Lymphocytes in action

Symposium on Lymphocyte Activation and Signalling

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Regulation of Th17-cell differentiation

The newly defined proinflammatory IL-17-expressing T cells, known as T-helper (Th) 17 cells, have important roles in autoimmune diseases and immunity (reviewed in Ivanov *et al*, 2007). Recent findings have shown that Th17 cells differentiate from naive T cells through a programme that is distinct from those of Th1 and Th2 cells (Ivanov *et al*, 2007). Differentiation of Th17 cells is directed by a crucial lineage-specific transcription factor, ROR- γ t, in collaboration with STAT3, in response to cytokines. A combination of IL-6 and TGF- β is sufficient for the generation of Th17 cells *in vitro*; however, *in vivo*, the Th17-cell response requires an additional cytokine, IL-23, presumably for the expansion or survival of committed Th17 cells. D. Littman (New York, NY, USA) and co-workers have generated a ROR- γ t-GFP reporter mouse strain by knock-in (Eberl *et al*, 2004); in these mice, ROR- γ t is expressed in both T cells and lymphoid tissue-inducer (LTi) cells, which are mediators of lymphoid organogenesis. In normal, unimmunized mice, Th17 cells are most abundant in the intestinal lamina propria; however, these intestinal Th17 cells are markedly reduced in ROR- γ t-deficient mice (Ivanov *et al*, 2006). T cells from ROR- γ t-knockout (KO) mice are unable to express IL-17 and their pathogenic capacity is greatly reduced when tested in induced autoimmune-disease models. Consistent with the *in vitro* data, Th17 cells are not detected in an IL-6-KO background. In addition, mice that are treated with antibiotics or kept under germ-free conditions do not develop Th17 cells. Therefore, the development of Th17 cells requires resident gut microflora and IL-6. TGF- β alone is known to induce both FOXP3 and ROR- γ t in TCR-stimulated T cells, and TGF- β is required for the differentiation of both regulatory T cells (T_{REG}) and Th17 cells. However, the parameters that direct T_{REG}-cell compared with Th17-cell differentiation have so far remained unclear. Littman showed that high concentrations of TGF- β facilitate FOXP3-mediated T_{REG}-cell differentiation by repressing IL-23-receptor expression, whereas low concentrations of TGF- β in combination with proinflammatory cytokines (such as IL-6, IL-21 and IL-23) favour Th17-cell differentiation by promoting IL-23-receptor expression (Zhou *et al*, 2008). Littman also showed that FOXP3 can inhibit the function of ROR- γ t, at least in part, by physical interaction. Consistent with this finding, a subset of T cells in the lamina propria that coexpressed FOXP3 and ROR- γ t produced less IL-17 than T cells that expressed ROR- γ t without FOXP3.

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See Glossary for abbreviations used in this article.

Introduction

Around 280 immunologists attended the Keystone Symposium on Lymphocyte Activation and Signaling, which provided a venue for leading scientists to present their most recent findings. A wide range of immunological topics was covered such as the development and differentiation of T and B lymphocytes, signal-transduction mechanisms of immune cells, antibody gene diversification and lymphomagenesis, and the genetic basis of immunological disorders. Here, we summarize the main themes that emerged during the meeting.

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Glossary

AID	activation-induced deaminase
APOBEC1	apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide 1
BIRC4	baculoviral IAP repeat-containing 4 protein
EAT2	Ewing's sarcoma-activated transcript 2
ERT	EAT2-related transducer
FOXP3	forkhead box P3 transcription factor
GFP	green fluorescent protein
IFN- γ	interferon- γ
IgE	immunoglobulin E
IL-17	interleukin-17
NFAT	nuclear factor of activated T-cells
NF- κ B	nuclear factor- κ B
NURD	nucleosome remodelling and histone deacetylase
RAG	recombination-activating gene
ROR- γ t	retinoic acid-related orphan receptor- γ t
SLAM	Signalling lymphocyte-activation molecule
STAT3	signal transducer and activator of transcription 3
STIM	stromal cell-interaction molecule
TCR	T-cell receptor
TGF- β	transforming growth factor- β
XIAP	X-linked inhibitor of apoptosis protein
XRCC4	X-ray repair cross-complementing group 4

On the basis of these results, Littman proposed a model in which T cells in the lamina propria initially have the potential to become T_{REG} or Th17 cells, and their lineage commitment is determined by the microenvironment (Fig 1).

