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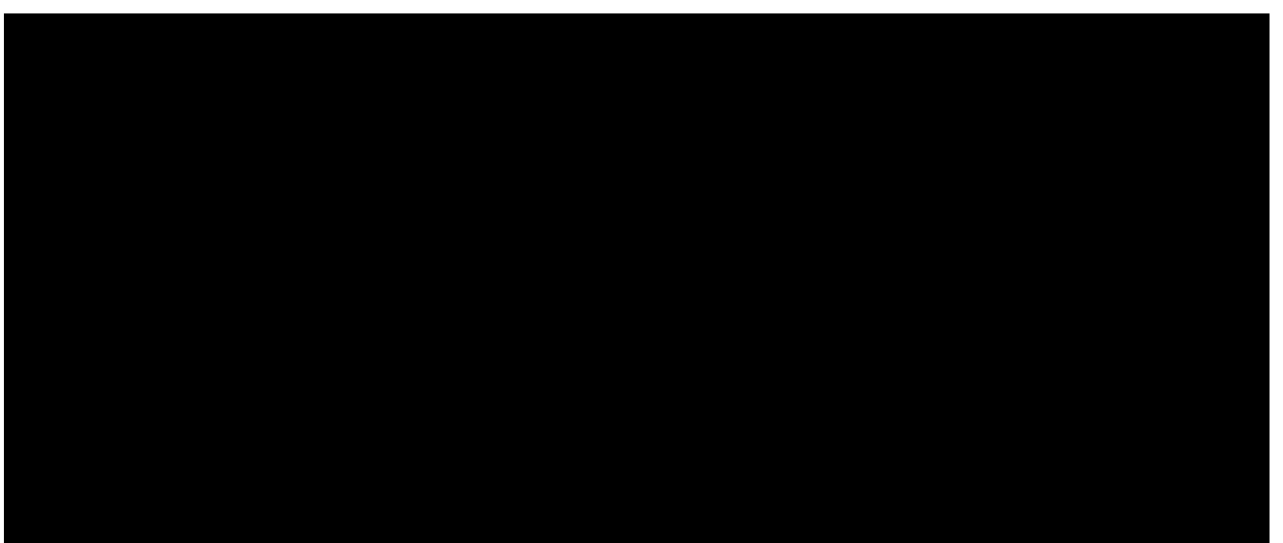

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Abstract

CHECKS AND BALANCES REGULATING

LYMPHOCYTE CYTOKINE PRODUCTION

Daniel B. Stetson

Cytokine production by lymphocytes is a key element of protective immunity. While much is known about the control of cytokine expression at the level of transcription, little is known about when and how cytokine producing cells emerge *in vivo*. We used bicistronic IL-4 reporter mice and MHC-peptide tetramers to track the emergence of an immunodominant response to *Leishmania major* infection, and found that IL-4-expressing cells emerge rapidly in the draining lymph nodes of infected mice, regardless of genetic predispositions to susceptibility or resistance.

Further studies of IL-4 and IFN-γ reporter mice revealed that NK T and NK cells, which are characterized by rapid activation of cytokine production after stimulation, activate lineage-specific cytokine transcription - but not translation - during development. These cells populate the periphery with constitutive cytokine mRNAs, which contribute to their rapid response upon activation.

Memory Th2 cells, which differ from their naive counterparts by the ability to rapidly produce IL-4 upon cognate stimulation, were found to contain constitutive IL-4 mRNAs *in vivo*, but no detectable protein. These IL-4 mRNAs were necessary, but not sufficient, for rapid protein production following restimulation. Polyribosome analysis of Th2 cells before and after restimulation in vitro revealed a global suppression of translation intitiation.

These studies highlight the multifaceted regulation of cytokine production. The rapid emergence of cytokine-expressing cells following infection, the correlation between constitutive cytokine mRNAs and rapid effector function, and the careful regulation of the global translation apparatus in effector T cells suggest that optimal cytokine production is achieved through control of both transcription and translation.

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Chapter 1:

Regulation of cytokine production by lymphocytes

Abstract

The matching of cytokine production by lymphocytes to the type of infectious challenge is a key element of immunity. CD4 T cells in particular differentiate from naive cells capable of producing a diverse array of cytokines to highly polarized effector cells which secrete a restricted set of effector cytokines. This chapter will review the literature relevant to the thesis, and will attempt to frame the questions we addressed in the context of current models which explain the mechanisms by which lymphocytes regulate their cytokine production.

Cytokines in Immunity

Cytokines are small, secreted effector molecules that facilitate cellular communication in multicellular organisms. A unifying theme that has emerged from the literature is the pivotal function that many of these proteins serve in immune system development and function. Because immune cells are numerous and migratory, they must be able to rapidly and efficiently convey information during occasions of cognate interaction. This information is often relayed in the form of specific combinations of cytokines, which are implicated in such diverse processes as stem cell mobilization, immunoglobulin class-switching, and phagocyte activation.

The identification of populations of CD4 T cells with the ability to produce either interferon-γ (IFN-γ) or interleukin-4 (IL-4), but rarely both, led to the Th1/Th2 paradigm that persists today (Kim et al., 1985; Mosmann et al., 1986). Th1 cells are involved in systemic immunity to intracellular pathogens, while Th2 cells mobilize mucosal defenses against extracellular pathogens like nematodes. When misregulated, Th1 cells cause autoimmunity and Th2 cells mediate asthma and allergy. Because these dichotomous effector fates originate from the same multipotent naive T cell and are largely irreversible once established, the factors underlying this cell fate decision have been intensely scrutinized (Abbas et al., 1996; Constant and Bottomly, 1997).

Recently, the Th1/Th2 paradigm has been extended to non-T cells and has blossomed into the broader concept of "Type 1" and "Type 2" immunity. Populations of CD8 T cells (Li et al., 1997), macrophages (Mills et al., 2000), B cells (Harris et al., 2000), NK cells (Loza and Perussia, 2001), and dendritic cells (Rissoan et al., 1999) have been identified with type 1 and type 2 behavior. Although the physiological relevance of

some of these studies remains to be determined, it seems appropriate to consider type 1 and type 2 immunity as the coordination of multiple cell types which conspire to protect against broad classes of pathogens. Regardless, IFN-γ and IL-4 remain the definitive cytokines of type 1 and type 2 responses, respectively.

Checkpoints that regulate Th1/Th2 polarization

The regulation of commitment to the Th1 or Th2 lineage has been a hotly debated subject in immunology. The arguments can be distilled into two main camps: some favor a stochastic mechanism of cytokine gene activation followed by selection of effector cells with the appropriate cytokine profile, while others suggest an instructive mechanism whereby the microenvironment dictates the initial activation of only the appropriate cytokine genes.

Recent data suggests that elements of both stochastic and instructive commitment to cytokine gene expression may underlie the polarization of T cells into IL-4- or IFN-γ-expressing cells. First, T cell receptor (TCR) and CD28 stimulation alone cause robust transcription of both IL-4 and IFN-γ within minutes of engagement (Grogan et al., 2001). This transcription does not require signaling by cytokine receptors, suggesting that cytokine genes reside in a "poised" state in naïve T cells, prior to antigen recognition. Second, subsequent stabilization of cytokine gene expression *in vitro* requires cytokine receptor signalling and activation of signal transducer and activator of transcription (STAT) proteins (Grogan et al., 2001). Thus, while early transcription of cytokine genes is indiscriminate and does not sense the cytokine microenvironment, stabilization of expression patterns is acutely responsive to the cytokine milieu (Grogan and Locksley, 2002).

Cytokines themselves have long been known to influence the polarization of CD4 T cells (Abbas et al., 1996). IL-12, made primarily by macrophages and dendritic cells, positively reinforces Th1 commitment and negatively regulates IL-4 production. Conversely, IL-4 stabilizes Th2 commitment while antagonizing IFN-γ production. These cytokines exert their effects through STAT proteins, and by induction of the key transcription factors T-bet and GATA-3, which when ectopically expressed are able to direct Th1 or Th2 polarization, respectively (Ho and Glimcher, 2002).

Another important checkpoint regulating lineage-specific cytokine expression is the epigenetic remodeling of the cytokine genes themselves. Expressed alleles are heritably marked such that daughters of polarized T cells maintain not only the same cytokine profile, but also expression from the same alleles (Bix and Locksley, 1998). At the level of the whole nucleus, this heritability can be visualized by the position of cytokine alleles with respect to heterochromatin, a marker of silenced DNA. While both IL-4 and IFN-γ loci are positioned apart from heterochromatin in naïve T cells, Th2 cells maintain IL-4 loci in active chromatin while shuttling IFN-γ loci to silenced heterochromatin (Grogan et al., 2001). Conversely, IL-4 loci associate with heterochromatin in differentiated Th1 cells. This dynamic interplay between activation and silencing is associated with the balance of acetylation versus methylation of cytokine genes (Avni et al., 2002; Fields et al., 2002). Acetylation of the expressed cytokine locus is dependent on STAT signaling and activity of T-bet in Th1 cells and GATA-3 in Th2 cells. Additionally, the IL-4 locus in differentiating Th2 cells becomes progressively demethylated (Lee et al., 2002a). Thus, cytokines, acting through STAT proteins and

transcription factors, link environmental cues to epigenetic remodeling of genes, and are critical for sustained expression of effector programs.

***Leishmania major* infection as an experimental model for T cell differentiation**

Leishmania major is a protozoan parasite endemic to the Middle East and regions bordering the Sahara desert in Africa. Infection of inbred mouse strains revealed that while most control infection with a Th1 response, a few strains, such as those on the BALB background, suffer progressive disease resulting from an inappropriate Th2 response (Reiner and Locksley, 1995). The pivotal role of IFN-γ and IL-4 in mediating protective versus pathogenic T cell responses is underscored by the phenotypes of knockout mice for these cytokines and their receptors. IFN- $\gamma^{-/-}$ and IFN- γreceptor$^{-/-}$ mice are unable to control infection, even on a resistant background (Swihart et al., 1995; Wang et al., 1994). Conversely, IL-4 -/- or IL-4R -/- mice on a BALB background heal infection with some (Kopf et al., 1996), but not all (Noben-Trauth et al., 1996), strains of *L. major*. Antibodies which block IL-4 function, when administered concomitant with infection, also reverse the susceptible phenotype of BALB/c mice (Sadick et al., 1990). CD4 T cells alone can account for the response, because transfer of purified CD4 T cells into severe combined immunodeficiency (Scid) mice recapitulates the course of disease (Holaday et al., 1991), and MHC Class II-/- mice succumb to infection (Locksley et al., 1993). This highly restricted response has made *L. major* infection an *in vivo* litmus test of T cell effector function, and has illuminated intriguing phenotypes in many transgenic and knockout mice (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002).

The importance of CD4 T cells and MHC Class II-mediated antigen presentation in the response to *L. major* fueled a search for parasite-derived peptide antigens and the T

cells which respond to them. Using an expression cloning strategy, Glaichenhaus and colleagues identified an I-A^d-binding peptide from a parasite protein termed LACK (*Leishmania* homolog of Receptor for Activated C Kinase) (Mougneau et al., 1995). Subsequently, it has been shown that LACK-specific T cells appear to nucleate the inappropriate IL-4 response in BALB/c mice. Immunization of LACK protein results in a burst of IL-4 message from Vα8/Vβ4+ CD4 T cells in draining lymph nodes detectable 16 hours after injection (Launois et al., 1997). Deletion of Vβ4+ T cells with a mouse mammary tumor virus superantigen results in the disappearance of the early IL-4 mRNA (Launois et al., 1997). Ectopic expression of LACK in the thymus of BALB/c mice results in protection from subsequent infection (Julia et al., 1996). Immunization with altered forms of the LACK peptide are also protective (Pingel et al., 1999). The latter two manipulations likely work by deleting or anergizing LACK-specific T cells. A response to LACK in resistant mice can also confer protection: mice with a monoclonal population of transgenic T cells specific for LACK on a B10.D2 background are able to restrain parasite growth (Reiner et al., 1998). These studies reveal that the response to *L. major* in mice of the d MHC haplotype is remarkably restricted to a single peptide epitope, at least early after infection. Considering that *L. major* is a pathogen with a 30 megabase genome, this restriction is striking from an immunological standpoint, and fortuitous for studies aimed at dissecting the parasite-specific T cell response.

It appears that a response to LACK can be protective on a resistant mouse background and pathogenic on a susceptible background. What, then accounts for the different responses? In early limiting dilution studies, it was found that BALB/c mice have a higher frequency of *L. major*-specific T cells than a number of resistant strains of

mice (Milon et al., 1986). In addition, transfer of small numbers of BALB/c CD4 T cells into immunodeficient hosts conferred protection from subsequent infection, while transfer of large numbers (>4 x 10^7) of T cells resulted in susceptibility (Varkila et al., 1993). These studies suggested that an enlarged precursor pool specific for *L. major* antigens was responsible for the early IL-4 response. Subsequently, one study implicated cross-reactivity of memory phenotype, LACK-specific T cells with endogenous gut antigens in BALB/c (but not B10.D2) mice, as an important factor leading to the exaggerated repertoire in susceptible mice (Julia et al., 2000). However, BALB/c mice maintained under gnotobiotic conditions remain susceptible to *L. major* infection (Julia et al., 2000).

The importance of LACK and LACK-specific T cells in the response to *L. major* suggests that methods to identify theseT cells *in vivo* would be informative in furthering our understanding of the events which occur early in infection.

Class II MHC-peptide tetramers

The identification of antigen-specific cells in a diverse population of lymphocytes is a difficult task, considering that a naive repertoire of B or T cells may contain one in three hundred thousand cells capable of recognizing a given antigenic shape (Blattman et al., 2002; McHeyzer-Williams and Davis, 1995). While the seeding of transgenic cells of known specificity greatly increases the naive precursor frequency and allows for their easy detection (Kearney et al., 1994), the diversity of an endogenous repertoire cannot be recreated using this approach. In the case of B cells, this problem was solved by considering that the affinity of an antibody for its antigen is quite high, with a dissociation constant (Kd) on the order of 10^{-9} M. Thus, simply labeling a protein antigen

with fluorophores allows the discrimination of B cells which bind with high affinity in tissue sections and by flow cytometry. T cell antigen recognition, however, adds two important obstacles to direct labeling. First, the T cell receptor (TCR) recognizes components of both the antigenic peptide and the MHC molecule, which must be in the correct orientation. Second, the affinity of a TCR for MHC/peptide is at least a thousand-fold lower than that of an antibody for its antigen.

Mark Davis and colleagues devised a reagent that solves both of these problems (Altman et al., 1996). First, soluble MHC Class I molecules were expressed in bacteria and refolded *in vitro* with β_2-microglobulin and specific peptide, creating a uniform population of MHC/peptide molecules. Second, a linear peptide recognition sequence for the *E. coli* biotin ligase BirA (Schatz, 1993) was appended to the c-terminus of the MHC molecule. An *in vitro* biotinylation reaction added a single biotin molecule per MHC/peptide. A tetrameric staining reagent was created by combining the biotinylated MHC/peptide molecules at a >4:1 molar ration to fluorophore-labeled streptavidin (which has four biotin binding sites). Because the avidity of three or more immobilized MHC/peptide molecules interacting with the surface of a T cell is considerably stronger than the sum of three individual MHC/peptide-TCR interactions in solution, the half-life of the interaction was sufficient to label antigen-specific T cells for flow cytometry.

