Lymphoid development: it's not 'all Greek to us' any more

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Lymphocyte lineage specification involves multiple regulatory factors that act in reciprocal fashion to ensure lineage committment and identity. New insights on how these factors interact were presented at the second Aegean Workshop on Gene Regulation in Lymphocyte Development.

Picture a turquoise blue sea, sunny, warm days, and good Greek food and wine. Couple this with several hours of cutting-edge science in the morning and early afternoon and you can imagine the experience of the recent Aegean Conference on Gene Regulation in Lymphocyte Development held 20–25 October 2004 on the Greek isle of Crete (**Fig. 1**). This workshop was first held 2 years ago on the island of Santorini, and both meetings were ably and thoughtfully organized by Ellen Rothenberg (Pasadena, USA) and Cornelis (Kees) Murre (La Jolla, USA). The Aegean Conferences, the successful brainchild of Greek-born University of Pennsylvania professor John Lambris, began in 2000 as a forum to convene scientists interested in innate immunity. Today the Aegean Conferences have grown to sponsor four to six meetings in several areas of investigation each year, and all are held on an island in the Aegean Sea. The overriding aim of these meetings is to present the latest scientific data in a relaxed and enjoyable venue, and the result is a gathering of approximately 80–100 investigators from all over the world who, over a 4-day span, interact closely, share unpublished data and get to know each other while also enjoying the cultural and gastronomic pleasures of Greece. This unique environment promotes communication and spawns promising collaborations.

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Regulation of antigen receptors

The focus of this meeting was gene regulation in lymphocyte development. B and T cell development share many features, the most well studied being the ordered rearrangement of immunoglobulin and T cell receptor (TCR) gene loci[1]. One of the main themes of this conference centered on chromatin modifications and their effects on immunoglobulin and TCR gene expression and rearrangement. Cornelis (Kees) Murre and Meinrad Busslinger (Vienna, Austria) both showed evidence for chromatin looping of immunoglobulin variable heavy-chain (V_H) gene segments to the diversity heavy-chain (D_H) region as a possible mechanism for the juxtaposition of

Figure 1 The lymphocyte development workshop was held near the ancient Minoan ruins of Knossos, located on the Greek island of Crete. Photograph by Barbara Osborne.