Along with ROR- γ t, STAT3 has been shown to have the following crucial roles in the differentiation of Th17 cells at various stages: transactivation of the *IL-21* gene in response to IL-6 (initial phase) and IL-21 (during an autocrine feedback loop); enhancement of IL-23 responsiveness by inducing the IL-23 receptor; and production of IL-17. This idea has been consolidated by two findings. First, A. Rao (Boston, MA, USA) showed that a plant alkaloid originally identified as an inhibitor of TGF- β signalling actually inhibits Th17-cell differentiation by inhibiting STAT3 phosphorylation in the late phase of the process *in vitro*. T cells expressing a constitutively active form of STAT3 were insensitive to the compound. Second, Y. Minegishi (Tokyo, Japan) and his group analysed the genetic basis of type I hyper-IgE syndrome (HIES), and showed that it is caused by sporadic dominant-negative mutations in the *STAT3* gene (Minegishi *et al*, 2007). Apart from high levels of serum IgE and atopic dermatitis, type I HIES patients are susceptible to bacterial and fungal infections. Recent findings have shown that T cells from type I HIES patients have a selective defect in the production of IL-17, but not of other inflammatory cytokines (Milner *et al*, 2008). The STAT3-mutant T cells from the patients were unable to differentiate into Th17 cells *in vitro*, showing impaired expression of ROR- γ t. These findings suggest that STAT3 might be a good target for Th17-cell-mediated immunity or immunopathology. However, it should be noted that type I HIES patients have skeletal abnormalities, suggesting that STAT3 inhibition might affect the skeletal system.

T_{REG} cells in immunity and tolerance

Most, but not all, FOXP3⁺ T_{REG} cells express CD25, and a subset of CD25⁺ T cells comprises FOXP3-negative effector T cells without T_{REG} function. Therefore, *in vivo* depletion of CD25⁺ T cells by

the injection of CD25 monoclonal antibodies was neither complete nor selective. To achieve a more reliable means of depleting FOXP3⁺ T_{REG} cells, A. Rudensky (Seattle, WA, USA) and colleagues generated a knock-in mouse strain in which human diphtheria toxin receptor (DTR) is expressed in FOXP3-positive T_{REG} cells (Kim *et al*, 2007). They showed that T_{REG} cells can be acutely depleted near completion in DTR mice by injecting diphtheria toxin. By using this method, they found that the deletion of T_{REG} cells in neonatal mice recapitulates the phenotypes of the *scurfy* mouse. When adult mice were depleted of T_{REG} cells by this regimen, there was an enormous expansion of the myeloid cell population, revealing the crucial role of T_{REG} cells in myeloid cell homeostasis. Rudensky also provided a new insight into the role of T_{REG} cells in local antiviral immune responses (Lund *et al*, 2008). Depletion of T_{REG} cells during intra-vaginal infection with herpes simplex virus 2 (HSV-2) increased viral titres in the vagina and the central nervous system, leading to more severe neurological damage and increased mortality. He showed that this result is due to the increased production of multiple chemokines by non-T cells in the draining lymph nodes, which, in turn, delays the migration of plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells into the vaginal tract where the viruses replicate. The reduced and delayed pDC and NK-cell responses allow HSV-2 to replicate in the vaginal tract in an uncontrollable manner, and to spread into the central nervous system. Therefore, T_{REG} cells can, paradoxically, contribute to an effective immune response against local infection with HSV-2.