Since their introduction in 1996, MHC/peptide tetramers have revolutionized the study of antigen-specific T cell responses. Several improvements have been made to the initial technology, and extended to the production of MHC Class II/peptide molecules. First, insect cell expression systems where the individual components are synthesized and secreted from the same cell bypass the need for laborious *in vitro* refolding reactions

(Crawford et al., 1998). Second, relatively unstable interactions between the α and β chains of certain MHC Class II molecules are reinforced by the introduction of complementary leucine zipper motifs (Scott et al., 1998). Third, peptides can be covalently linked via a serine-glycine linker to the N-terminus of the MHC β chain, allowing for folding stability during biosynthesis (Kozono et al., 1994). Thus, while not as simple as labeling a monoclonal antibody, the technology for producing MHC/peptide tetramers has become less prohibitive.

Most of the progress in studying antigen-specific T cell responses with MHC/peptide tetramers has involved the visualization of CD8 T cell responses to pathogens. Ahmed and colleagues used MHC tetramers linked to immunodominant peptide epitopes of lymphocyte choriomeningitis virus (LCMV) to show that at the peak of the splenic response to infection, as many as one in two CD8 T cells is specific for a single peptide (Murali-Krishna et al., 1998). This remarkable statistic reflects an antigen-specific expansion of approximately ten thousand-fold over the naive precursor frequency during one week (Blattman et al., 2002).

The utility of Class II MHC/peptide tetramers has proven more challenging (Ferlin et al., 2000). The primary reason for this difficulty lies in an inherent property of CD4 T cells: they tend to divide less extensively than CD8 T cells, and thus the peak of a CD4 response is not nearly as dramatic (Kotzin et al., 2000; Rees et al., 1999). Soluble MHC Class II/peptide molecules are also less stable and more difficult to produce in large quantities than Class I MHC/peptide reagents (Scott et al., 1998).

We took advantage of the restriction of the pathogenic *L. major* response in BALB/c mice to a single I-A^d binding epitope and generated soluble I-A^d/LACK

molecules in insect cells. A similar reagent had previously been reported and used with success in mice transgenic for the β chain of a LACK-specific T cell receptor (Malherbe et al., 2000). However, we were interested in visualizing the LACK-specific T cell response in wild-type mice, so we devised a flow cytometry strategy to detect rare, antigen-specific T cells. Chapter II of this thesis describes our findings using I-A^d/LACK tetramers to study the emergence of LACK-specific T cells in susceptible and resistant mice. Surprisingly, we found that the early LACK-specific response is not as polarized as originally thought, and that activation of the IL-4 gene is identical in the draining lymph nodes of susceptible and resistant mice. Thus, healer mice contain infection despite an early type 2 immune response. These findings led us to consider ways of regulating the production of IL-4 after its transcriptional activation in draining lymph nodes.

Translation and T cells

Cellular function requires translation of mRNAs into proteins, and T cells are no exception. Initiation of translation is a carefully regulated process, and involves many distinct factors which ensure specificity and fidelity (Sachs et al., 1997). In the case of lymphocytes, the delivery of effector molecules involves their rapid translation and secretion. Thus, the coordination of the translation apparatus and the secretory compartment of lymphocytes is an essential, but perhaps underappreciated, component of their effector function.

Translation can be attenuated in several ways, all of which respond to the perturbation of the cellular environment. For instance, poliovirus encodes a protein which cleaves the eukaryotic initiation factor (eIF) 4G, and drastically inhibits cap-

dependent host cell translation intitiation in a matter of hours after infection (Johannes et al., 1999). Cellular mechanisms exist which are also capable of rapidly remodeling the translation apparatus (Sachs et al., 1997). One such mechanism is the phosphorylation of eIF2. eIF2 delivers the charged methionyl tRNA to the nascent ribosome, which is required for initiation of most cap-dependent mRNA translation. Phosphorylation of eIF2 on serine 51 of its alpha subunit prevents reloading of methionyl tRNA, thus reducing initiation events. The importance of this regulatory mechanism is highlighted by the phenotype of mice with a knockin mutation replacing serine 51 of eIF2α with an an alanine (Scheuner et al., 2001). These mice die of hypoglycemia 18 hours after birth because of deficient pancreatic β cell function. Thus, translation regulation is an important component of cellular homeostasis, especially in secretory cell types.

Most cytokines are thought to be regulated primarily by transcription, and their mRNAs are considered short-lived, owing to the presence of an AU-rich element (ARE) in their 3' untranslated regions (Chen et al., 2001). Thus, robust transcriptional induction is thought to overcome the rapid degradation of cytokine mRNAs, allowing for their translation. However, new data suggests that the ARE can also recruit stabilizing factors under certain conditions (Neininger et al., 2002), which suggests that cytokine mRNA turnover may not be as uniformly rapid as previously thought.

Recent studies have begun to explore the role of translation regulation in the immune system. Perhaps the most striking example is in B cells, which require the transcription factor XBP-1 for their terminal differentiation into plasma cells (Reimold et al., 2001). In this study, Glimcher and colleagues used $RAG^{-/-}$ blastocyst complementation to generate mice devoid of XBP-1 in T and B cells (the full knockout is

embryonic lethal). Although XBP-1$^{-/-}$ B cells developed normally, proliferated in response to antigen, and were capable of immunoglobulin (Ig) class-switching, a severe defect in secretion of all isotypes of antibodies was observed. Ectopic expression of XBP-1 was sufficient to drive plasma cell differentiation in immature B cells. The authors concluded that the targets of XBP-1 were essential for B lymphocyte terminal differentiation; thus making XBP-1 the "master regulator" of plasma cell fate.

An intriguing pair of papers published soon after the XBP-1-/- B cell study shed more light on the role of XBP-1, not only in B cells but in all cell types (Calfon et al., 2002; Shen et al., 2001). XBP-1 was found to be the ortholog of yeast Hac-1, a transcription factor which mediates the cellular response to endoplasmic reticulum (ER) stress (Patil and Walter, 2001). This stress-activated pathway, called the unfolded protein response (UPR), coordinates the biosynthesis of components of the translation, chaperoning, and secretory machinery, including more of the ER membrane itself (Cox et al., 1997; Travers et al., 2000). In yeast grown under steady-state conditions, Hac-1 mRNA is present, but inefficiently translated (Cox and Walter, 1996). Upon induction of the UPR, cytoplasmic Hac-1 mRNA is cleaved by the ER resident kinase/endonuclease IRE-1 and then ligated by tRNA ligase, resulting in the removal of an intron and subsequent translation of the mature transcription factor (Ruegsegger et al., 2001; Sidrauski et al., 1996). ER stress decreases the viability of yeast deficient in upstream components of the UPR, revealing the importance of this pathway in maintaining homeostasis (Patil and Walter, 2001).

XBP-1 mRNA is regulated similarly to yeast Hac-1: it is cleaved by IRE-1 and then spliced in an unconventional cytoplasmic reaction to yield an active transcription

factor (Lee et al., 2002b). Placed in the context of XBP-1's role as the transcriptional gateway to the UPR, the failure of XBP-1-/- B cells to differentiate into plasma cells is informative. The cardinal property of a plasma cell is the elaboration of an extensive endoplasmic reticulum to accommodate secretion of over 2000 Ig molecules per second. Without the ability to make new ER, B cells cannot terminally differentiate, thus linking the mammalian UPR to B cell effector function.

While the recently appreciated role of translation regulation in B cells is clear, less is known about its importance in T cells. Unlike plasma cells, which secrete antibody constitutively for up to several years after differentiation (Slifka et al., 1998), T cells secrete effector molecules in a more carefully regulated, episodic fashion (Slifka et al., 1999). Although most work has focused on the regulation of the transcriptional machinery driving cytokine gene expression, a recent study has shown that the chemokine RANTES is regulated post-transcriptionally in memory T cells, and translation of stable RANTES mRNA is activated upon TCR stimulation (Swanson et al., 2002). We therefore wondered whether the cardinal cytokines of Type 1 and Type 2 immunity, IFN-γ and IL-4, are regulated by a similar process. In Chapters III and IV we provide evidence that translational control of IFN-γ and IL-4 is an important checkpoint which regulates the effector function of NK cells, NK T cells, and memory Th2 cells. Such a level of regulation would allow for rapid and efficient cytokine production during cognate recognition of a partner cell by lymphocytes.

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Chapter II:

Rapid expansion and IL-4 expression by *Leishmania*-specific naive helper T cells *in vivo*

Abstract

CD4 T cells are pivotal for effective immunity, yet their initial differentiation into effector subsets after infection remains poorly defined. We examined CD4 T cells specific for the immunodominant *Leishmania major* LACK antigen using MHC/peptide tetramers and IL-4 reporter mice. Comprising ~15 cells/lymph node in naïve mice, LACK-specific T cells expanded over 100-fold and 70% acquired IL-4 expression by 96 hours. Despite their pathogenic role in susceptible mice, LACK-specific precursor frequency, expansion and IL-4 expression were comparable between susceptible and resistant mice. When injected with unrelated antigen, *Leishmania* efficiently activated IL-4 expression from naïve antigen-specific T cells. CD4 subset polarization in this highly characterized model occurs independently from IL-4 expression by naïve T cells, which is activated indiscriminately after parasitism.

Introduction

The differentiation of naïve helper T cells to cytokine-expressing effector cells is important for the orchestration of immunity. Th1 and Th2 cells, which express the canonical cytokines IFN-γ and IL-4, respectively, mediate defense against distinct types of challenges, with Th1 cells more typically involved in systemic and Th2 cells in mucosal immunity. Despite this dichotomy, it is not entirely clear where or when these distinct cytokine responses become established. Current models envision dendritic cells to be central in mediating these responses through their capacity to recognize conserved pathogen-specific recognition motifs by innate receptors, including Toll-like receptors (Reis e Sousa, 2001). Upon maturation and migration to lymph nodes, dendritic cells can subsequently influence T helper subset differentiation by the elaboration of specific gene programs, including cytokines and receptors (Huang et al., 2001). Distinct classes of dendritic cells may also influence Th development (Liu et al., 2001).

Examination of naïve CD4 T cell responses *in vivo* has been hampered by the inability to identify the low numbers of cells within the endogeneous precursor population reactive to a given antigen. Methods for studying early antigen-specific CD4 T cell responses typically rely on the seeding of transgenic T cells of known specificity into animals prior to challenge (Garside et al., 1998; Reinhardt et al., 2001) or on analysis of effector functions revealed upon restimulation several days after challenge (Whitemire et al., 1998; Topham and Doherty, 1998). Modulation of activation markers has also been examined on normal T cells to define specificity, but few correlations with cytokine expression have been made (McHeyzer-Williams and Davis, 1995; McHeyzer-Williams et al., 1999; Panus et al., 2000). While such studies have elegantly documented the

anatomy and dynamics of CD4 effector/memory T cells, the initial expansion and acquisition of effector fates after infection remain incompletely characterized.

Murine *Leishmania major* infection remains an exceptional model for T helper subset development because the outcome has been linked definitively to effector fate - resistant mice make a protective Th1 response and susceptible mice make a non-protective Th2 response (Reiner and Locksley, 1995). Further, the susceptible response in BALB/c mice is nucleated by an aberrant Th2 response to an immunodominant peptide from the parasite LACK antigen (Mougneau et al., 1995). Deleting or tolerizing T cells that respond to LACK protects subsequently infected BALB/c mice from infection by enabling the development of a protective Th1 response (Julia et al., 1996; Launois et al., 1997, Pingel et al., 1999). Despite the utility of this model, the events that specify the development of protective versus pathogenic T cells remain elusive because they occur early in infection, before significant expansion of rare naïve precursors (Reiner and Locksley, 1995).

Here we use a flow cytometry strategy to visualize rare LACK-specific T cells and study their behavior after *L. major* infection in normal mice. Using bicistronic IL-4 reporter mice, we characterize the initial expression of IL-4 by these cells in the context of their early expansion. We find that despite the highly polarized responses that ultimately develop, early IL-4 expression is strikingly similar in MHC congenic susceptible and resistant mice. Indeed, *Leishmania* parasites inoculated with an unrelated antigen efficiently induced IL-4 expression from naïve, antigen-specific T cells, suggesting that these parasites might activate the elusive 'Th2-inducing' pathway in antigen presenting cells.

Results

Generation of LACK-specific tetramers and detection of LACK-specific CD4 T cells

The immunodominant peptide comprising amino acids 156-173 from the *L. major* LACK antigen (Pingel et al., 1999) was linked with the I-A^d β chain and used to generate peptide-specific tetramers after expression with I-A^d α chains in insect cells as described (Schatz, 1993; Scott et al., 1998). Soluble I-A^d/LACK adsorbed to tissue culture plates stimulated the proliferation of LACK-specific, but not ovalbumin-specific, transgenic T cells, demonstrating that these molecules retain their specificity *in vitro* (Figure 1a).

To detect LACK-specific T cells by flow cytometry in normal mice, we simultaneously stained single-cell suspensions with nonsaturating concentrations of I-A^d/LACK tetramers coupled to both streptavidin-phycoerythrin (SA-PE) and SA-allophycocyanin (APC) (Figure 1b). Because PE and APC are excited by different lasers and their emission spectra do not overlap, a T cell must meet two independent criteria to be tetramer-positive. This approach reduced the lower limit of detection to 1 in 3 x 10^5 CD4 T cells in normal mice. The lower limits of detection of PE- and APC-labeled tetramers used alone were 1 in 10^3 and 1 in 10^4, respectively. The frequency of DO11.10 transgenic T cells specific for ovalbumin peptide in I-A^d that stained with I-A^d/LACK tetramers was fewer than 1 in 4 x 10^6 (D. S., data not shown), confirming the specificity of the reagent revealed by the functional studies (Figure 1a).

We tested the sensitivity of this approach *in vivo* by analyzing the LACK-specific recall response eight weeks after primary immunization with LACK or ovalbumin proteins in adjuvant. In both BALB/c and B10.D2 mice that were primed and rechallenged with LACK protein, we observed a 220-fold increase in both the total

number of LACK-specific T cells and their frequency per 10^5 CD4 T cells (Figure 1c, 1d). Even at the peak of the memory response to saturating antigen doses, the frequency of LACK-specific T cells was fewer than 1 in 1000 CD4 T cells, a number that was undetectable with PE-labeled tetramers alone (Figure 1d). In contrast, LACK-specific T cell numbers remained at resting levels of 11-15 cells per lymph node in mice immunized and rechallenged with ovalbumin (Figure 1d). In mice immunized with LACK but rechallenged with ovalbumin, we detected resting, LACK-specific memory T cells that were 10- to 20-fold more numerous than in naïve mice (Figure 1d). Thus, I-A^d/LACK tetramers detect an immune response after both primary and secondary immunization, and retain their sensitivity and specificity even during the peak response to immunization with an unrelated antigen.

Figure 1. Specificity and sensitivity of I-A^d/LACK tetramers.