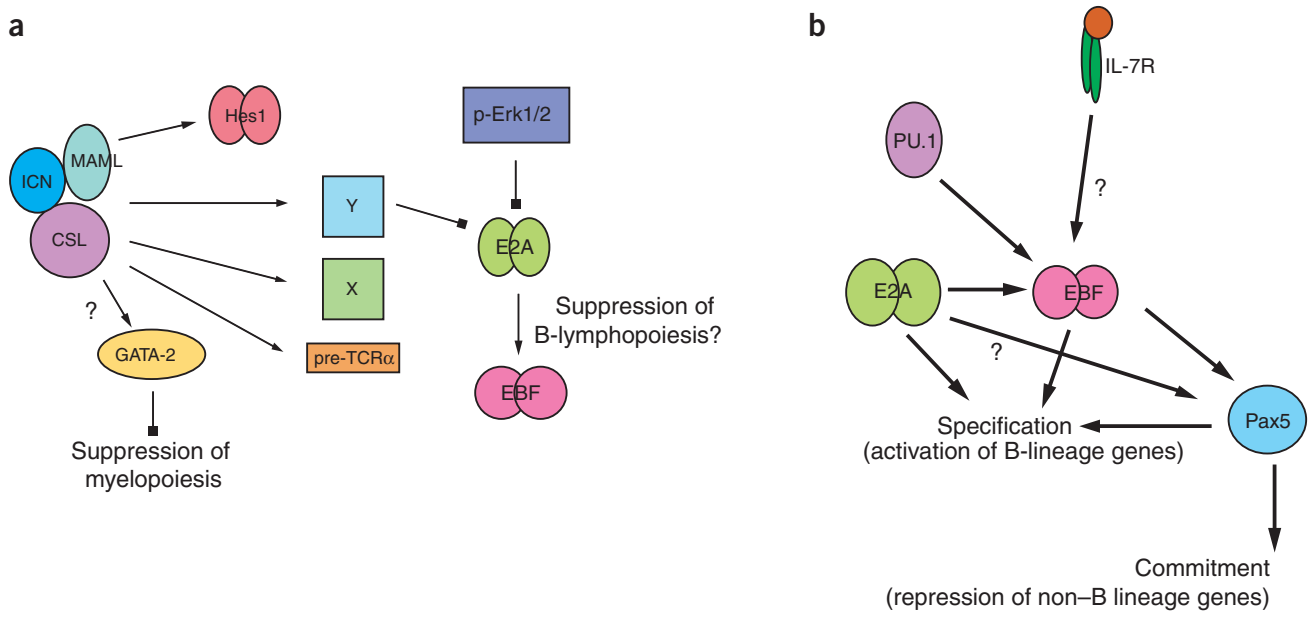

Figure 2 Molecular events involved in B and T lineage specification and commitment. (**a**) In T lineage commitment, activation of Notch (ICN) and subsequent nuclear translocation and interaction with CSL and the coactivator mastermind (MAML) are required events. In results presented at this conference, Hes1, a known target of this complex, was shown to be induced in hematopoietic stem cells cultured on OP9-DL1 before activation of most T lineage–associated genes. GATA-2 also was induced in the presence of DL1 suppressing the development of macrophages from hematopoietic stem cells. The pre-TCRα gene is also a target of CSL-ICN-MAML, but additional targets ('X' and 'Y') also must be induced, and some of these may function in collaboration with active mitogen-activated protein kinases (such as phosphorylated Erk1 and Erk2 (p-Erk1/2)) to partially degrade E2A proteins, an event suggested to be involved in the antagonization of B lymphopoiesis by preventing the activation of EBF. (**b**) E2A, EBF and Pax5 operate in a transcriptional hierarchy required for B lineage specification and commitment. PU.1- and interleukin 7 receptor (IL-7R)–derived signals may also be involved in regulating EBF expression. Moreover, EBF rescues B lineage specification and probably commitment in lymphoid progenitors lacking PU.1, interleukin 7 or E2A. Arrows indicate activation by an upstream factor; square blocks represent inhibitory interactions; question marks indicate that the mechanism is not yet known.

these gene segments during recombination. Ann Feeney (La Jolla, USA) demonstrated that preferential histone H3 acetylation at the S107 V_H1 gene segment, rather than V_H11 or V_H13, may explain the biased use of V_H1 in the S107 family. She also showed that the transcription factor Pax5 may recruit the recombination activating gene (RAG) proteins and target recombination to distal V gene segments, such as V_H1 and V_H11. Ranjan Sen (Baltimore, USA) showed that the chromatin around D_H and joining heavy-chain (J_H) loci early in B cell development is more 'active', as characterized by DNAse I hypersensitivity and histone hyperacetylation. An epigenetic study of the immunoglobulin light chain κ locus *Igk* by Yehudit Bergman (Jerusalem, Israel) showed that progressive histone modification, nuclear localization and replication timing may determine which allele is likely to undergo recombination. Using cell lines with conditional deletions of the E2A transcription factors, Yuan Zhuang (Durham, USA) demonstrated direct involvement of E2A in germline transcription and rearrangement of *Igk*. Mark Schlissel (Berkeley, USA) addressed the question of κ allelic exclusion using targeted insertion of cDNA encoding green fluorescent protein proximal to $J_κ1$ that acts as a measure of chromatin accessibility. The data presented indicate that activation of *Igk* is a rare event that can be modulated by ectopic expression of transcription factors, such as E2A, that are capable of binding to the κ enhancers. In a transgenic model system designed to measure RAG recombinase activity, Rachel Gerstein (Worcester, USA) demonstrated that RAG is active in the earliest lymphoid precursors in bone marrow before T and B lineage commitment and that E2A has a chief function in inducing RAG activity. Pierre Ferrier (Marseille, France) provided evidence that the 3′ TCRβ enhancer ($E_β$) regulates chromatin remodeling, which in turn influences $D(J)_β$ rearrangement. Additionally, $E_β$ is required for proper regulation of chromatin complexes at the $D_β1$ promoter. These studies demonstrated the essential involvement of specific chromatin modifications and transcription factor activity in mediating the accessibility and recombination of antigen receptor loci.