It has been shown that FOXP3 and NFAT cooperate to regulate the expression of FOXP3 target genes (Rudensky *et al*, 2006). Therefore, abrogation of the sustained Ca²⁺ signal (upstream of NFAT) should impair development and/or the function of FOXP3⁺ T_{REG} cells. The Rao group ablated genes encoding STIM1 or STIM2, individually or in combination, in mice (Oh-Hora *et al*, 2008). STIM1 and STIM2 detect the depletion of intracellular Ca²⁺ storage and activate calcium release-activated calcium channels (CRACs) to allow the entry of extracellular Ca²⁺. T cells lacking either STIM1 or STIM2 showed varying degrees of impairment in CRAC activities. Both knockouts led to severe defects in the nuclear translocation of NFAT and cytokine production. These results confirm the overlapping roles of STIM1 and STIM2 in calcium signalling and T-cell activation. However, the T-cell-specific knockout of both STIM1 and STIM2 caused lymphoproliferative phenotypes owing to a selective impairment of T_{REG}-cell development. A few FOXP3⁺ T_{REG} cells did appear in the periphery, but they did not show any suppressor functions. These *in vivo* findings strongly support the idea that FOXP3–NFAT cooperation is crucial for the development and function of FOXP3⁺ T_{REG} cells.

The intestinal mucosa is a unique environment in which the immune system must tolerate antigenic challenges from the intestinal commensal flora and food antigens, while still fighting against pathogens. F. Powrie (Oxford, UK) described a role in mucosal tolerance for an intestinal DC subset that expresses CD103 (also known as α _E β , integrin or E cadherin; Coombes *et al*, 2007). Compared with T cells stimulated with CD103⁺ DCs, T cells activated by CD103⁺ DCs produced less IFN- γ , expressed higher levels of gut-homing chemokine receptor CCR9 and were more likely to become T_{REG} cells. The enhanced generation of T_{REG} cells was, in part, due to a high level of retinoic acid produced by CD103⁺ DCs. Powrie also described the role of IL-23 in inflammatory bowel disease (IBD; Izcue *et al*, 2008). IL-23 promotes Th1 and Th17 responses, and suppresses T_{REG}-cell development

Fig 1 | Differentiation of Th17 cells and inducible T_{REG} cells. Naive T cells express low levels of FOXP3 and ROR- γ t on stimulation in the presence of TGF- β , presumably maintaining the potential to develop into either Th17 or T_{REG} cells. Microenvironments with high levels of TGF- β , IL-2 and retinoic acid favour FOXP3-expressing T_{REG} cells, whereas a low concentration of TGF- β with high levels of IL-6, IL-21 and IL-23 promotes Th17 cells. IL-2, IL-6, retinoic acid and IL-23 have opposing effects on Th17 compared with T_{REG} cells. STAT3 has crucial roles at the initial programming (by IL-6), as well as at the autocrine positive-feedback stage (by IL-21) of the Th17 pathway. Ag, antigen; DC, dendritic cell; FOXP3, forkhead box P3 transcription factor; IFN- γ , interferon- γ ; IL-2, interleukin-2; iT $_{\text{REG}}$, inducible regulatory T cells; ROR- γ t, retinoic acid-related orphan receptor- γ t; STAT3, signal transducer and activator of transcription 3; TGF- β , transforming growth factor- β ; Th17, T-helper 17; T_{REG} , regulatory T cells.

and/or function. IBD was not induced in IL-23-deficient animals; however, in the absence of T_{REG} cells, IL-23 is not required for the induction of IBD. On the basis of these data, Powrie proposed that the main role of IL-23 in IBD is to overcome T_{REG} -cell-mediated immunosuppression.