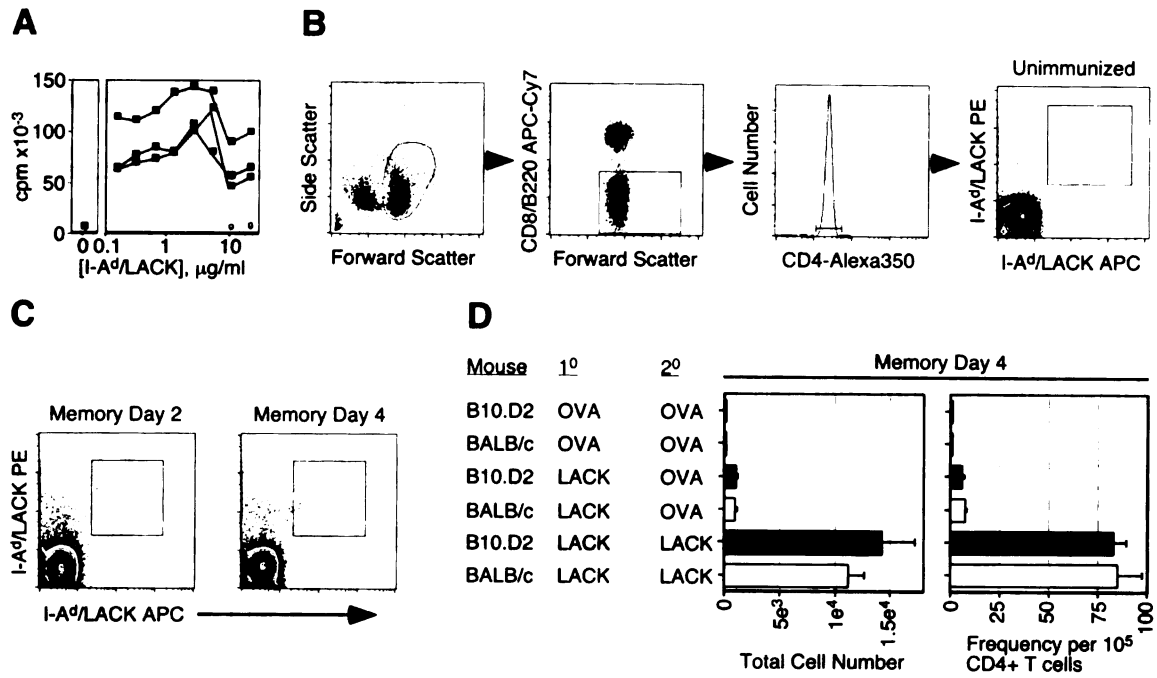
a. BALB/c ABLE TCR-Cα-/- (black squares) and DO11.10 TCR-Cα-/- (white circles) transgenic T cells were stimulated with plate-bound I-A^d/LACK at the indicated concentrations for 72 hours, and proliferation was measured by incorporation of ^{3}H-thymidine. Data for three independently-produced batches of soluble I-A^d/LACK are shown.

b. Flow cytometry strategy for detection of LACK-specific T cells. Intensity of I-A^d/LACK-PE and I-A^d/LACK-APC staining was monitored on [CD8/B220]-, CD4+ lymphocytes. The right panel shows staining of 50,000 CD4 T cells from unimmunized BALB/c mice.

c. BALB/c mice immunized eight weeks earlier with LACK protein were rechallenged, and the emergence of LACK-specific T cells in the draining lymph nodes at days 2 and 4 of the recall response was enumerated. Each panel shows tetramer staining for 90,000 CD4 T cells.

d. At day 4 of the recall response to the indicated proteins, the number and frequency of LACK-specific T cells in the draining lymph nodes of BALB/c and B10.D2 mice were enumerated. Mean and standard deviations of data from 3-4 mice per strain for each treatment are represented.

Figure 1


A
B
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cpm x10-3
150
100
50
0
0 0.1 1 10
[I-Ad/LACK], µg/ml
Side Scatter
Forward Scatter
CD8/B220 APC-Cy7
Forward Scatter
Cell Number
CD4-Alexa350
Unimmunized
I-Ad/LACK PE
I-Ad/LACK APC
Memory Day 2
Memory Day 4
I-Ad/LACK PE
I-Ad/LACK APC
Mouse
1°
2°
B10.D2 OVA OVA
BALB/c OVA OVA
B10.D2 LACK OVA
BALB/c LACK OVA
B10.D2 LACK LACK
BALB/c LACK LACK
Memory Day 4
0
5e3
1e4
1.5e4
Total Cell Number
0
25
50
75
100
Frequency per 10^5
CD4+ T cells

I-A^d/LACK tetramers detect a biologically relevant population of precursor CD4 T cells

The dual tetramer approach to identify LACK-specific T cells in normal mice is sensitive to frequencies comparable to estimates of naïve CD4 T cell precursors derived from direct sequencing approaches, suggesting numbers approximating 15 cells per lymph node or 200-250 per animal (McHeyzer-Williams and Davis, 1995; Casrouge et al., 2000). Recent estimates of naive CD8 T cell precursor frequencies suggest similar numbers (Blattman et al., 2002). The concern remains that this approach might fail to detect LACK-specific T cells in resting lymph nodes because the reagent lacks sufficient sensitivity to identify the true positives. Indeed, the electronic gates we use impose an arbitrary cutoff that excludes T cells with tetramer staining approaching background levels (Figure 1b, 1c). Activation of T cells can increase binding avidity to MHC-peptide tetramers (Fahmy et al., 2001), raising the possibility that resting naïve T cells might not be reliably detected with the reagent. However, naïve, LACK-specific transgenic T cells stained brightly with I-A^d/LACK tetramers, and the staining intensity did not change with proliferation *in vivo* (Figure 3c, below). To address this issue further, we sorted CD4 T cells from naïve BALB/c mice and excluded cells positive for both PE- and APC-labeled tetramers using our stringent gates. We reconstituted TCR Cα-/- BALB/c mice with the I-A^d/LACK tetramer-depleted cells and infected the mice with *L. major*. Depletion of the few I-A^d/LACK tetramer-positive cells prior to infection significantly ameliorated the outcome of infection in reconstituted TCR Cα-/- BALB/c mice, as evidenced by reduced footpad swelling ($p<0.02$ at 7 weeks) and parasite burdens, when compared to mice reconstituted with the same number of total CD4 T cells (Figure 2). Given the stringent gating strategy, we were not surprised to recover I-A^d/LACK tetramer-positive cells after

7 weeks in the depleted mice, although these were reduced in number as compared to mice receiving total CD4 T cells (data not shown). Taken together, however, the data support our ability to detect a biologically relevant population of naïve I-A^d/LACK-specific T cells that contributes to the susceptibility of BALB/c mice (Julia et al., 1996; Launois et al., 1997).

Figure 2

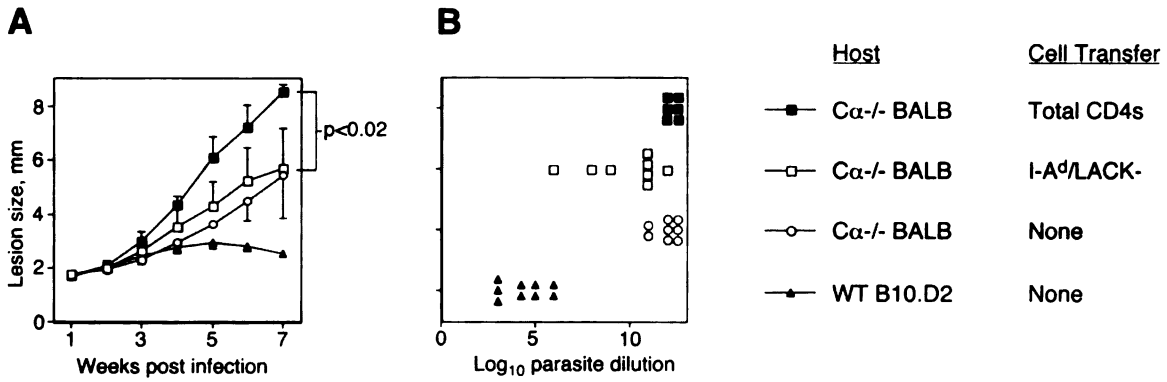


Figure 2. Depletion of I-A^d/LACK-specific T cells from the BALB/c CD4 repertoire ameliorates the outcome of *L. major* infection.

Progression of footpad lesions (**a**) and parasite burdens in infected footpads (**b**) seven weeks after infection in BALB/c TCR-Cα-/- mice receiving no cells (white circles), or 10^7 BALB/c CD4 T cells (black squares) or 10^7 BALB/c CD4 T cells depleted of I-A^d/LACK-tetramer+ cells i.v. 24 hours prior to infection. Wild-type, resistant B10.D2 mice were infected concurrently (black triangles). Left panel depicts mean and standard deviation for footpad lesions with parasite cultures represented as individual symbols from each infected mouse in right panel. Data represent one of three comparable experiments. P values were calculated using the Student t-test.

LACK-specific CD4 T cells have a naïve phenotype and expand rapidly after infection

Prior to infection, both BALB/c and B10.D2 mice had an average of 11-15 LACK-specific T cells per lymph node. These cells were uniformly naïve with respect to surface expression of the activation markers CD25, CD62L and CD69 (Figure 3a). We focused on the LACK-specific response in the popliteal lymph nodes during the first week following *L. major* infection because treatments that reverse the susceptible phenotype of BALB/c mice are most effective when administered during this period (Reiner and Locksley, 1995). During the first 48 hours, a marginal increase in the number of LACK-specific T cells accompanied a three-fold increase in popliteal lymph node total cellularity. Between 48 and 72 hours, however, LACK-specific T cell numbers expanded fifteen-fold in BALB/c mice and fifty-fold over resting levels (Figure 3b). Expansion was not as pronounced in B10.D2 mice at 72 hours, potentially reflecting more efficient restriction of parasite dissemination to lymph nodes at early time periods in resistant mice (Laskay et al., 1995). By 96 hours, however, LACK-specific T cells peaked at similar numbers in BALB/c and B10.D2 mice and remained the same at 120 hours (Figure 3b). No increase in the numbers of LACK-specific T cells was detected in either mouse strain in spleen or cervical lymph nodes cells at this time (data not shown). By 192 hours, a decline in LACK-specific T cells occurred, consistent with migration from the draining lymph node as previously defined (Garside et al., 1998; Reinhardt et al., 2001).

To confirm our ability to identify naïve LACK-specific T cells, we adoptively transferred CFSE-labeled, LACK-specific, transgenic T cells into BALB/c mice and monitored cell division at 24 hour intervals after infection by following the reduction in CFSE fluorescence intensity. Despite a >1000-fold increase in precursor frequency, cell

division of the transferred population was minimal for the first 48 hours (Figure 3c). The transferred T cells divided extensively between 48 and 72 hours, followed by rapid disappearance from the draining lymph node, and was essentially restricted to the draining lymph node as compared to distal nodes (Figure 3c). Thus, the kinetics and expansion of transgenic T cells of known specificity mirrored the kinetics and expansion of rigorously gated, tetramer-positive T cells. Taken together, these data suggest that we have accurately identified the response of naïve, LACK-specific, CD4 T cells *in vivo*.

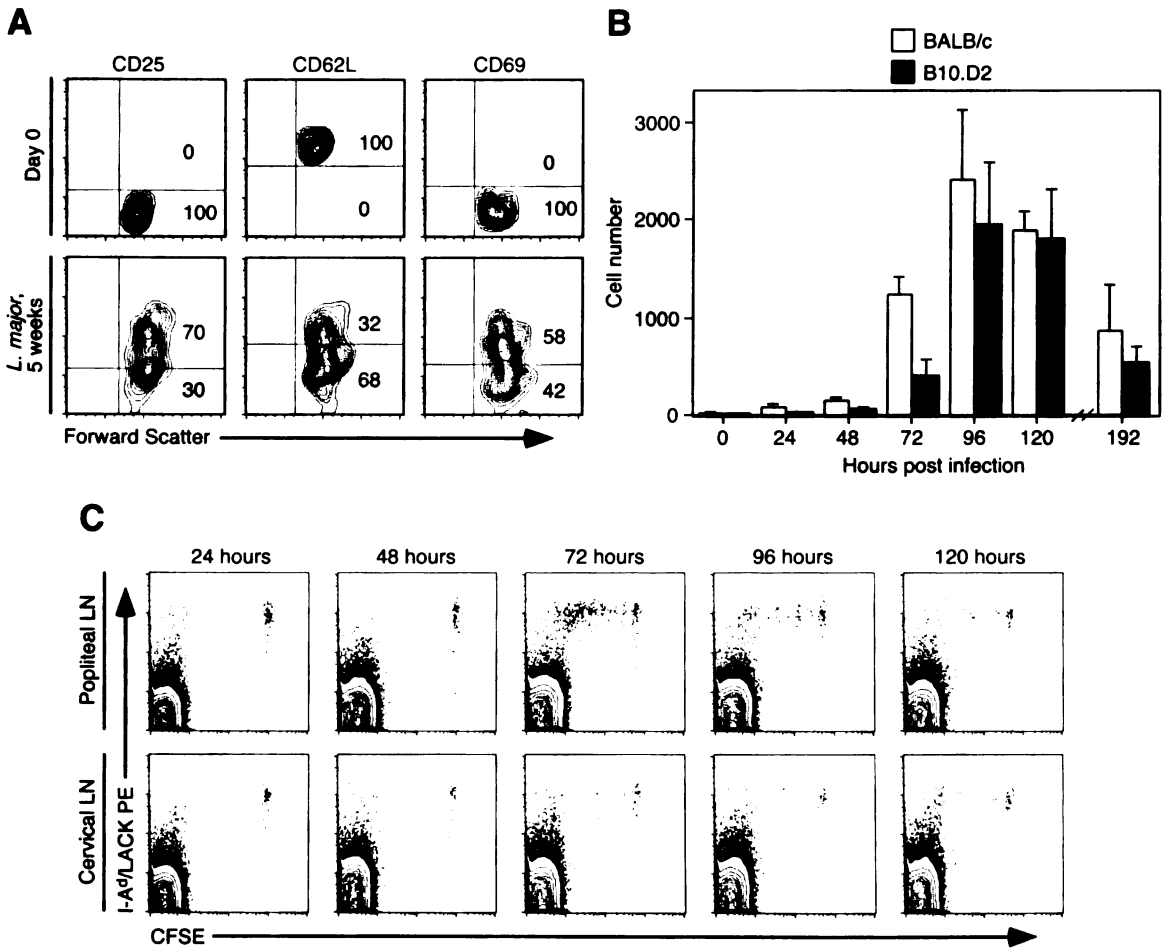
Figure 3. Kinetics of LACK-specific T cell expansion after *L. major* infection.

a. Surface expression of CD25, CD62L and CD69 was compared on I-A^d/LACK+ T cells from uninfected (top panels) and 5-week infected (bottom panels) BALB/c mice. Cells were pooled from peripheral and mesenteric lymph nodes and spleen.

b. Quantitation of LACK-specific T cell numbers in the draining, popliteal lymph nodes of BALB/c (white bars) and B10.D2 (black bars) mice at 24 hour intervals after infection with *L. major*. Data represent the mean and standard deviation of 4-11 mice of each strain per timepoint.

c. 10^6 CFSE-labeled, BALB/c ABLE TCR-Cα-/- T cells were transferred intravenously into normal BALB/c recipients 24 hours prior to infection, and dilution of CFSE fluorescence intensity in the I-A^d/LACK tetramer+ donor T cells was analyzed at 24 hour intervals. Plots represent 10^5 [CD8/B220]- T cells, and are representative of four mice per timepoint.