Notch and hematopoiesis

Both B and T lymphocytes undergo recombination of antigen receptor genes. However, these cells develop from common lymphoid progenitors or early thymic progenitors in distinct environments and differ in their requirement for specific transcription factors and signaling through Notch receptors[2–4]. The mechanism(s) by which Notch influences lymphocyte cell fate decisions was another main focus of discussion at the meeting (**Fig. 1a**). Notch1 is essential for T cell development and, in its absence, B lymphocytes develop in the thymus. Conversely, constitutive Notch1 activation inhibits B lineage development and promotes T lymphopoiesis and transformation in the bone marrow[5,6]. Juan Carlos Zuniga-Pflucker (Toronto, Canada) showed that stromal cell monolayers derived from fetal thymic organ cultures fail to support T lymphopoiesis because they lose expression of the Notch ligands Delta-like 1 (DL1) and DL4. He also showed data indicating that the commitment of hematopoietic stem cells to myelopoiesis is suppressed on the stromal cell line OP9 expressing transgenic DL1 (OP9-DL1) because of Notch1-induced expression of the transcription factor GATA2. Bianca Blom (Amsterdam, Netherlands) showed that Notch signaling inhibits the development human plasmacytoid dendritic cells *in vitro*. She identified the Ets-related protein SpiB as

