytopenia. Leukocytosis and lymphadenopathy are characteristic and $CD4^+$ T cells secrete excessive cytokines. To test the hypothesis that mutations of the human *FOXP3* gene are the cause of IPEX we examined 7 unrelated IPEX families and found seven novel mutations including deletions and missense and nonsense mutations affecting the forkhead domain, the termination codon, or mRNA stability. The study of these families demonstrate that IPEX is due to mutations of the *FOXP3* gene.

Griscelli Disease: genotype-phenotype correlation in an array of clinical heterogeneity

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Griscelli disease (GD) is a rare autosomal recessive disorder characterized by diffuse pigmentary dilution and cellular immune deficiency leading to increased susceptibility to certain infections and development of a life-threatening hemophagocytic syndrome (HS). Recently, two closely linked genes (MYO5A and RAB27A) on human 15q21 region encoding myosin-Va and a Rab protein (Rab27a), respectively have been found to be responsible for the Griscelli disease.Here, we report the clinical presentation, laboratory features, and outcomes classified according to the gene defect, of 16 unrelated patients with Griscelli Disease who were evaluated and followed at a single center over 14 years.

Mutation analysis was performed in 12 patients, eight of whom showed homozygous mutations in the RAB27A gene. MYO5A gene mutations were identified in two unrelated patients displaying primary neurological impairment. Based on the findings in our patients, it seems that there are four types of GD which differ in clinical severity, development of HS and outcome.

Group 1 (patients with mutation in RAB27A gene): 149delG is the most common mutation and associated with the most severe form of the disease with early entrance into the HS and rapid progression. Children with other mutations in RAB27A, C346T and T217G, presented with a relatively later entrance into HS and with later entrance to HS and good response to chemotherapy respectively.

Group 2 (patients with MYO5A deficiency): These patients presented with primary neurological impairment in the absence of infection susceptibility and HS.

Group 3: There is no known mutation in RAB27A gene and the onset of HS is as late as at 8 years of age.

Group 4: There is no known mutation in RAB27A gene and the patients have only typical pigmentary hair changes in the absence of primary neurological symptoms or development of HS with a prolonged life span.

This genotype-phenotype correlation suggests that the natural course of the disease and outcome is dictated by the site and type of the genetic mutation.

Pyk2 regulatory function on MTOC translocation and T cell response through an adapter role for Fyn during superantigen stimulation

David Sancho, María Montoya, Alicia Monjas, Takuya Katagiri, Mónica Gordón, Diana Gil, Reyes Tejedor, Balbino Alarcón and Francisco Sánchez-Madrid

The relocation of kinases during binding of a T cell to an appropriate antigen-presenting cell (APC) is essential for lymphocyte activation. Immunofluorescence and live fluorescence microscopy showed that the proline-rich tyrosine kinase-2 (PYK-2) is rapidly translocated to the cell-cell contact area upon T cell specific recognition of superantigen pulsed APCs. The activation with anti-CD3-coated latex microspheres was sufficient for Pyk2 reorientation, and CD28 co-engagement gave an additive effect. The absence of functional Lck, but not of ZAP-70, prevented TCR-mediated Pyk2 rearrangement but not its activation. Further, analysis of chimeric molecules of CD8 containing in its cytoplasmic region one immunoreceptor tyrosine-based activation motif (ITAM) from TCR-zb or CD3e, or a non-functional ITAMe demonstrated that lck-mediated Pyk2 translocation and activation required the presence of at least one intact ITAM. In addition, Pyk2 colocalized with Fyn at the MTOC of T cells. Overexpression of a kinase-dead mutant of Pyk2H did not affect TCR-induced Pyk2/MTOC translocation, suggesting that Pyk2 reorientation was independent of its activation. In contrast, a mutant Pyk2H-Y402F, which associates normally with the MTOC but is unable to bind Fyn, significantly inhibited MTOC complex translocation and IL-2 production, but not the induction of CD69 expression in response to superantigen stimulation. Therefore, Pyk2 plays a critical role in T cell activation by coupling Fyn to the MTOC complex, which mediates the early reorientation of the MTOC and its associated signaling complex towards the APC.

RFX complex containing mutated RFXANK protein binds to MHC II promotor but does not lead to MHCII expression

Wojciech Wiszniewski

MHC class II deficiency or bare lymphocyte syndrome is a combined immunodeficiency caused by defects in MHC specific regulatory factors that control MHC II expression at transcriptional level. MHC II expression is controlled at least by four trans-acting genes: CIITA, RFXANK, RFX5 and RFXAP. RFXANK encodes a subunit of the tripartide RFX transcription complex that functions in the assembly of multiple transcription factors on MHC II promoters. It has four ankyrin repeats important for interaction with RFXAP and CIITA. So far seven different RFXANK mutations have been reported in 26 unrelated patients. The most frequent mutation – deletion of 26 bp (752delG-25) was identified in 17 patients. The other mutations are nonsense or splice site mutations leading to proteins lacking all or part of the ankyrin repeat region. Here we report two novel point mutations of RFXANK gene: D121V and R212X found in two families with BLS and additional studies on tyrosine residues 224Y and 235 Y located in fourth ankyrin domain. We found D121V allele is expressed in vitro but is unable to form, with other factors, stable RFX complex. The experimental mutant Y224A is able to form RFX complex which binds to the MHC II promoter in vitro but cannot reverse BLS phenotype. These data the importance of the D121 and Y224 residues deduced from modelling studies.

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The lectures summarized in this publication were presented by their authors at a workshop held on the 3rd through the 5th of December, 2001, at the Instituto Juan March.

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