Figure 3



Activation of IL-4 expression from LACK-reactive T cells is comparable among resistant and susceptible mice infected with *L. major*

We next sought to track the emergence of IL-4-expressing cells during this early period of T cell expansion. To enumerate these cells *in vivo*, we used mice with a bicistronic IL-4/IRES/eGFP gene knocked into the IL-4 locus. These mice, designated 4get (IL-4 GFP enhanced transcript), maintain IL-4 production under endogenous regulation, and cells that activate IL-4 accumulate eGFP in their cytoplasm, allowing identification without the need for restimulation (Mohrs et al., 2001). 4get mice backcrossed onto BALB/c and B10.D2 backgrounds recapitulated the biology of infection when compared to their wild-type counterparts, as assessed by kinetics of footpad swelling, parasite burden, and frequency of IL-4- and IFN-γ -producing cells four weeks after infection (Figure 4, and data not shown). We first characterized the emergence of IL-4 producing cells in susceptible, 4get BALB/c mice. Using I-A^d/LACK tetramers, we failed to detect a single LACK-specific, eGFP+ T cell in the spleen and pooled lymph nodes (including mesenteric) of uninfected 4get BALB/c mice (Figure 5a). Post-infection, none of the LACK-specific T cells were eGFP+ after 48 hours (Figure 5a). By 72 hours, coincident with their abrupt proliferation (Figure 3b), 50% of I-A^d/LACK+ T cells were eGFP+ (Figure 5a). At 96 hours, this frequency peaked at 70% and remained similar at 120 hours (Figure 5a, b).

We next compared the frequency of eGFP-expressing T cells in susceptible 4get BALB/c and resistant 4get B10.D2 mice. Unexpectedly, the frequency of LACK-specific, eGFP-expressing cells in 4get B10.D2 mice was not different from 4get BALB/c mice at the peak of cellular expansion in the draining lymph node (Figure 5b, left panel).

Moreover, the percentage of tetramer-negative T cells that activated IL-4 expression was even higher in infected 4get B10.D2 mice (Figure 5b, right panel). Neither strain of mice developed significant numbers of tetramer-positive, eGFP+ CD4 T cells in the spleen during this period (D.S.; data not shown). To assess whether the IL-4-expressing cells from susceptible or healer mice expressed differing amounts of IFN-γ, the eGFP+ CD4+ T cells were sorted from the lymph nodes of 120-hour infected 4get BALB/c and 4get B10.D2 mice. Total RNA was isolated and used for reverse-transcriptase-polymerase chain reaction (RT-PCR) using fluorogenic probes. Quantitative RT-PCR revealed that IL-4 and IFN-γ transcripts were comparable, respectively, in the eGFP+ T cells from 4get BALB/c and 4get B10.D2 mice, and similar to the transcript abundance from Th2 cells generated *in vitro* (Figure 5c). Thus, eGFP+ CD4+ T cells from healer mice isolated early after infection contain cytokine transcripts consistent with their identification as Th2 cells.

We next examined the emergence of eGFP expression among LACK tetramer-positive and -negative cells in susceptible BALB/c 4get mice treated at the time of infection with neutralizing IL-4 antibody. A cohort of mice was followed for 8 weeks and confirmed the anti-IL-4 intervention conferred a healer phenotype (data not shown). When examined at 96 hours after infection, IL-4 expression from tetramer-positive cells was slightly, but insignificantly, attenuated in mice that received anti-IL-4 compared to control mice (Figure 5d). In contrast, IL-4 expression from tetramer-negative cells was reduced four-fold, consistent with a role for LACK-specific cells in nucleating a broader Th2 response.

Figure 4

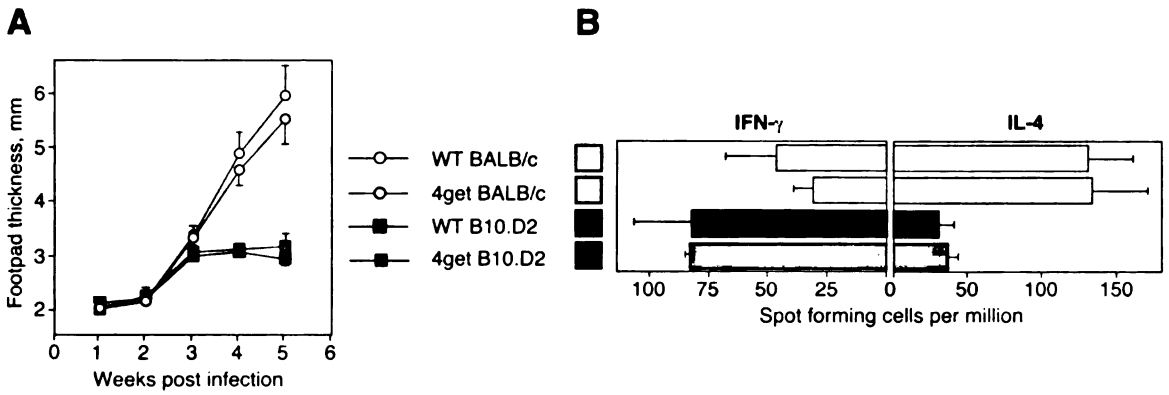


Figure 4. Characterization of 4get BALB/c and 4get B10.D2 mice.

a. Progression of footpad lesions after *L. major* infection of BALB/c and B10.D2 mice with wild-type or 4get homozygous *Il-4* alleles. Mean and standard deviation of four mice per group are shown, and are representative of three experiments.

b. The frequency of IL-4 and IFN-γ-secreting cells from dissociated popliteal lymph nodes was determined by ELISPOT four weeks after infection. Mean and standard deviations of four mice per group are shown.

Figure 5. Emergence of IL-4 producing cells *in vivo.*

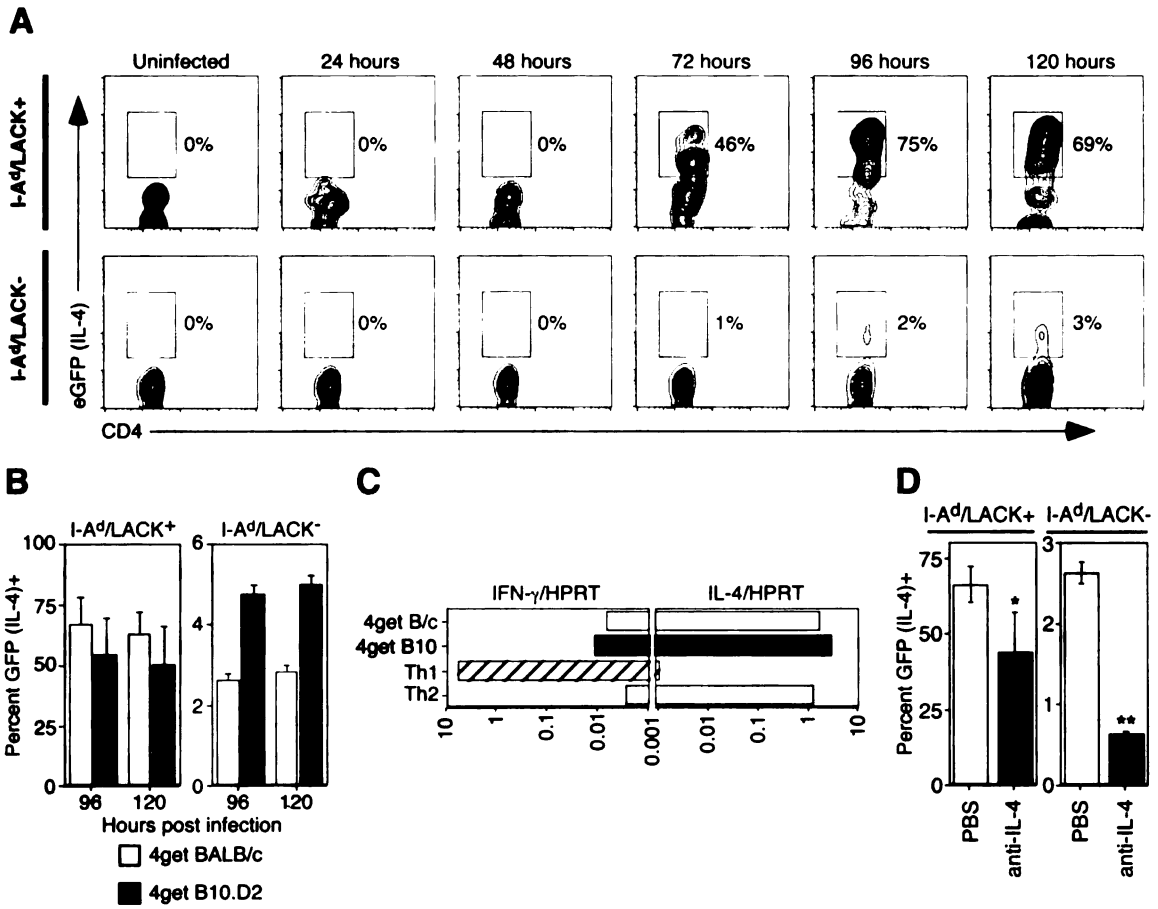
a. Representative FACS plots of eGFP expression in LACK-tetramer positive (top panels) and LACK-tetramer negative (bottom panels) CD4 T cells from 4get BALB/c mice before and at 24 hour intervals after infection. Data are representative of 4-12 mice at each timepoint.

b. The percentage of eGFP+, LACK-tetramer positive (left panel) and LACK-tetramer negative (right panel) CD4 T cells in infected fifth backcross 4get BALB/c (white bars) and 4get B10.D2 (black bars) at 96 and 120 hours after infection. Data represent means and standard deviations for four mice per strain at each timepoint.

c. 5' nuclease fluorogenic RT-PCR for IL-4 and IFN-γ was performed on purified CD4+ eGFP+ T cells from 4get BALB/c and 4get B10.D2 mice infected 120 hours earlier with *L. major*. Data are represented as the ratio of cytokine message to HPRT message and depict data from one of two comparable experiments. Established Th1 and Th2 cell lines generated as described (Grogan et al., 2001) were compared for reference.

d. Cohorts of 4get BALB/c mice were treated with neutralizing IL-4 antibody or vehicle control the day of infection. After 4 days, draining lymph node cells were harvested and analyzed for eGFP expression among LACK tetramer-positive and tetramer-negative populations. Means and standard deviations are shown for four mice per group, and are representative of three experiments. P values were calculated using the Student t-test: *p=0.11;**p=0.0005.

Figure 5


A
Uninfected
24 hours
48 hours
72 hours
96 hours
120 hours
I-Ad/LACK+
I-Ad/LACK-
eGFP (IL-4)
CD4
0%
0%
0%
46%
75%
69%
0%
0%
0%
1%
2%
3%
B
I-Ad/LACK+
I-Ad/LACK-
Percent GFP (IL-4)+
Hours post infection
4get BALB/c
4get B10.D2
C
IFN-γ/HPRT
IL-4/HPRT
4get B/c
4get B10
Th1
Th2
D
I-Ad/LACK+
I-Ad/LACK-
Percent GFP (IL-4)+
PBS
anti-IL-4

Leishmania activate antigen-dependent IL-4 expression from naïve T cells

Prior studies have suggested that the early IL-4 made by CD4 T cells after *Leishmania* infection derives from cross-reactive memory cells that might more efficiently activate cytokine gene expression (Julia et al., 2000). Three observations in the 4get mice suggested that *Leishmania* efficiently activated IL-4 expression from naïve T cells. First, the few (~0.5%) spontaneously eGFP+ T cells in resting 4get mice were uniformly $CD62L^{lo}$, consistent with their identification as effector/memory cells (Figure 6a). Second, eGFP expression early after infection occurred exclusively among cells that were $CD62L^{hi}$, consistent with their naïve phenotype, as demonstrated definitively for the LACK tetramer-positive subpopulation (Figure 6a, 3a). Finally, none of the eGFP+ cells that appeared in the lymph nodes over the initial 120 hours stained with α-galactosylceramide/CD1 tetramers which identify canonical NK T cells (D.S.; data not shown)(Matsuda et al., 2000). NK T cells can rapidly secrete IL-4 after activation, although these cells are unlikely to contribute to the early IL-4 production after *L. major* infection (Brown et al., 1996).

Whereas the specificity of the tetramer-negative, eGFP-expressing cells is not known, their phenotype is consistent with a *de novo* response to parasite antigens distinct from LACK. To assess directly whether *L. major* infection could elicit IL-4 expression from naïve CD4 T cells of known but unrelated specificity, naïve, ovalbumin-specific D011.10 x 4get T cells were transferred into BALB/c recipients before challenge with ovalbumin in adjuvant or with the pathogens *L. major* or *Listeria monocytogenes*. *Listeria* is a potent inducer of IL-12 from antigen-presenting cells (Hsieh et al., 1993). Draining lymph node cells were recovered after 96 hours and donor T cells were assessed for

specificity using the clonotypic mAb KJ1-26 and for IL-4 gene activation by eGFP fluorescence (Figure 6b, 6c). *L. major* potently induced IL-4 expression from OVA-specific T cells when co-injected with ovalbumin, but not when injected in the absence of ovalbumin. Whereas CFA or RIBI adjuvants elicited intermediate levels of IL-4 expression when injected with ovalbumin, *Listeria* co-injection resulted in no IL-4 expression from OVA-specific 4get T cells. Interestingly, co-inoculation of *Listeria* with *L. major* and ovalbumin completely abrogated the IL-4 response elicited by *L. major* alone (Figure 6c). Thus, activation of the IL-4 gene is not the result of bystander stimulation of 4get T cells in lymph nodes, but is powerfully activated by *Leishmania* parasites by an antigen-dependent process.