Regulation of class-switch recombination

During an immune response, B cells in germinal centres undergo somatic hypermutation (SHM), by which point mutations are introduced at a high rate into the variable regions of expressed immunoglobulin genes, allowing for the selection of B cells with higher antibody affinities. Mature B cells also undergo class-switch recombination (CSR) to rearrange the DNA segments encoding the constant region of the antibody, so that the B cells change antibody isotypes from IgM to IgG, IgA or IgE (Fig 2). CSR occurs within large, repetitive G/C-rich sequences, known as S regions, which are parts of germline-transcription units termed 'C_H genes'. Each C_H gene comprises promoters, S regions and individual IgH constant-region exons (reviewed in Chaudhuri *et al*, 2007).

The B-cell-specific enzyme, AID, has an essential role in both CSR and SHM (Muramatsu *et al*, 2000; Revy *et al*, 2000). Although the function of AID in CSR and SHM was the subject of considerable debate at the symposium, the current view is that AID functions as a DNA deaminase that catalyses the deamination of cytidine residues in transcribed S regions and V genes during CSR and SHM, respectively (Chaudhuri *et al*, 2007). T. Honjo (Kyoto, Japan) and colleagues, however, upheld their original hypothesis that AID acts not on DNA but on RNA. Their view, based on the homology of AID to the RNA editing enzyme APOBEC1, is that AID edits an unknown messenger RNA, which subsequently encodes a protein—possibly an endonuclease—essential for CSR and SHM (Muramatsu *et al*, 2000). To

provide evidence against the DNA-driven model, V. Shivarov (Kyoto, Japan) from the Honjo laboratory presented data that uncoupled the DNA-deamination activity of AID from its ability to mediate CSR. He showed that several AID mutants—especially an N51A mutant—which were expressed in an *in vitro*-translation system had significantly reduced *in vitro* DNA-deamination activity, but could still mediate notable levels of CSR. The lack of correlation between DNA deamination and CSR activity was presented as evidence against the DNA-deamination model. The next step in these studies will surely be the identification of mRNA targets of AID.

Germline transcription through S regions has an essential role in CSR; however, the molecular mechanisms and factors that regulate this transcription remain poorly understood. M. Sellars (Illkirch, France) presented evidence that the transcriptional repressor Ikaros has a crucial role in CSR by regulating S-region transcription. Splenic B cells taken from mice with a hypomorphic mutation in the *Ikaros* gene (*IK^{L/L}*), which leads to a reduction of Ikaros expression to 10% of the wild-type level, underwent CSR to IgG2b and IgG2a at threefold to tenfold higher levels than wild-type cells. Increased CSR is probably due to a tenfold or higher upregulation of Sy2b and Sy2a germline transcripts. Consistent with the fact that Ikaros associates with the histone deacetylase complex NURD and that histone H3 acetylation correlates with S-region transcription, chromatin-immunoprecipitation experiments showed that H3 acetylation in promoters of Sy2b and Sy2a was increased threefold to fourfold in *IK^{L/L}* cells compared with wild-type cells. Ikaros was found to be associated with the S-region promoters and the IgH 3' regulatory sequence, indicating that it might function by interacting directly with these elements and recruiting the deacetylase complex. These results identify Ikaros as a novel and crucial regulator of CSR, which functions by repressing transcription at Sy2b and Sy2a.

Fig 2 | Mechanisms of immunoglobulin class-switch recombination. Transcription through S regions (that are G-rich on the non-template strand) generates RNA:DNA hybrid structures such as R-loops in which the RNA (orange line) hybridizes stably to the template strand, looping out the non-template strand as single-stranded DNA (step 1). AID deaminates cytidines to uridines on single-strand DNA (step 2). The DNA-glycosylase, UNG, removes uracil bases from DNA to create abasic sites that are subsequently processed by APE1 to generate nicks. Mismatch-repair proteins can also process deaminated residues, and generate nicks and gaps in the DNA. Closely spaced nicks on opposite strands generate DSBs (step 3). The mechanism that deaminates the template strand is not well understood. DSBs on two S regions are synapsed and joined both by components of the classical non-homologous end-joining proteins and by a robust microhomology-mediated alternative end-joining pathway (step 4). The activity of AID is regulated at several levels. AID phosphorylated at Ser 38 interacts with the single-strand DNA-binding replication protein A to promote CSR (step 5). MicroRNA 155 (miR-155) binds to the 3' untranslated region (UTR) of AID messenger RNA (mRNA) to regulate AID levels (step 6). Ikaros participates in regulating germline transcription of Sy2b and Sy2a (step 7). AID, activation-induced deaminase; APE1, apurinic/apyrimidinic endonuclease 1; CSR, class-switch recombination; DSBs, double-strand breaks; IgH, immunoglobulin H; MMR, mismatch repair; NHEJ, non-homologous DNA end-joining; ORF, open reading frame; RPA, replication protein A; UNG, uracil-DNA glycosylase.