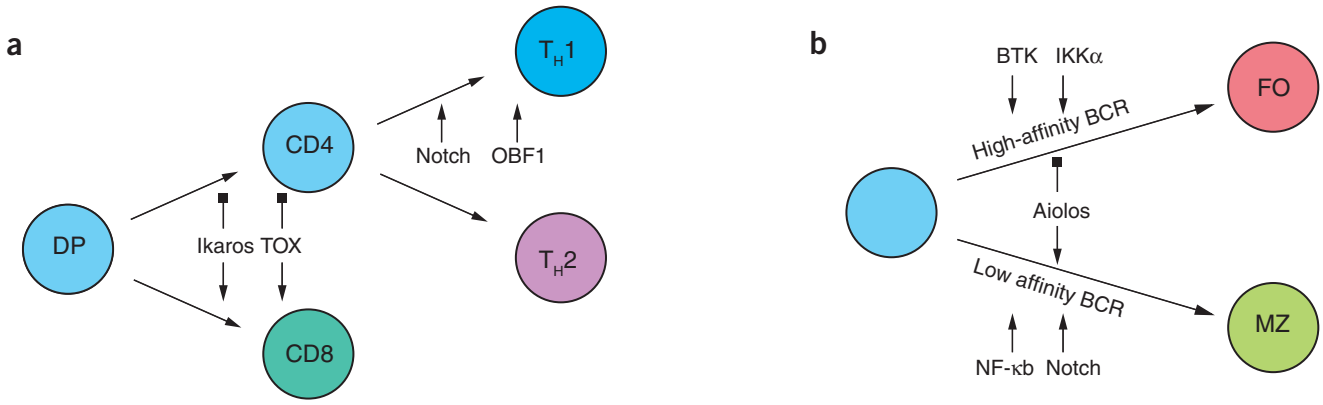

Figure 3 Transcriptional control of mature lymphocyte cell fates. The development of mature lymphocyte subpopulations is under the control of many transcriptional regulatory proteins and signaling pathways, some which were discussed at this conference. (**a**) The HMG-box transcription factor TOX is involved in promoting the development of CD8 single-positive cells, possibly by influencing *Cd4* silencing. Additionally, hypersensitivity sites in *Cd8* are important for the activation of *Cd8* expression, possibly through Ikaros. The polarization of CD4+ T cells to a T_H1 phenotype is influenced by the transcription factor OBF1, a coactivator for octamer-binding proteins, previously thought to be exclusively expressed in B lineage cells. Notch is also involved in T_H1 development, possibly through regulation of the T_H1 transcription factor T-bet. (**b**) Mature B lymphocytes can be activated to differentiate into cells with a marginal zone (MZ) or follicular (FO) phenotype. The development of these two cell types is differentially affected by the transcription factor Aiolos, which promotes marginal zone cell development. In addition, the strength of B cell receptor (BCR) signaling may be involved in the follicular–versus–marginal zone cell fate. High-affinity BCRs promote follicular development and are dependent on Bruton's tyrosine kinase (BTK) and IκB kinase-α (IKKα), whereas low-affinity BCR promote marginal zone cell development in an NF-κB- and Notch-dependent way.

an essential plasmacytoid dendritic cell transcription factor and found that SpiB can rescue plasmacytoid dendritic cell development in the presence of activated Notch1.

Ellen Rothenberg presented a time course of gene expression after plating of hematopoietic stem cells on OP9-DL1 cells. Hes-1, a 'downstream' target of Notch, was induced within 24 hours, whereas T lineage–associated gene expression was delayed until day 3 of culture. Notably, approximately 10% of double-negative DN1 ($CD4^-CD8^-CD44^+CD25^-$) cells that persist for more than 5 days in these cultures retain B lymphocyte potential when transferred to OP9 cells lacking DL-1, suggesting that activation of Notch1 does not irreversibly commit the cell to the T cell fate. Warren Pear (Philadelphia, USA) discussed the possible timing of the Notch signal in T lymphopoiesis. He showed that Notch signaling is required for development of the earliest subset of thymocytes, DN1a and DN1b[7], using a dominant negative mutant of the CSL (named for CBF1/RBP-Jκ in mammals, suppressor of hairless in drosophila and Lag-1 in *Caenorhabditis elegans*) coactivator mastermind (MAML). These results suggest that progenitors see Notch ligands before entering the thymus. Consistent with this hypothesis, T lymphocyte progenitors ($Thy1^+CD25^{lo}$) were found in spleen colonies at day 12 after bone marrow transplantation, and their development was dependent on activation of Notch. Many downstream targets of Notch signaling have been shown to influence T and B lymphopoiesis, although the mechanism by which Notch modulates cell fate decisions is not known. Xiao-Hung Sun (Oklahoma City, USA) presented evidence that Notch might promote T lymphopoeisis in part by degrading E2A proteins, which are essential for the induction of early B cell factor (EBF) and B lymphopoiesis (**Fig. 2a**). Mice carrying mutations in the serine residues of E2A, which are required for Notch-induced degradation, had normal T and B lymphocyte development, indicating that this degradation pathway is not essential for T lymphopoiesis *in vivo*. However, culture of bone marrow progenitors on a mixed stromal cell layer of OP9 and OP9-DL1 resulted in development of some B lymphocytes from E2A mutant but not wild-type bone marrow cells. Although these data are intriguing, more work is needed to determine whether degradation of E2A has a relevant function in Notch-induced T lymphopoiesis[8].