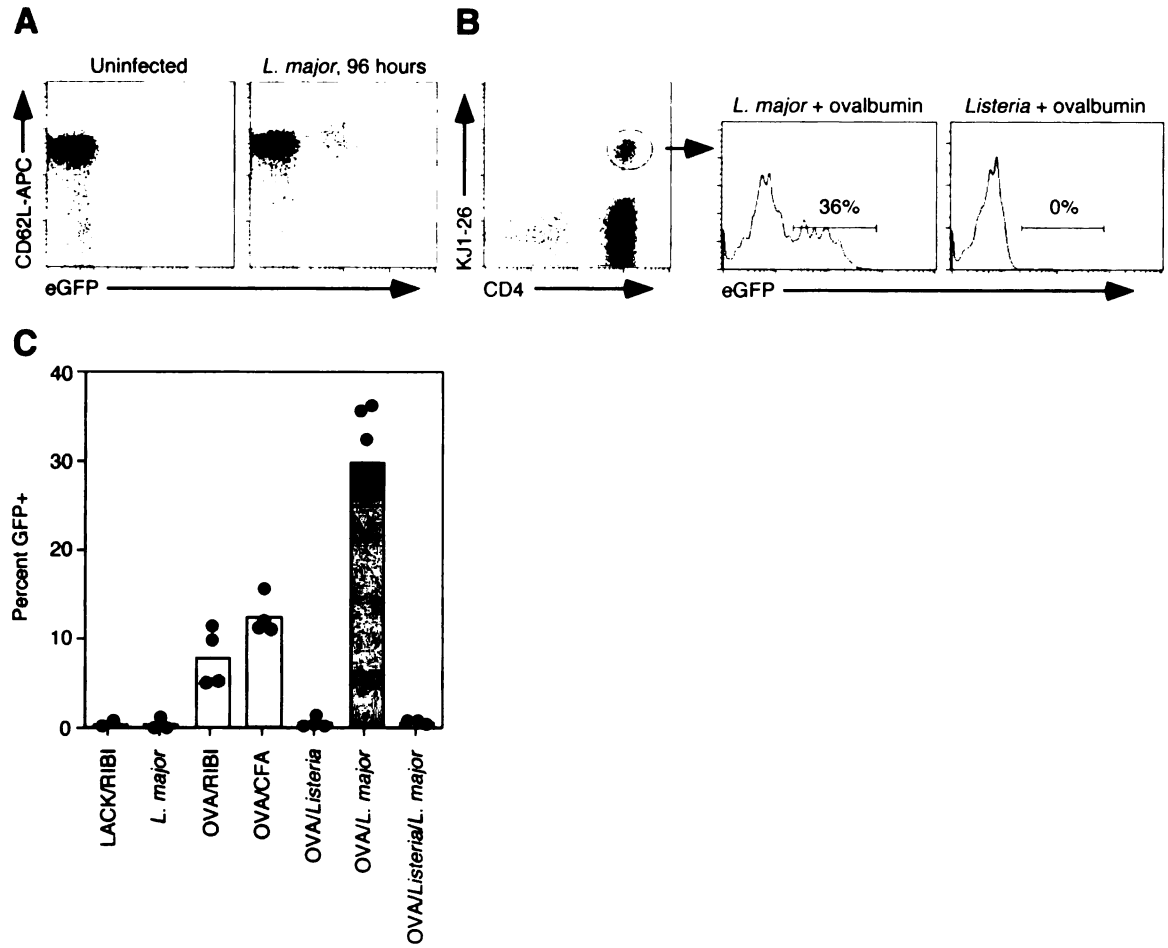
Figure 6. *Leishmania* activate IL-4 expression from naïve T cells.

a. CD62L and eGFP expression were examined on CD4 T cells from uninfected (left panel) and 96-hour infected (right panel) 4get BALB/c mice. 10,000 cells per plot are shown, and are representative of eight mice per group. Analysis of 4get B10.D2 mice gave identical results.

b. CD4+, KJ1-.26+ donor T cells were enumerated from the draining lymph nodes of wild-type BALB/c mice that received 10^6 highly purified, naïve DO11.10 4get T cells 24 hours before inoculation. FACS plot on the left is of [CD8/B220]- lymphocytes and indicates the gate used for analysis of eGFP expression (right panels).

c. eGFP (IL-4) expression by KJ1-26+ DO11.10 T cells was analyzed 96 hours after inoculation with the indicated antigens. Bars are means and dots represent individual mice.

Figure 6


A
Uninfected
L. major, 96 hours
CD62L-APC
eGFP
B
KJ1-26
CD4
L. major + ovalbumin
Listeria + ovalbumin
36%
0%
eGFP
C
Percent GFP+
40
30
20
10
0
LACK/RIBI
L. major
OVA/RIBI
OVA/CFA
OVA/Listeria
OVA/L. major
OVA/Listeria/L. major

Discussion

L. major infection results in a highly stereotyped host response characterized by a protective Th1 response in resistant inbred strains and an aberrant Th2 response in susceptible strains. The use of MHC congenic mice and analysis of a dominant peptide-specific response allowed careful assessment of host-specific determinants that might ultimately underpin the dichotomous T helper subset differentiation that determines the outcome of disease. A number of novel findings were uncovered. First, we use a dual fluorescent labeling approach to identify a biologically relevant naïve CD4 T cell precursor population for the LACK epitope that comprises approximately 15 cells in the popliteal lymph node of resting mice. Second, we identify the kinetics of early expansion, peaking at approximately 125-fold by 4 days, and demonstrate that cell expansion is coordinately regulated with IL-4 gene expression in the majority of peptide-specific responding cells. Third, despite the clear pathogenic nature of these cells in susceptible mice as shown here (Figure 2) and elsewhere (Julia et al., 1996; Launois et al., 1997; Pingel et al., 1999), we could discern no differences in the precursor frequency, expansion, or IL-4 gene expression in comparing susceptible and resistant mice. Lastly, *Leishmania* parasites efficiently activate IL-4 gene expression from naïve T cells even when administered with exogenous antigen. Taken together, these findings suggest that the ultimate differentiation into Th1 and Th2 cells that determines the outcome of infection must occur independently from the initial activation of naïve T cells in draining lymph nodes.

Our flow cytometry approach allowed us to characterize the initial expansion of LACK-specific T cells in the draining lymph nodes of normal mice. During the first 48

hours after infection, LACK-specific T cells expanded minimally, and none activated IL-4 expression (Figure 3b, 5a). Seeding recipient mice with a >1,000-fold excess of antigen-specific cells did not enable significant cell division during the first two days (Figure 3c). These data suggest that transit of antigen presenting cells to the draining lymph node and maturation of MHC-peptide complexes on the surface of APC, rather than T cell precursor frequency, are rate-limiting during the initiation of the response to *L. major* in both susceptible and resistant mice. Additionally, *Leishmania* does not appear to interfere with MHC class II/LACK peptide presentation *in vivo* (Prina et al., 1996).

Using sensitive IL-4 reporter mice, we found that >70% of LACK-specific T cells in draining lymph nodes activated IL-4 expression by 96 hours in both susceptible and resistant mice. These findings are in agreement with earlier studies demonstrating that a broad range of cytokines, including IL-4, are induced at early timepoints after *L. major* infection, regardless of genetic background (Morris et al., 1992; Reiner et al., 1994; Sommer et al., 1998), although we add quantitative insights to these earlier analyses. The finding that fully 5% of lymph node T cells activate IL-4 in resistant mice suggests alternative roles for this cytokine in the subsequent response. IL-4 promotes the maturation and survival of myeloid dendritic cells (Rissoan et al., 1999) and has been implicated in paradoxically enhancing production of the prototypic type 1-enhancing cytokine, IL-12, from dendritic cells (Hochrein et al., 2000). Indeed, pre-treatment of BALB/c mice with a high dose of recombinant IL-4 protein directed development of stable protective immunity, presumably through effects on dendritic cells (Biedermann et al., 2001). IL-4-deficient mice have impaired type 1 immune and cytotoxic CD8 T cell

responses in some experimental systems (Schuler et al., 1999; Schuler et al., 2001), including *L. major* infection (Noben-Trauth et al., 1996). Such data suggest that early IL-4 production may serve an important role in enhancing elements of antigen presentation required not only for type 2, but also type1, responses. Importantly, exogenous IL-4 at the time of infection does not reverse the inherent resistance of healer mice (Sadick et al., 1991).

We focused our studies on LACK-reactive cells because of prior evidence that IL-4 production by these cells is required for the BALB/c susceptible phenotype (Julia et al., 1996; Launois et al., 1997; Pingel et al., 1999). Despite their pathogenic role, these cells comprise less than 5% of the total CD4 T cells that express IL-4 early after infection. How then to explain their central role? As shown here, treatment with anti-IL-4 had a greater impact on IL-4 expression from the LACK tetramer-negative population than the LACK tetramer-positive population, which was little affected (Figure 5d). These findings are consistent with the interpretation that naive LACK-reactive cells may rely less on exogenous IL-4 for their initial activation of Th2 cytokine expression. In turn, these cells might serve to prime the spread of IL-4 production to CD4 clones of other specificities, as demonstrated elsewhere (Launois et. al., 1997). It is also possible that differences may occur at the level of IL-4 mRNA translation into protein. Since the 4get mice were generated to mark transcriptional activation of the IL-4 gene, it is conceivable that translational differences occur amongst LACK-specific and non-specific cells, or when comparing resistant and susceptible strains. Further work will be required to address these possibilities.

These studies leave open the question of when and from which precursors do protective IFN-γ-producing Th1 cells emerge in resistant mice? Quantitative RT-PCR analysis of transcripts from eGFP+ cells from resistant and susceptible mice revealed no significant IFN-γ mRNA, consistent with the activation of *bona fide* Th2 cells in both inbred strains (Figure 5c). Moreover, in preliminary experiments, the transfer of eGFP+ cells from 120 hour-infected B10.D2 mice into TCR Cα-/- B10.D2 recipients did not confer protection upon subsequent infection (D.S., data not shown). These data suggest that IFN-γ-expressing effector cells either diverge from a common precursor before detectable IL-4 expression in resistant mice, or emerge independently from the IL-4-expressing cells. Our findings differ from prior studies using single-chain LACK-specific TCR transgenic mice demonstrating that IL-4 expression correlated inversely with TCR affinity (Malherbe et al., 2000). Although we do not quantitate TCR affinity/avidity here, we find no differences in activation of the IL-4 gene when comparing either LACK-tetramer-specific cells or total CD4 T cells between resistant and susceptible mice. Despite these unexpected findings, we emphasize that the 4get backcrossed mice respond faithfully to *L. major* infection (Figure 4) and that we identify rigorously a biologically important subset of T cells as defined by their capacity to mediate disease progression in BALB/c mice (Figure 2).

Together with data from the infections, our experiments with DO11.10 x 4get T cells demonstrate that *Leishmania* parasites powerfully activate IL-4 expression from naïve T cells. It is thus tempting to speculate that elements of *L. major* direct a Th2-inducing pathway in antigen presenting cells. Co-inoculation of *L. major* with *Listeria* completely abrogated IL-4 expression from naïve T cells *in vivo*, presumably through a Toll-like

receptor (TLR)-dependent response to bacterial products. We are investigating whether the IL-4 response induced by *L. major* reflects a default response in the absence of TLR-dependent activation, or is an active process that is suppressed by Toll-like receptor recognition (Schnare et al., 2001). Despite the capacity of *L. major* to activate indiscriminately an IL-4 response from naïve T cells, resistant mice are able to constrain parasite growth by developing a polarized Th1 response while extinguishing the early Th2 response we document. Whether this switch is kinetically displaced or played out in peripheral tissues will be important issues that could be addressed with similarly designed IFN-γ reporter mice.

Experimental Methods

Mice. 4get mice containing a mutated knockin gene consisting of IL-4 linked through a viral IRES element to enhanced green fluorescent protein (eGFP) were generated as described (Mohrs et al., 2001). Mice were backcrossed 5 generations to either BALB/c or B10.D2 mice and mated to achieve homozygosity. BALB/c ovalbumin-peptide-specific DO11.10 T cell receptor transgenic mice (Murphy et al., 1990) and LACK-peptide-specific ABLE T cell receptor transgenic mice (Reiner et al., 1998) were crossed to 10th backcross BALB/c x TCR-Cα-deficient mice prior to use. DO11.10 mice were crossed to N8 4get BALB/c mice. BALB/c and B10.D2 mice (Jackson Laboratories, Bar Harbor, ME) were maintained under specific pathogen-free conditions in the animal care facility at the University of California San Francisco according to institutional guidelines.

Parasites and infection. *L. major* (strain WHOM/IR/-/173) was prepared and injected as 10^6 metacyclic promastigotes in Hank's balanced salt solution into the hind footpads of mice as described (Fowell et al., 1997). Designated mice were treated the day of infection with 3 mg neutralizing IL-4 antibody, 11B11, or isotype control. The course of disease was followed by measuring footpad thickness using a metric caliper. At the end of the experiments, dispersed popliteal lymph nodes, footpads and spleens were serially diluted into culture media and assayed for parasite growth after 2 weeks as described (Fowell et al., 1997).

I-A^d/LACK tetramers. Expression constructs for $A\alpha^d$ and $A\beta^d$ were as described (Scott et al., 1998). The $A\beta^d$ cDNA was modified to contain coding sequence for the LACK peptide - ICFSPSLEHPIVVSGSWD - followed by an SGSGS linker preceding the N-terminus, and for biotinylation sequence number 85 at the C terminus (Schatz, 1993).

For protein expression, stable lines of transfected Drosophila SC2 cells were expanded to 13 liters in 850 cm^2 roller bottles in Insect Xpress serum-free media (Biowhittaker, Walkersville, MD) by splitting at a 1:1 ratio every two days. After induction of protein expression by 0.7 mM copper sulfate for 3 days, cells were cleared by centrifugation and the supernatants were concentrated to 400 ml by tangential flow. Soluble I-A^d-peptide molecules were purified by affinity chromatography after overnight incubation at 4° C with Ni-NTA agarose beads, and further purified by anion exchange chromatography on a Resource Q column (Pharmacia, Peapack, NJ). Elution fractions were checked by SDS-PAGE for purity and stoichiometric expression of each chain. Following buffer exchange to 10 mM Tris/50 mM arginine, soluble I-A^d-peptide molecules were biotinylated at 1.8 mg/ml for 24 hours at 27° C according to manufacturer's instructions (Avidity, Denver, CO). The extent of biotinylation was determined following extensive buffer exchange to PBS using the HABA colorimetric reagent (Pierce, Rockford, IL) and depletion with streptavidin beads followed by SDS-PAGE. Biotinylation was 70-100% efficient for each batch of protein. Tetrameric complexes were generated by incubation of biotinylated monomers with streptavidin-PE (Biosource, Camarillo, CA) or streptavidin-APC (Molecular Probes, Eugene, OR) at a molar ratio of 6:1.

To assess T cell activation, biotinylated I-A^d-peptide molecules and purified anti-CD28 (37N51.1, 5 μg/ml) in PBS were incubated overnight at 4° C in 96-well u-bottom plates. After washing, 2 x 10^5 DO11.10 x TCR-$C\alpha^{-/-}$ or ABLE x TCR-$C\alpha^{-/-}$ T cells were added to each well in complete RPMI media (10% FCS, penicillin/streptomycin, L-glutamine, 2-mercaptoethanol) with 50 U/ml recombinant human IL-2. ^{3}H-thymidine

was added after 48 hours of incubation at 37° C, and cells were harvested 18 hours later for analysis on a scintillation counter.

Cells and procedures. For immunizations, 140 μg recombinant LACK protein, prepared as described (Mougneau et al., 1995), or ovalbumin (Sigma Chemical Co., St. Louis, MO), were injected subcutaneously at the base of the tail in MPL+TDM RIBI adjuvant (Corixa, Seattle, WA). Mice were rested for eight weeks before re-immunization with the same dose of the designated protein in adjuvant. Following immunization or infection, mice were killed at the indicated timepoints and single-cell suspensions were prepared from draining lymph nodes by dispersal through a 70 μm mesh. Live cell counts were estimated by trypan blue exclusion and cells were washed and resuspended at 4 x 10^8/ml in 5% FCS/PBS. Tetramers were prepared as described above and optimal, nonsaturating concentrations were determined for each batch by staining of ABLE x TCR-C$\alpha^{-/-}$ transgenic T cells specific for I-A^d/LACK. PE- and APC-labeled tetramers were mixed at [2x] and added to cells at a 1:1 ratio for a final cell concentration of 2 x 10^8/ml. Cells were stained at 25° C for 45 min before transfer to ice and addition of the following antibodies: $Alexa_{350}$-GK1.5 (anti-CD4; Molecular Probes), FITC-anti-CD25 (Pharmingen, San Diego, CA), APC-Cy7-anti-CD8 and APC-Cy7-anti-B220 (Caltag, Burlingame, CA). Cells were incubated with antibodies for 45 min on ice, washed, and resuspended at 4 x 10^7/ml in 5% FCS/PBS. Data acquisition and sorting were performed using a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO) using an ultraviolet laser for excitation of $Alexa_{350}$, a 488 nm laser for excitation of FITC, PE, and Cy5-PE, and a 647 nm krypton-argon laser for APC and APC-Cy7 excitation. Elliptical beam shaping optics were used to minimize physical overlap in laser interrogation of the

stream, and cross-beam compensation was performed electronically. For each sample, a 500 k event file was collected to set gates. A second file was then collected in which 100% of dual tetramer-positive events were saved with 1.0% of events that failed the gating criteria to allow accurate enumeration of tetramer-positive cells in a file of manageable size. Routinely, 3-10 million live events were analyzed per sample. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

For analysis of ovalbumin specific responses, DO11.10 x 4get CD4+, GFP-negative, T cells were sorted and an aliquot was stained with KJ1-26 to determine the frequency of clonotypic T cells in the sorted population. Cell volumes were adjusted to contain 10^6 KJ1-26+ cells in 250 µl PBS, and were injected intravenously into BALB/c recipients. After 1 day, mice were immunized in the hind footpad with 100 µg ovalbumin protein mixed with RIBI or CFA adjuvants, 10^4 *Listeria monocytogenes*, or 10^6 *L. major*, in a final volume of 50 µl. Popliteal lymph nodes were harvested after 96 hours and prepared for FACS analysis as described above.