Overexpression of AID converts it into a general mutator and deregulated AID activity has been implicated in the ontogeny of mature B-cell lymphomas. AID activity is highly regulated at the transcriptional level (it is expressed almost exclusively in activated B cells) and intracellular localization (it is primarily cytoplasmic; Chaudhuri *et al*, 2007). M. Nussenzweig (New York, NY, USA) presented data to show that AID mRNA levels are regulated by microRNA 155 (miR-155; Dorsett *et al*, 2008). miR-155 is highly expressed in germinal centre B cells, and the 3'-untranslated region (UTR) of AID mRNA contains an evolutionarily conserved miR-155 binding site. The Nussenzweig group created a knock-in mouse with a mutation in the putative miR-155 binding site in the 3' UTR of AID (AID¹⁵⁵). AID¹⁵⁵ mice have a twofold higher level of AID mRNA in germinal centres, and activated splenic B cells derived from these mice had levels of AID protein that were approximately threefold higher. Functionally, this led to a slight increase in CSR and SHM. Notably, mutation of the miR-155 binding site on the 3' UTR of AID mRNA resulted in a threefold to sixfold increase in IgH-c-Myc translocations compared with wild-type controls, indicating that miR-155 has a crucial role

in restricting oncogenic translocations in mature B cells. Finally, in B cells deleted for miR-155, the rate of translocations was more than 15-fold higher than that observed in control B cells, suggesting that miR-155 might have additional targets that function to restrict translocations. Determining whether these translocations lead to mature B-cell lymphomas, and identifying additional miR-155 targets that protect such oncogenic translocations and B-cell transformation, will be of great interest.

The non-homologous DNA end-joining (NHEJ) pathway has an essential role in joining double-strand breaks (DSBs) generated by the RAG proteins during V(D)J recombination; however, the requirement for the NHEJ pathway in CSR has not been rigorously tested. F. Alt (Boston, MA, USA) described that, in mature B cells conditionally deleted for XRCC4 or DNA ligase IV (both of which are essential and specific components of NHEJ), CSR is decreased to 20–50% of wild-type levels, indicating that NHEJ does have a role in CSR (Yan *et al*, 2007). Notably, an alternative end-joining pathway—which is markedly biased towards microhomology joins—supports CSR at unexpectedly robust levels in

XRCC4-deficient or ligase IV-deficient cells. Although 30–60% of wild-type B cells have direct $\Sigma\mu$ – $\Sigma\gamma 1$ joins and the remainder have 1–4 base pair homology, none of the XRCC4-deficient B cells have direct joins. Instead, most of the XRCC4-deficient joins appeared to be microhomology mediated, with an increased distribution of junctions having longer microhomologies in comparison to wild-type controls. In addition, 20–30% of XRCC4-deficient B cells activated in culture had IgH breaks, approximately 20% of which participate in translocations, possibly using the same alternative end-joining machinery that completes S-region DSBs during CSR in the absence of classical end-joining factors. Notably, all the IgH breaks and translocations are AID dependent, as the chromosomal lesions were not observed in AID-deficient B cells. Overall, these results provide convincing evidence of an alternative end-joining pathway that is robust in joining DSBs during CSR. The identification of components of this pathway, which differs from the classical pathway by the use of a different ligase as well as different ends, is eagerly awaited.