Early B cell development

Early B lymphopoiesis is dependent on activation of a transcription factor hierarchy: E2A activates EBF, which in turn activates Pax5, perhaps in concert with E2A (**Fig. 2b**). This transcriptional cascade is essential for both B lineage specification, the turning on of the program of B lineage–specific genes, and commitment, the turning off of alternative developmental programs. E2A and EBF are required for B lineage specification and commitment and have been shown to cooperatively regulate essential B lineage genes, including those encoding λ5, Vpre-B and Igα[9]. Pax5, in contrast, is not required for B lineage specification but is essential for commitment, as specified pro–B lymphocytes in *Pax5*$^{-/-}$ mice express many non–B lineage genes and differentiate to alternative lineages when cultured in appropriate conditions[10,11]. One conclusion that emerged from this meeting was that EBF not only is essential for B lymphopoiesis but also may be the primary determinant of B lineage specification and is involved in B lineage commitment. Chris Klug (Birmingham, USA) showed that forced expression of EBF in the hematopoietic system results in an increased number of B lineage cells in peripheral blood and a decrease in T lymphocyte and natural killer cell progenitors, suggesting that EBF may antagonize the development of non–B lineage lymphoid cell types. Harinder Singh (Chicago, USA) showed that EBF rescues B lymphopoiesis *in vitro* from fetal liver progenitors lacking PU.1, a transcription factor required for the development of multiple myeloid and lymphoid cell types. Data were also presented showing that EBF promotes B lymphopoiesis from a subset of hematopoietic stem cells cultured on OP9-DL1, demonstrating that EBF expression overrides Notch inhibition of B lymphopoiesis. Barbara Kee (Chicago, USA) showed that ectopic expression of EBF is sufficient to promote B lineage specification and commitment from fetal liver progenitors that lack E2A protein. Survival of E2A-deficient EBF-transduced cells and expression of B lineage genes remain dependent on the E protein

activity provided by E2-2 and HEB, which are less abundantly expressed than wild-type E2A. The data presented suggest that E2A is required to achieve sufficient E protein activity for the induction of EBF, whereas reduced E protein abundance or activity is sufficient for synergy with EBF to promote B lineage gene expression. Therefore, EBF is central in the induction of the B lineage program and has the potential to suppress non–B lineage cell fates.

In addition to the transcriptional hierarchy controlling B lymphopoeisis, interleukin IL-7 is essential for survival and expansion of the earliest B lineage progenitors[12]. Sheila Dias (Paris, France) showed that bone marrow common lymphoid progenitors from IL-7–deficient mice can differentiate into T lymphocytes and natural killer cells but have lost B lineage developmental potential when analyzed in the presence of IL-7. These IL-7–deficient common lymphoid progenitors fail to express EBF, and forced expression of EBF resulted in restoration of B lineage potential. Therefore, EBF seems to be an essential determinant of the B lineage cell fate whose expression may be regulated by IL- 7 signaling. Jim Hagman (Denver, USA) provided insight into the possible mechanism by which EBF controls B lymphopoiesis. In elegant experiments using a plasmacytoma cell line and primary cells from mouse bone marrow, he showed that both E2A and EBF contribute to demethylation of CpGs in the promoter of the gene encoding mb-1 (*Cd79a*) and are required for transcription of this gene, which encodes Igα, by Pax5-containing complexes. These findings suggest that E2A and EBF may be required upstream of Pax5 to impart epigenetic alterations in DNA, thus allowing Pax5 and other transcription factors to activate gene expression. Marianne Waterman (Los Angeles, USA) added to our understanding of how Pax5 might regulate expression of LEF1, a high-mobility group (HMG) box transcription factor, in pro–B lymphocytes. She characterized two promoters of *Lef1* and showed that Pax5 specifically regulates promoter 1 and can act in synergy with LEF1 to activate this regulatory region. Mikael Sigvardsson (Lund, Sweden) presented a microarray analysis of 14 B lineage cell lines that showed a direct correlation between EBF expression and known EBF target genes. In addition, he showed that activated Notch1 antagonizes EBF DNA binding and activation of a luciferase reporter. Kamala Kesavan (Vienna, Austria), using *Pax5*$^{-/-}$ mice, showed that Pax5 is required to suppress Notch-induced T lymphopoiesis in B lineage–specified cells. Specifically, *Pax5*$^{-/-}$ pro–B lymphocyte lines begin to express T lineage genes and diminish expression of B lineage genes after transfer to OP9-DL1 stromal cells. The data presented suggest that Pax5 is required to suppress Notch-induced T lymphopoiesis in pro–B lymphocytes and that EBF may also be involved as a target and/or a modulator of Notch signaling.