Staining with α-galactosylceramide/CD1 tetramers was performed as described (Matsuda et al., 2000).

Quantitative fluorogenic RT-PCR for cytokines. Cells of the desired phenotype were sorted to >99% purity, and RNA was prepared using RNAzol according to manufacturer's instructions (Biotecx, Houston, TX). RT reactions were performed using Sensiscript RT (Qiagen, Valencia, CA). Primers and probes for quantitative PCR were as described (Grogan et al., 2001). Quantitative PCR was performed on an ABI-Prism 7700 DNA sequence analyzer, and signals for each cytokine were normalized to HPRT levels.

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Chapter III:

Rapid cytokine production by NK T and NK cells correlates with constitutive cytokine mRNAs

Abstract

NK T cells and NK cells are tissue lymphocytes that secrete cytokines rapidly upon stimulation. Here, we show that one mechanism underlying this rapid response is the presence of constitutive cytokine mRNAs in mature NK T and NK cells. Unlike conventional T cells, NK T cells accumulate both IL-4 and IFN-γ mRNA transcripts during thymic development, and populate the periphery with both cytokine loci modified by histone acetylation. Similarly, NK cells transcribe and modify the IFN-γ gene during development in the bone marrow. Developmental activation and maintenance of lineage-specific cytokine transcription suggests these lymphocytes enter their resident tissues poised for a rapid effector response.

Results and Discussion

Effective immunity involves regulated cytokine production by lymphocytes. At the site of infection, dendritic cells collect antigens and information regarding the nature of the invading pathogen using receptors that recognize conserved pathogen-specific motifs, and migrate to lymphoid organs to convey that information to naïve T cells (Guermonprez et al., 2002; Mellman and Steinman, 2001). Following selection among rare, naïve, antigen-specific precursors in the lymphoid organs, clonally expanded T cells migrate to the site of infection, where they provide effector function and long-term, antigen-specific memory (Masopust et al., 2001; Reinhardt et al., 2001). To fill the lag prior to antigen-specific effector function during the activation and expansion of adaptive immunity, several lymphocyte populations reside at sites of pathogen entry, where they are able to secrete cytokines within minutes to hours of infection. These cell types include NK T and NK cells in the liver, spleen and bone marrow (Bendelac et al., 1997; Cerwenka and Lanier, 2001). While recent studies have elegantly demonstrated how these cells are activated (Kronenberg and Gapin, 2002; Lanier, 1998; Natarajan et al., 2002), the mechanism by which they rapidly secrete cytokines remains unknown.

To enumerate cytokine-expressing cells directly, we generated gene knockin bicistronic reporter mice for IL-4 and IFN-γ. CD4 T cells from these mice, designated 4get (IL-4 GFP enhanced transcript) and Yeti (yellow enhanced transcript for IFN-γ), faithfully activate eGFP or eYFP expression, respectively, when cultured under Th2 or Th1 conditions . Moreover, the production of the endogenous cytokine is preserved (Mohrs et al., 2001), allowing for functional studies not possible in conventional knockin mice, which replace the cytokine gene with a heterologous marker gene.

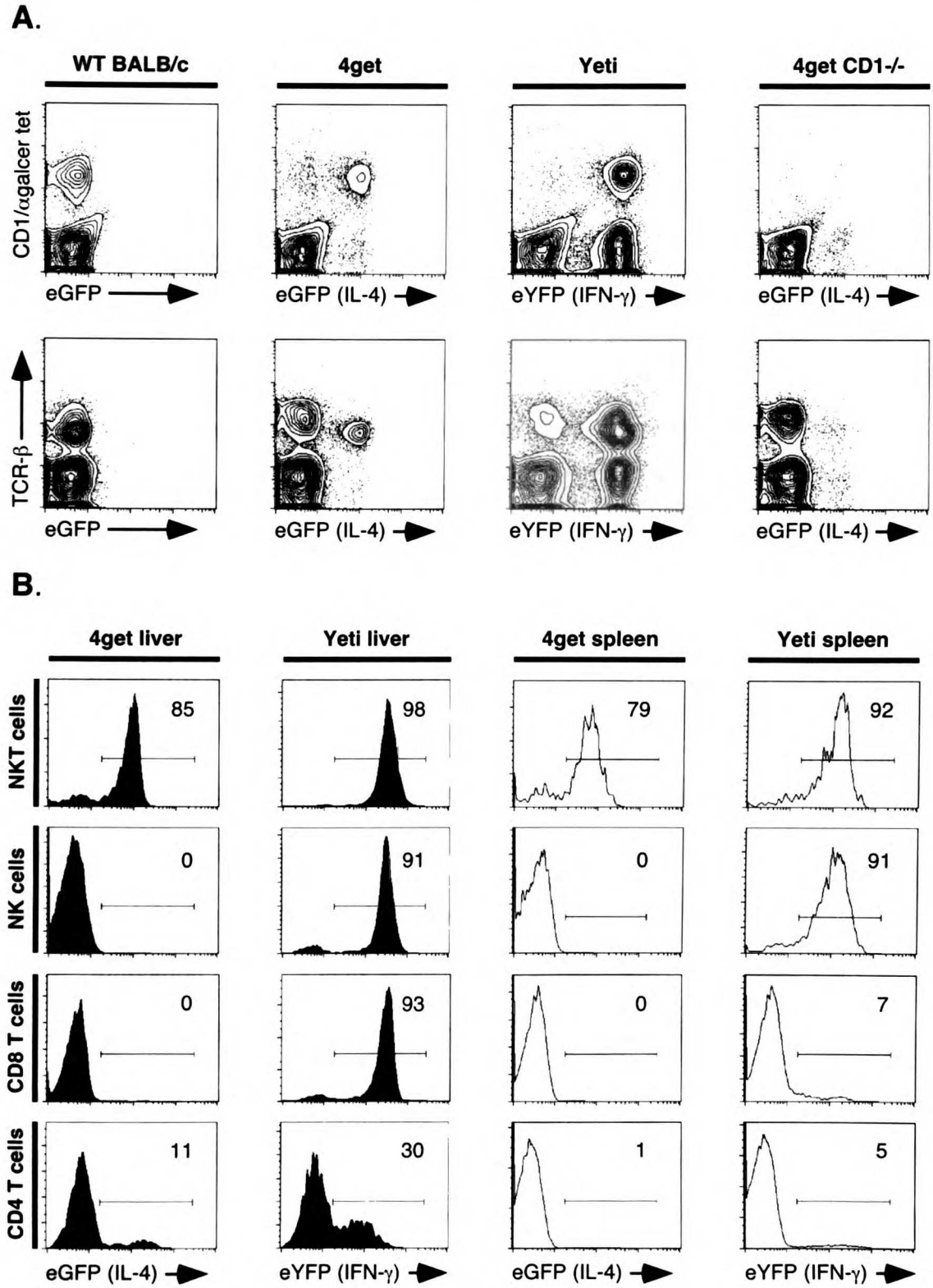
When examining the livers of naive, unchallenged, 4get and Yeti we found many resident lymphocytes were spontaneously fluorescent in the absence of specific immunization (Fig. 1). NK T cells with an invariant Vα14 arrangement, which recognize the glycolipid α-galactosylceramide (αGal/Cer) presented by CD1d, were the predominant eGFP-expressing cells in the livers of 4get mice (Fig. 1A). More than 80% of liver NK T cells expressed the IL-4 reporter (Fig. 1B). 4get x CD1-deficient mice lack NK T cells, and few residual eGFP+ cells remained in the liver of such mice (Fig. 1A). In contrast, Yeti mice had several populations of cells that spontaneously expressed eYFP, including NK T cells, NK cells and tissue CD8 T cells, as well as small numbers of tissue CD4 T cells (Fig. 1A, 1B). Since few CD8 T cells in the spleen expressed high-level eYFP fluorescence (Fig. 1B), we speculate that the high frequency of eYFP+ CD8 T cells represents differentiated effector cells that entered the liver in response to environmental antigens, or, alternatively, cells marked for death and removal in hepatic tissue (Crispe et al., 2000). In contrast, the cytokine profile of NK T cells and NK cells was similar in the spleen as assessed using the indicator mice: the majority of NK T cells were positive for both IL-4 and IFN-γ reporters, and NK cells contained IFN-γ but not IL-4 (Fig. 1B). The majority of conventional CD4 and CD8 T cells in the spleen expressed neither IL-4 nor IFN-γ markers (Fig. 1B). These data demonstrate that the majority of tissue effector cells in the liver, a critical organ for surveying pathogens from both systemic and portal blood circulations, spontaneously express cytokine reporters.

Figure 1. Spontaneous fluorescence in peripheral NK T and NK cells in naïve cytokine reporter mice.

a. Liver cells isolated from wild-type, 4get, Yeti and 4get x CD1d$^{-/-}$ mice were stained with CD1d/αGal/Cer tetramers and antibodies to TCRβ. The top panel displays CD1d/αGal/Cer tetramer staining versus the fluorescent cytokine reporter, and the bottom panel depicts TCRβ expression versus the fluorescent marker. FACS plots represent 5% contours with outliers, and are representative of 7-10 mice per group.

b. Liver and spleen cells were stained with phenotypic markers to identify major lymphocyte populations. The following phenotypes were used to identify the different cell populations: NK T cells: CD8- TCRβ+ CD1d/αGal/Cer tetramer+; NK cells: CD3- CD8- CD4- CD122+; CD8 T cells: CD4- CD1d/αGal/Cer tetramer- TCRβ+ CD8+; CD4 T cells: CD8- CD1d/αGal/Cer tetramer- TCRβ+ CD4+. Histograms are representative of three experiments. The electronic gates were set to contain < 1% of wild-type, non-(auto)-fluorescent controls.

Figure 1


A.
WT BALB/c
4get
Yeti
4get CD1-/-
CD1/αgalcer tet
TCR-β
eGFP
eGFP (IL-4)
eYFP (IFN-γ)
B.
4get liver
Yeti liver
4get spleen
Yeti spleen
NKT cells
NK cells
CD8 T cells
CD4 T cells
85
98
79
92
0
91
0
91
0
93
0
7
11
30
1
5
eGFP (IL-4)
eYFP (IFN-γ)

These observations were puzzling given that neither NK T cells nor NK cells store or secrete detectable cytokine protein before cognate stimulation (Kim et al., 2002; Matsuda et al., 2000a). So why are the cells from the bicistronic reporter mice so uniformly bright for the fluorescent markers, which also require translation of the bicistronic mRNA? We considered the design of the reporter mice, which have a viral IRES element driving translation of the fluorescent marker. IRES elements potentiate translation of mRNAs under conditions where global host translation is repressed, as in the case of poliovirus infection (Johannes et al., 1999). Moreover, a subset of endogenous cellular mRNAs also contain IRES elements, which allow their translation during times of repressed protein synthesis (Sachs et al., 1997). IRES-mediated translation involves initiation factors distinct from those required for conventional, cap-mediated translation (Sachs et al., 1997). Thus, the nature of the bicistronic reporter allows translation of eGFP in 4get mice and eYFP in Yeti mice under conditions where 5'-cap-dependent translation of cytokine mRNAs might be repressed. In support of this model, we found that eGFP fluorescence in Th2 cells from 4get mice required active translation and decayed with a half-life of 16 hours following incubation with protein synthesis inhibitors (D.B.S., unpublished data).

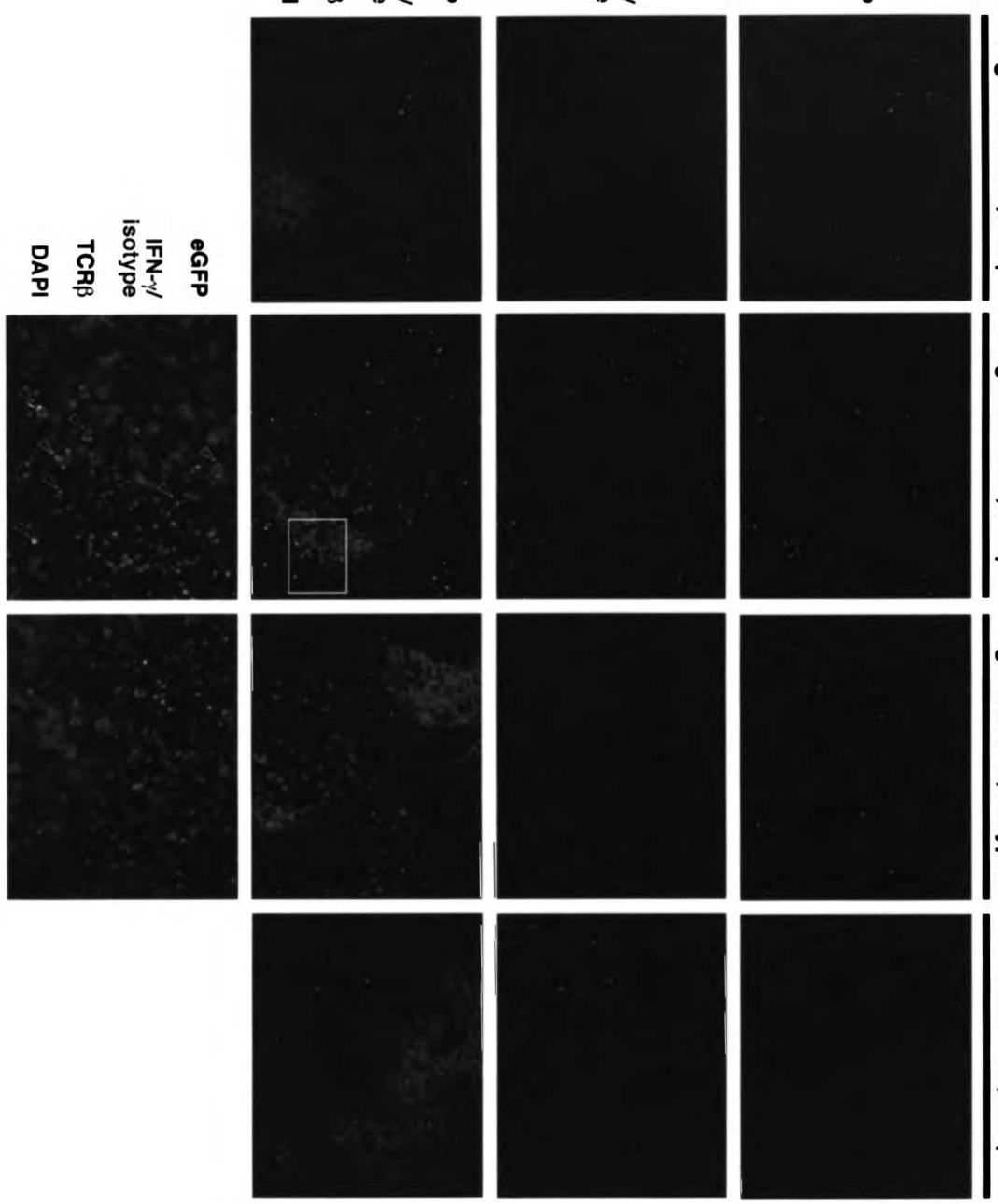
Activation of NK T cells *in vivo* using systemically administered anti-CD3 or αGal/Cer accounts for a burst of early cytokine production detected in tissues and serum (Yoshimoto and Paul, 1994), and these cells tend to make both IL-4 and IFN-γ (Matsuda et al., 2000a), consistent with a 'poised' effector state maintained by pre-existing cytokine transcripts. The vast majority of spontaneously eGFP+ cells in the spleens of unimmunized 4get mice are NK T cells, and analysis of Yeti mice suggests that these