SLAM, SAP and XLP

SLAM-associated protein (SAP) is an adaptor protein utilized by most members of the SLAM family of costimulatory receptors, and is deficient in human patients with X-linked lymphoproliferative disease (XLP; also known as XLP1). P. Schwartzberg (Bethesda, MD, USA) showed that SAP can signal through Fyn-dependent and Fyn-independent pathways (Cannons *et al.*, 2006). By using the R87A-mutant form of SAP, which specifically lacks the ability to bind to Fyn, it was shown that SAP can mediate NF- κ B activation and IL-4 induction in a Fyn-dependent manner. However, the R87A SAP mutant can still support T cell–B cell conjugate formation, germinal centre reactions and antibody production independently of Fyn. Schwartzberg also reported on the initial characterization of CD84-deficient mice. R. Germain (Bethesda, MD, USA) presented new insights into the cellular basis of impaired germinal centre reactions in SAP-deficient mice. By imaging interactions between T and B cells in the lymph nodes, he showed that SAP-deficient T cells cannot make stable contacts with cognate B cells at multiple steps during the evolution of T-cell-dependent humoral responses, preventing the effective delivery of ‘help’ to the B cells. A. Veillette (Montreal, Canada) and co-workers generated T-cell-specific and B-cell-specific SAP-KO mice (Veillette *et al.*, 2008), and also mice deficient in two SAP-related adaptor proteins: EAT2 and ERT. By using the SAP-KO mice, Veillette formally demonstrated that the defect in humoral immunity in SAP-deficient mice is T-cell intrinsic and independent of SAP deletion in B cells. The Veillette laboratory has previously shown that SAP-KO NK cells display reduced cytotoxicity (Roncagalli *et al.*, 2005). By contrast, the cytotoxicity of EAT2 and ERT knock out NK cells was augmented towards some targets but reduced towards others, pointing to a complex interplay between various SLAM receptors and adaptors during NK–target interaction.

S. Latour (Paris, France) and colleagues uncovered a genetic basis for XLP2, which is a human immune disorder related to XLP1 (Rigaud *et al.*, 2006). XLP2 patients display symptoms similar to those of XLP1 patients—namely, a lymphoproliferative disorder with haemophagocytic syndrome and hypogammaglobulinaemia in response to Epstein–Barr virus (EBV) infection. In addition, XLP2 patients are characterized by a lack of NKT cells (natural killer T cells), similar to XLP1 patients, but have normal NK-cell cytotoxicity,

which is defective in XLP1 patients. The cause of this disease has been elusive because the *SAP* gene is not affected in XLP2 patients. Latour showed that null mutations in the anti-apoptotic gene *BIRC4/XIAP* located on the X chromosome caused XLP2. As expected, the lymphocytes of XLP2 patients, but not of XLP1 patients, are more prone to apoptosis. As the absence of NKT cells is the only common cellular defect associated with XLP1 and XLP2, Latour proposed that the lack of NKT cells in XLP1 and XLP2 might explain the aberrant immune responses to EBV infection.

Concluding remarks

This meeting provided an excellent opportunity for immunologists from a range of disciplines to discuss the most recent discoveries in the fields of lymphocyte development, differentiation, and signalling in health and disease. The most impressive progress was made in the field of Th17/T_{REG}-lineage commitment; the roles of local cytokine environments and antigen-presenting cells in Th17-cell compared with T_{REG}-cell differentiation are now more clearly defined. Significant advances were also reported in dissecting the signalling mechanisms of the SLAM–SAP family of costimulatory receptors and adaptors. The next challenge is to solve the apparent redundancy and interplays between the members of the SLAM and SAP families in the collaboration between B and T cells, NK-cell cytotoxicity and the development of NKT cells. Finally, several groups touched on various aspects of AID function and regulation during CSR. However, one of the outstanding issues in the field—identifying the mechanism that targets AID to the immunoglobulin locus—was left unresolved and will surely be a major topic of discussion at future meetings.

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