Wnt, PTEN and lymphopoiesis

Signaling through the Notch receptor is essential for T cell development; however, the Wnt signaling pathway is also important in this process. Wnt proteins bind to a receptor, composed of Frizzled proteins and the low-density lipoprotein receptor–related proteins LPR5 and LPR6, resulting in dissociation of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β from a complex containing β-catenin. The dissociation of this complex leads to stabilization and accumulation of β-catenin in the cytosol and in the nucleus, where it functions as a coactivator for the TCF, (also known as LEF1) family of transcription factors[13]. Jyoti Sen (Baltimore, USA) demonstrated that Wnt1 and Wnt4 are important in promoting thymocyte population expansion throughout T cell development. In contrast, T lineage–specific deletion of β-catenin results in decreased thymic cellularity, with a partial developmental arrest at the DN3 stage. Fotini Gounari (Boston, USA) described the phenotype of mice carrying a mutation in *Apc* (*Apc*$^{\Delta 468}$) in T lineage cells that results in loss of binding of APC to β-catenin and increased β-catenin stabilization. These mice have a block at the DN4 stage of T cell development and total thymocyte numbers are decreased. However, chromosome counts indicate that the defect may be due to the involvement of APC in chromosome segregation rather than Wnt signaling, as APC$^{\Delta 468}$ is also unable to bind microtubules. Hergen Spits (Amsterdam, Netherlands) showed that conditional deletion of phosphatase and tensin homolog (PTEN) which represses phosphatidylinositol 3 kinase signaling, can rescue thymus cellularity in common γ-chain–deficient and CD3γ-deficient mice and results in CD4$^+$CD8$^+$ thymocytes in mice deficient in both common γ-chain–deficient and RAG2. In the case of mice deficient in both PTEN and CD3γ, there is no evidence of β-selection, suggesting that the loss of PTEN may lead to survival of cells that would otherwise die in the absence of CD3γ signaling. Therefore, activation of phosphatidylinositol 3 kinase by γ-chain-containing cytokine receptors and CD3γ, as well as signaling through Frizzled receptors to stabilize β-catenin are important elements of the pathways required for expansion of T lymphocyte progenitor populations in the thymus.

Mature lymphoid development

In addition to the numerous discussion of early lymphopoiesis, many talks focused on gene regulation at later stages of lymphocyte development (**Fig. 3**). Jonathan Kaye (La Jolla, USA) discussed the involvement of TOX, an HMG-box protein with promiscuous DNA-binding capabilities, in the CD4-CD8 lineage decision. TOX-transgenic mice have an expanded CD8$^+$ cell population, independent of major histocompatibility complex class I expression, and increased expression of the transcription factor Runx3, indicating that TOX may influence *Cd4* gene silencing. Data presented by Dimitris Kioussis (London, UK) demonstrated once again the importance of chromatin structure in regulating gene activity. Targeted deletion of DNAse I hypersensitivity sites I and II in the *Cd8a* promoter resulted in decreased numbers of CD4$^+$CD8$^+$ cells, increased numbers of CD4$^+$ cells (with low TCR expression) and no CD8$^+$ cells, suggesting that loss of these sites inhibits *Cd8* expression. In addition, he presented evidence that the transcription factor Ikaros is involved in regulating these hypersensitive sites at the *Cd8* locus.

Martin Janz (Berlin, Germany) presented data on the extinction of the B cell phenotype in classical Hodgkin lymphoma through an examination of gene expression profiles for several independently derived human classical Hodgkin lymphoma cell lines. He showed that the B lineage transcription factors Pax5, EBF and E2A are expressed in classical Hodgkin lymphoma cells; however, Id2 and ABF-1, both repressors of E2A, are increased and may be responsible for the loss of B lineage characteristics in these lymphomas. Shiv Pillai (Boston, USA) presented data that addressed the derivation of follicular B cells versus marginal zone B cells. He showed that the transcription factor Aiolos blocks the development of mature follicular cells while promoting the development of marginal zone B cells. Using mice engineered to express B cell receptors that bind antigen with defined affinity, he showed that B cells that bind self antigen poorly are selected to become marginal zone B cells and that this requires both Notch and NF-κB (p50). In contrast, B cells that recognize self antigen with higher affinity become follicular B cells and this process depends on the expression of Bruton's tyrosine kinase and IκB kinase-α.