cells also contain IFN-γ transcripts (Fig. 1). To confirm that NK T cells are the source of early IFN-γ , we injected 4get mice with a dose of anti-CD3 mAb demonstrated to activate cytokine secretion from NK T cells (Yoshimoto and Paul, 1994), and used immunohistochemistry to examine IFN-γ protein expression from the unmodified, wild-type *Ifng* gene in eGFP+ cells. In resting 4get spleen, eGFP-expressing cells were distributed outside the T cell zones, as delineated by TCRβ staining (Fig. 2, left column). The low level of spontaneous IFN-γ protein did not co-localize with eGFP-expressing cells in untreated mice. After anti-CD3 injection, a ring of IFN-γ protein-expressing cells appeared at the periphery of the periarteriolar lymphoid sheath (PALS) (Fig. 2, second column). In contrast to untreated mice, the majority of this IFN-γ protein signal co-localized with eGFP-expressing cells, consistent with their identification as NK T cells (Fig. 2, arrows). An isotype control antibody for IFN-γ did not stain eGFP-expressing cells from anti-CD3 treated 4get mice (Fig. 2, third column), and IFN-γ+ splenocytes from treated wild-type mice were not eGFP+ (Fig. 2, fourth column), confirming the specificity of the staining. These *in situ* data localize the NK T cell response to the periphery of the PALS, and support a model of poised mRNA transcripts nucleating a rapid cytokine response following stimulation *in vivo*.

Figure 2. *In situ* localization of the NK T cell cytokine response.

Spleen sections from untreated or anti-CD3 injected mice of the indicated genotypes were fixed and stained with antibodies to eGFP, IFN-γ or isotype control, and TCRβ. The first row indicates eGFP staining, the second row depicts IFN-γ protein staining, and the third row shows merged images of eGFP, IFN-γ or isotype control, TCRβ, and DAPI fluorescence. Magnification of the first three rows is 100x, and is 400x for the detail images in the fourth row. The white arrows in the high magnification image of anti-CD3 treated 4get spleen indicate eGFP (IL-4) co-staining with IFN-γ protein.

Figure 2


4get untreated, IFN-γ
4get anti-CD3, IFN-γ
4get anti-CD3, isotype
WT anti-CD3, IFN-γ
eGFP
IFN-γ/ isotype
eGFP
IFN-γ/ isotype
TCRβ
DAPI
eGFP
IFN-γ/ isotype
TCRβ
DAPI

One potential caveat of the reporter mice is that the bicistronic cytokine mRNA may be more stable than the wild-type mRNA because its 3'-untranslated region (UTR) differs from the endogenous 3'-UTR, which contains stability-regulating elements (Kontoyiannis et al., 1999; Neininger et al., 2002). However, comparison of 4get and wild-type IL-4 mRNAs in Th2 cells revealed comparable abundance and stability of the two messages (D.B.S., unpublished data). To further address this issue, we sorted NK T and NK cells from wild-type C57BL/6 mice and isolated RNA for quantitative RT-PCR. We compared the abundance of IL-4 and IFN-γ mRNA to that of highly purified, naïve CD4 T cells sorted from lymph nodes of the same mice. Consistent with the results from the reporter mice, NK T cells from wild-type mice contained over 1000-fold more IL-4 message and 100-fold more IFN-γ message than naïve CD4 T cells (Fig. 3A). NK cells also contained over 100-fold more IFN-γ transcripts than naïve CD4 T cells, but only background levels of IL-4 mRNA (Fig. 3A). The abundance of cytokine message in these cells approached that of Th1 and Th2 cells stimulated *in vitro* under optimal conditions for inducing these polarized cytokines (Fig. 3A). These data confirm findings in the reporter mice and suggest that NK T cells and NK cells reside in tissues poised with cytokine transcripts at levels comparable to *in vitro* differentiated effector CD4 T cells.

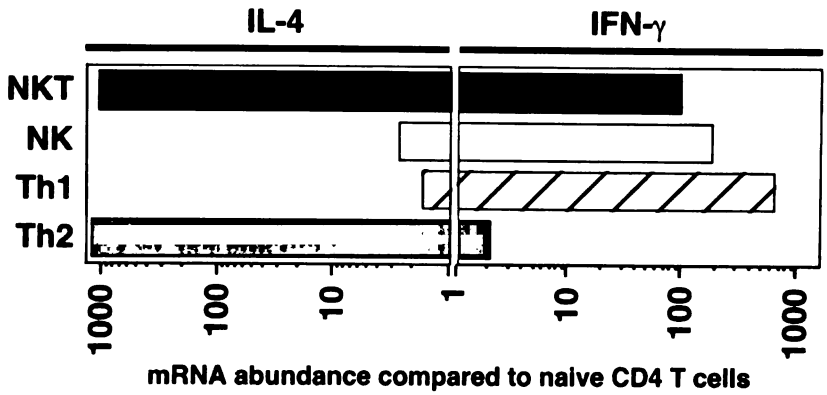
Figure 3. Analysis of spontaneous cytokine mRNAs and chromatin modifications in wild-type NK T and NK cells.

a. NK T and NK cells (see 1B for gating) from spleens of C57BL/6 mice were sorted to >99% purity and 5'-nuclease fluorogenic RT-PCR was performed on isolated total RNA. The abundance of cytokine mRNA for each sample was normalized to HPRT message and the ratio compared to the cytokine:HPRT ratio of highly purified, naïve CD4 T cells sorted from lymph nodes of the same animals. Th1 and Th2 cells were generated by *in vitro* stimulation of naïve DO11.10 x TCR-C$\alpha^{-/-}$ T cells with OVA peptide and polarizing cytokines on irradiated antigen presenting cells.

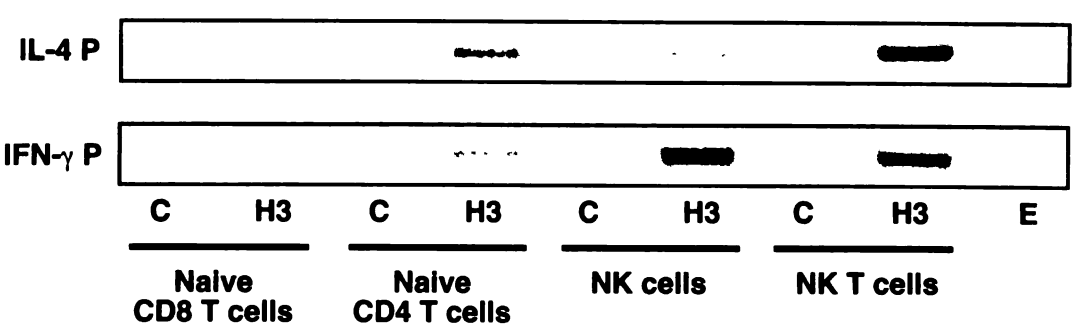
b. 10^7 cells of the indicated populations were sorted from the spleens and lymph nodes of C57BL/6 mice. Chromatin immunoprecipitations were performed using control rabbit IgG (C) or antibodies to acetylated histone 3 (H3). PCR was performed using primers to the IL-4 and IFN-γ promoters (Avni et al., 2002; Fields et al., 2002), and signals were normalized to input DNA. E: no template control for PCR.

Figure 3

A.


IL-4
IFN-γ
NKT
NK
Th1
Th2
1000
100
10
1
10
100
1000
mRNA abundance compared to naive CD4 T cells

B.


IL-4 P
IFN-γ P
C
H3
C
H3
C
H3
C
H3
E
Naive CD8 T cells
Naive CD4 T cells
NK cells
NK T cells

In CD4 T cells, commitment to the Th1 or Th2 lineage is accompanied by chromatin remodeling at the respective cytokine loci (Grogan et al., 2001). During Th1 differentiation, histone proteins surrounding the IFN-γ promoter become acetylated to promote accessibility to transcription factors; conversely, Th2 cells demonstrate acetylated histones at the IL-4 locus but not the IFN-γ gene (Avni et al., 2002; Fields et al., 2002). Because the amount of cytokine mRNA in NK T and NK cells is comparable to that of differentiated CD4 effector T cells (Fig. 2A), we analyzed the status of the chromatin surrounding the IL-4 and IFN-γ promoters. Sorted NK T and NK cells from unimmunized wild-type mice were used for chromatin immunoprecipitations with an antibody to the acetylated form of histone 3 (H3), a marker of accessible chromatin. Naïve CD8 T cells had minimal histone acetylation surrounding either cytokine promoter, and naive CD4 T cells had only low basal levels of acetylated H3 at both cytokine promoters (Fig. 3B). In contrast, NK cells had high levels of acetylated H3 at the IFN-γ promoter, but not the IL-4 promoter (Fig. 3B). NK T cells demonstrated high levels of H3 acetylation at both the IL-4 and IFN-γ promoters (Fig. 3B). These data show that NK T and NK cells in wild-type mice have evidence for chromatin modifications at cytokine genes that correlate with the presence of cytokine mRNAs in the respective lineages.

Since peripheral NK T and NK cells are spontaneously fluorescent in the reporter mice, we tracked their development in the thymus and bone marrow, respectively, to determine when these cytokine transcript profiles become established. Following stochastic rearrangement of the canonical Vα14 TCR and selection from double positive precursor thymocytes (Gapin et al., 2001), NK T cells undergo a maturation process characterized by sequential up-regulation of CD44 and NK1.1 (Benlagha et al., 2002).

We examined the thymi of two-week old 4get and Yeti mice, which have populations of both immature and mature NK T cells. Bendelac and colleagues recently demonstrated that NK T cells undergo a shift in αGal/Cer-stimulated cytokine production from IL-4 to IFN-γ as they mature to $CD44^{hi}$ $NK1.1^{hi}$ cells (Benlagha et al., 2002). In support of this model, we find that the fluorescence profile in 4get and Yeti thymic NK T cells precisely mirrored this shift (Fig. 4A). $CD44^{lo}$ $NK1.1^{lo}$ cells expressed high levels of eGFP (IL-4) and low levels of eYFP (IFN-γ). Concomitant with maturation to $CD44^{hi}$ $NK1.1^{hi}$ cells, eGFP (IL-4) expression was progressively down-regulated, and eYFP (IFN-γ) expression increased (Fig. 4A). The IL-4 message abundance in mature thymic NK T cells was low, but still higher than conventional CD4 T cells, which do not activate IL-4 transcription during thymic development (D.B.S., unpublished data). These data suggest that the developmental shift in cytokine production defined functionally by Bendelac and colleagues results from dynamic transcriptional regulation of IL-4 and IFN-γ during NK T cell maturation. Moreover, since the majority of NK T cells in the periphery express both IL-4 and IFN-γ mRNA (Fig. 1), these data support a model whereby NK T cells exit the thymus before terminal differentiation, which then occurs in peripheral tissues (Benlagha et al., 2002).

In conventional CD4 T cells, Stat6 signals are required to stabilize IL-4 production during Th2 differentiation (Grogan and Locksley, 2002). We examined 4get x Stat6-deficient NK T cells to determine if Stat6 has a similar role in NK T development. However, neither the percentage of liver NK T cells containing IL-4 mRNA nor the accumulation of these cells in the liver were affected by the absence of

Stat6 (Fig. 4B). Similar results were observed in the spleens of 4get x Stat6-deficient mice, and in 4get x IL-4 receptor α-deficient mice (D.B.S., unpublished data).

We next examined NK cell development in the bone marrow of two week-old Yeti mice. The earliest committed NK precursor is CD3-negative and expresses the β chain of the IL-2/15 receptor (CD122), but none of the other markers associated with the NK lineage, including NK1.1 and the immunomodulatory Ly49 and NKG2 receptors (Kim et al., 2002). We found that almost half of these earliest identifiable NK precursors (CD122+, NK1.1^{lo}) had already begun to express eYFP (IFN-γ) (Fig. 4C). Upon up-regulation of NK1.1, bone marrow NK-lineage cells became indistinguishable from their mature peripheral counterparts at the level of IFN-γ message expression (Fig. 4C and 1A). Up-regulation of IFN-γ transcription also preceded acquisition of other NK lineage markers, including α2 integrin, CD11b, and Ly49 receptors (D.B.S., unpublished data).

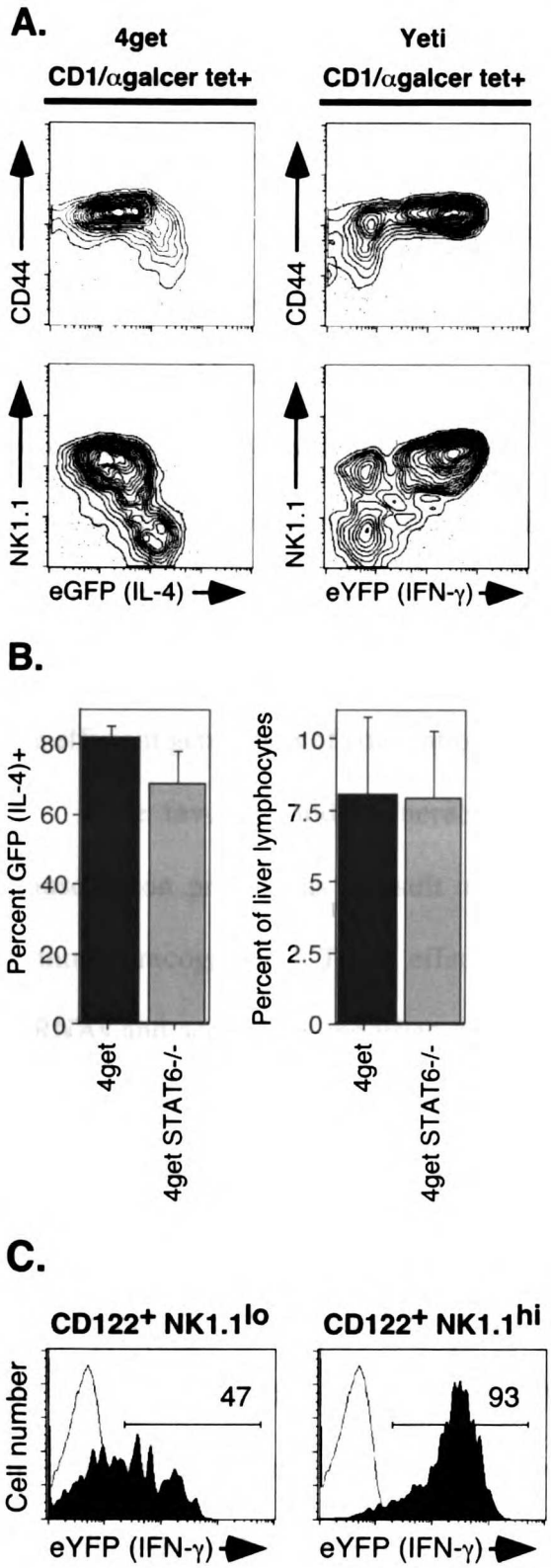
Figure 4. Developmental activation of cytokine mRNAs in NK T and NK cells.

a. Thymi were isolated from 2 week-old 4get and Yeti mice, and CD24 (HSA)-expressing cells were removed with biotinylated antibodies followed by streptavidin MACS bead depletion (Miltenyi Biotec, Auburn, CA). Gated CD1d/αGal/Cer tetramer+ cells were examined for expression of CD44, NK1.1, and the fluorescent cytokine reporter. FACS plots depict 5% probability contours with outliers, and are representative of three mice from each group.

b. Liver lymphocytes were isolated from 4get mice or 4get x Stat6-/- mice. In the left panel, the percentage of CD1d/αGa./Cer tetramer+ cells expressing eGFP (IL-4) was compared. In the right panel, the percentage of NK T cells in the liver was enumerated.

c. Bone marrow cells were isolated from the femurs of 2 week-old Yeti mice. NK lineage cells were identified as [CD3, TCRβ, CD4, CD8, B220]- CD122+. eYFP (IFN-γ) expression was examined in NK1.1- and NK1.1+ NK lineage cells (filled black histograms) and compared to the same cells from wild-type mice (white histograms). Data are representative of five animals.