OBF1 is a B lymphocyte–specific transcriptional coactivator capable of stimulating transcription through its interaction with Oct-1 or Oct-2, proteins that bind the octamer sequence in the promoters and enhancers of immunoglobulin genes. OBF1 is expressed early in B cell

development, and deletion of this coactivator results in reduced numbers of splenic B cells, severely impaired T cell–dependent antibody responses and a complete absence of germinal centers[14]. Patrick Matthias (Basel, Switzerland) presented data suggesting that SpiB is a 'downstream' target of OBF1 in B cells. He showed this by several criteria, including upregulation of SpiB in OBF1-transgenic mice as well as occupancy of the SpiB promoter by OBF1 by chromatin immunoprecipitation analysis. He concluded that the reduced abundance of SpiB in OBF1-deficient mice was at least in part responsible for the lack of germinal centers in these mice. OBF1 was thought to be expressed only in B cells; however, a closer examination of OBF1-deficient mice by Thomas Wirth (Ulm, Germany) showed a previously unknown and unexpected function for OBF1 in T helper type 1 (T_H1) cell development. Using *in vitro* polarization assays, he demonstrated that OBF1 promotes T_H1 development and that OBF1-deficient mice produce less of the T_H1 'signature' cytokine interferon-γ. He also showed that OBF1-deficient mice do not clear infection with *Leishmania major*, another important indication that OBF is involved in regulating T_H1 function.

Data presented by Barbara Osborne (Amherst, USA) indicated that Notch is another gene required for proper T_H1 cell development. She found that blocking of Notch activation by either *in vitro* or *in vivo* treatment with a γ-secretase inhibitor, which inhibits the enzyme required for Notch cleavage and activation, prevents T_H1 cell development. Additionally, she presented chromatin immunoprecipitation data demonstrating that Notch and its nuclear partner CSL can directly bind to the promoter of the T_H1-specific transcription factor T-bet. Finally, *in vivo* administration of γ-secretase inhibitor blocked the development of experimental autoimmune encephalomyelitis, a disease known to be mediated by T_H1 cells. The meeting came to a riotous conclusion with a special presentation of 'The Lymphocyte Rap', written and convincingly performed by Shiv Pillai (**Supplementary Note** online).

Conclusion

In summary, the Aegean Workshop on Gene Regulation in Lymphocyte Development covered topics ranging from the epigenetic regulation of gene expression to transcriptional control of lymphocyte lineage determination and regulation of mature lymphocyte effector functions. Our understanding of the complex process of gene regulation in lymphocyte development has moved beyond analysis of transcription factor binding sites to an appreciation of the involvement of histone and DNA modifications, chromatin localization and conformation, as well as replication timing. In addition, the transcriptional networks controlling cell fate decisions are increasingly more apparent and the means by which these networks are altered by extracellular signals is beginning to be demonstrated. The essential function of EBF in the network controlling B lineage specification, and how EBF expression and activity are controlled, was a chief topic of discussion at this meeting. The central involvement of Notch in T lineage specification as well as in lineage decisions in more mature T and B cell populations was another important aspect of several presentations. How Notch regulates lineage decisions in these cells is still poorly understood. The next meeting, in 2006, promises to provide even greater insight into the mechanisms controlling gene regulation and differentiation in this fascinating developmental system while combining productive scientific interactions with the beautiful surroundings of the Greek islands and Aegean Sea.

Note: Supplementary information is available on the Nature Immunology website.

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