Figure 4


A.
4get
CD1/αgalcer tet+
Yeti
CD1/αgalcer tet+
CD44
CD44
NK1.1
NK1.1
eGFP (IL-4)
eYFP (IFN-γ)
B.
Percent GFP (IL-4)+
80
60
40
20
0
4get
4get STAT6-/-
Percent of liver lymphocytes
10
7.5
5
2.5
0
4get
4get STAT6-/-
C.
CD122+ NK1.1lo
CD122+ NK1.1hi
Cell number
47
93
eYFP (IFN-γ)
eYFP (IFN-γ)

We have demonstrated that NK T cells and NK cells, distinguished by their ability to mobilize effector cytokines rapidly following immunization or infection, reside in the periphery spontaneously poised with abundant cytokine transcripts. Modification of the respective cytokine loci in a manner promoting constitutive access by transcription factors correlates with the presence of cytokine mRNAs. Unlike conventional T cells, NK T and NK cells activate transcription of cytokine genes during early development in the thymus and bone marrow, respectively. In the case of IL-4 for NK T cells, neither the percentage of IL-4+ cells nor the tissue localization of NK T cells was affected by the absence of Stat6 or the IL-4 receptor (Fig. 3B; D.B.S., unpublished data). These data suggest that the lineage-specific developmental modification of the cytokine loci proceeds by a process independent of these pathways, which, for IL-4, seem critical for the efficient generation of conventional tissue CD4 T cells (Fields et al., 2002).

We favor a model whereby NK T and NK cells activate distinct cytokine transcription profiles as a result of lineage-specific developmental cues, rather than pathogen recognition. These effector lymphocytes, by virtue of pre-formed cytokine mRNAs and accessible, modified cytokine loci, arm the periphery with the capacity for rapid, programmed responses that may serve two important functions. First, their immediate, stereotyped effector function could provide protection at sites of pathogen entry during the time required for the expansion and migration of antigen-specific, conventional T cells. Second, the cytokines elaborated by these cells may in turn affect the differentiation of conventional T cells (Kronenberg and Gapin, 2002). It will be interesting to determine if other populations of tissue lymphocytes, including intraepithelial CD8$\alpha\alpha$ T cells (Leishman et al., 2002), and dendritic epidermal $\gamma\delta$ T cells

(DETCs) (Jameson et al., 2002), arm mucosal and epithelial barriers with pre-formed cytokine transcripts. We speculate that conventional memory T cells may adopt a similar fate following differentiation and migration into tissues, thus layering the capacity for antigen specificity onto the rapid memory response .

Experimental Methods

Generation of IFN-γ reporter mice. A 7 kb ClaI-BamHI fragment was isolated from a 129/SvJ BAC clone (Research Genetics, Huntsville, AL) containing exons 2-4 and 2.5 kb of 3' untranslated sequence of the *Ifng* gene. BamHI and SalI sites were introduced downstream of the translational stop and upstream of the endogenous polyadenylation site using PCR-mediated mutagenesis, and the mutated fragment was inserted into pgkTK containing herpes simplex thymidine kinase for negative selection (Tybulewicz et al., 1991). A bicistronic reporter cassette was used containing an ECMV IRES element, modified as described (Mohrs et al., 2001), which was cloned 5' of eYFP followed by a bovine growth hormone polyadenylation signal (Clontech, Palo Alto, CA). A loxP-flanked neomycin resistance cassette, derived from pL2neo2 (Gu et al., 1993) was placed at the 3' end to generate the final selectable cassette, which was cloned into the BamHI and SalI sites in the mutated Ifng gene to generate the final targeting construct. The NotI-linearized construct was electroporated into PrmCre ES cells, which express Cre recombinase under control of the protamine promoter (O'Gorman et al., 1997) and selection was achieved using 400 μg/ml G418 and 2 μM gancyclovir. Resistant ES clones were screened using Southern blotting and targeted clones were injected into C57BL/6 blastocyts to create chimeric mice. The neomycin resistance cassette is deleted in the male germline by Cre-mediated recombination. Chimeric males were bred to wild-type C57BL/6 and BALB/c mice and offspring were selected using Southern blotting for the mutated knockin *Ifng* allele and for deletion of the neomycin cassette. Mice were backcrossed four generations to C57BL/6, and used as heterozygotes. These mice will be

described in more detail elsewhere (M. M. Mohrs, K. Mohrs, D. B. Stetson, R. L. Reinhardt, R. M. Locksley, manuscript in preparation.).

Liver lymphocyte isolation and FACS analysis. Livers from the indicated mice were perfused with phosphate buffered saline (PBS) and mashed through a 400 μm mesh screen before centrifuging over a 40%/60% Percoll step gradient. Lymphocytes isolated from the interface were washed and resuspended in staining buffer (5% fetal calf serum in PBS) supplemented with Fc Block (BD PharMingen) and 25 μg/ml neutravidin (Molecular Probes). CD1/αgalcer tetramers were generated as described (Matsuda et al., 2000b), and used at optimal concentration to stain cells for 20 minutes at room temperature. Following cooling on ice, cells were stained for an additional 25 minutes with the indicated antibodies. Antibodies to the following cell surface markers were used in this study to identify lymphocyte populations, and were purchased from BD PharMingen, unless otherwise indicated: TCRβ-APC, CD122-PE, CD122-biotin, NK1.1-PE, NK1.1-biotin, DX5-APC, Ly49A/D-PE, Ly49G2-APC, CD11b-APC, CD62L-APC, CD8α-biotin, CD19-biotin, CD24-biotin, CD4-TriColor (Caltag), CD4-APC-Cy7 (Caltag), CD8α-APC-Cy7 (Caltag), streptavidin-APC (Molecular Probes).

In situ localization of NK T cells. Mice were injected with 1.33 μg of anti-CD3 monoclonal antibody (2C11, BD PharMingen) intravenously in PBS via the tail vein 90 minutes prior to sacrifice. Spleens were dissected, placed directly into 4% paraformaldehyde, and incubated at 4°C for two hours. Following an overnight wash in PBS, organs were frozen in O.C.T. embedding compound. Seven μm sections were cut with a Leica CM 1850 cryomicrotome (Leica Microsystems Inc., Deerfield, IL), and placed directly onto charged glass slides (Fisher Scientific, Pittsburgh, PA). Endogenous

peroxidase activity was quenched by incubation in 1% H2O2 and 0.1% azide for 1 hour, followed by Fc-block with 1% mouse and rat serum, and avidin and biotin (Vector Laboratories). Sections were then incubated with rabbit anti-GFP polyclonal antibody (ab 6556, Novus Biologicals, Littleton, Colorado), followed by biotinylated donkey anti-rabbit F(ab')2 (Jackson ImmunoReseach,West Grove,PA), streptavidin-peroxidase, and FITC tyramide from the TSATM -fluorescein kit according to manufacturer's instructions (Perkin Elmer,Boston, MA). Sections were quenched and blocked as described above and incubated with biotinylated anti-TCRβ antibody (H57) followed by streptavidin-Cy5 (Caltag). Biotin was again blocked by treatment with avidin and biotin, and sections were incubated sequentially with biotinylated anti-IFN-γ antibody (XMG1.2, BD PharMingen), streptavidin-peroxidase, and biotinyl tyramide. Deposited biotin was detected by streptavidin-Cy3 (Caltag), and nuclei were counterstained for five minutes with a 10 μg/ml solution of 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Roche/Boehringer-Mannheim, Indianapolis, IN) in PBS. Sections were mounted in vectashield (Vector Laboratories). Digital images in the DAPI, FITC, Cy3, and Cy5 channels were collected using the Marianas digital microscopy workstation (Intelligent Imaging Innovations, Denver, CO). Images were converted to RGB, colored, and overlaid using Adobe Photoshop 5.5 software. IFN-γ levels and eGFP levels were set against isotype control (biotinylated rat IgG1) and wild type BALB/c tissue, respectively.

Quantitative RT-PCR. NK T and NK cells were sorted from the spleens of C57BL/6 mice to >99% purity using a MoFlo high speed cell sorter (Cytomation, Inc., Fort Collins, CO). Th1 and Th2 cells were generated by stimulating DO11.10 x TCR Cα-/-

transgenic T cells for five days as described (Grogan et al., 2001). Total RNA was isolated using RNAzol (Biotecx, Houston, TX), and quantitative RT PCR was performed using primer/probe sets specific for IL-4, IFN-γ, and HPRT as described (Grogan et al., 2001).

Chromatin immunoprecipitations. Spleen cell suspensions from wild-type C57BL/6 mice were labeled with biotinylated antibodies to CD8α, CD19, and CD24; these cells were magnetically depleted using strepdavidin BioMag particles (Qiagen, Valencia, CA). The remaining cells were stained as decribed above, and NK T and NK cells were sorted to >99% purity. Naive, $CD62L^{hi}$ CD4 and CD8 T cells were sorted from the lymph nodes of the same animals. 10^7 cells of the indicated populations were fixed and sonicated with three one minute pulses to shear genomic DNA. Chromatin immunoprecipitations using control rabbit IgG or antibodies to the acetylated form of histone 3 were performed according to manufacturer's instructions (Upstate Biotechnology, Lake Placid, New York). Recovered DNA was resuspended in 50 μl of 10mM Tris, pH 8.4, and 4 μl was used to template a 35 cycle PCR using primers to the IL-4 promoter (Avni et al., 2002) or the IFN-γ promoter (Fields et al., 2002). Products were resolved on 1.9% agarose gels, and images were captured with a Gel Doc equipped with Molecular Analyst software (BioRad). Input DNA from the control immunoprecipitation was amplified using the same primer sets to verify equal amounts of starting material for each cell population.

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Chapter IV:

A checkpoint between IL-4 transcription and translation regulates Th2 cell effector function

Abstract

Transcriptional control of cytokine genes in CD4 T cells has been intensely scrutinized, yet little is known about the relative contribution of translation to cytokine production. Here we show that most memory Th2 cells contain abundant, stable IL-4 transcripts which are not translated until cognate restimulation. Polyribosome analysis confirmed that translation efficiency of IL-4 is regulated in Th2 cells in a T cell receptor-dependent manner, and revealed a global suppression of translation initiation in poised Th2 cells. Phosphorylation of the eukaryotic initiation factor eIF2α on serine 51, a negative regulatory site, correlated with translation inhibition. Introduction of dominant negative forms of two eIF2α kinases, PERK and GCN2, into Th2 cells revealed a role for the integrated stress response in effector T cell survival. Global and cytokine-specific regulation of translation initiation are key elements of Th2 cell effector function.

Results and Discussion

The differentiation of naive T cells into cytokine-producing effector cells is important for immunity. In order to be effective, cytokine production must be rapid and focused to the microenvironment where it is needed. Systemic or inappropriate elaboration of cytokines can result in pathology (Kontoyiannis et al., 1999). While much work has focused on the dynamic interplay between transcription factors which specify or antagonize cytokine gene expression programs (Ho and Glimcher, 2002), still little is known about control of cytokine translation and its contribution to T cell effector function.

We recently found that NK T and NK cells, two lymphocyte populations capable of rapid cytokine production, are poised with preformed cytokine mRNAs (Chapter III). We therefore wondered if conventional memory T cells, distinguished in part from their naive precursors by their ability to rapidly produce cytokines, adopt a similar fate after differentiation. We examined the lymph nodes of unimmunized 4get mice and found a population of memory ($CD62L^{lo}$) CD4 T cells with spontaneous eGFP fluorescence (Figure 1a). These cells did not express NK1.1 or stain with CD1/αgalcer tetramers, and thus were not NK T cells (data not shown). We sorted these cells, along with naive (GFP-, CD62Lhi) and non-GFP+ memory cells, and tested their ability to produce IL-4 protein in ELISPOT assays (Figure 1a). In the absence of TCR restimulation, none of the three populations produced detectable amounts of IL-4 protein. Following TCR restimulation, neither the naive T cells nor the GFP- memory cells were capable of producing significant amounts of IL-4 protein. In contrast, the number of IL-4 protein producing cells in the GFP+ memory population increased over 500-fold during a four

hour TCR restimulation (Figure1a). We confirmed these results in wild type mice and found that comparable numbers of memory cells, although not separable by eGFP expression, produced IL-4 protein (Figure 1a, right panels). These data suggest that more than 90% of memory phenotype Th2 cells, defined by their ability to rapidly produce IL-4 protein with only TCR restimulation, contain preformed IL-4 mRNAs.

We next examined IL-4 mRNA and protein expression in Th2 cells generated *in vitro* from wild-type mice. Six days after antigen stimulation, IL-4 mRNA detected by quantitative RT-PCR was approximately 1000-fold more abundant in "poised" Th2 cells than in naive T cells, but IL-4 protein producing cells were virtually undetectable (Figure 1b, c). These IL-4 transcripts were spliced and polyadenylated, because we used oligo dT to prime the reverse transcriptase reaction and primers which spanned introns in the quantitative PCR assay. Upon restimulation, IL-4 message abundance increased 30-fold, but the number of cells which produced protein increased over 500-fold (Figure 1b, 1c). When we included the transcriptional inhibitor actinomycin D during the restimulation of T cells, the thirty fold induction of IL-4 message was ablated, but the preformed mRNAs did not decay, suggesting that the IL-4 transcripts in the poised state are relatively stable. Interestingly, actinomycin D competely inhibited the production of IL-4 protein during restimulation. Cycloheximide, an inhibitor of translation, also blocked the increase in IL-4 protein production (Figure 1c). Taken together, these data suggest that preformed IL-4 mRNAs are necessary, but not sufficient, for rapid IL-4 protein production.

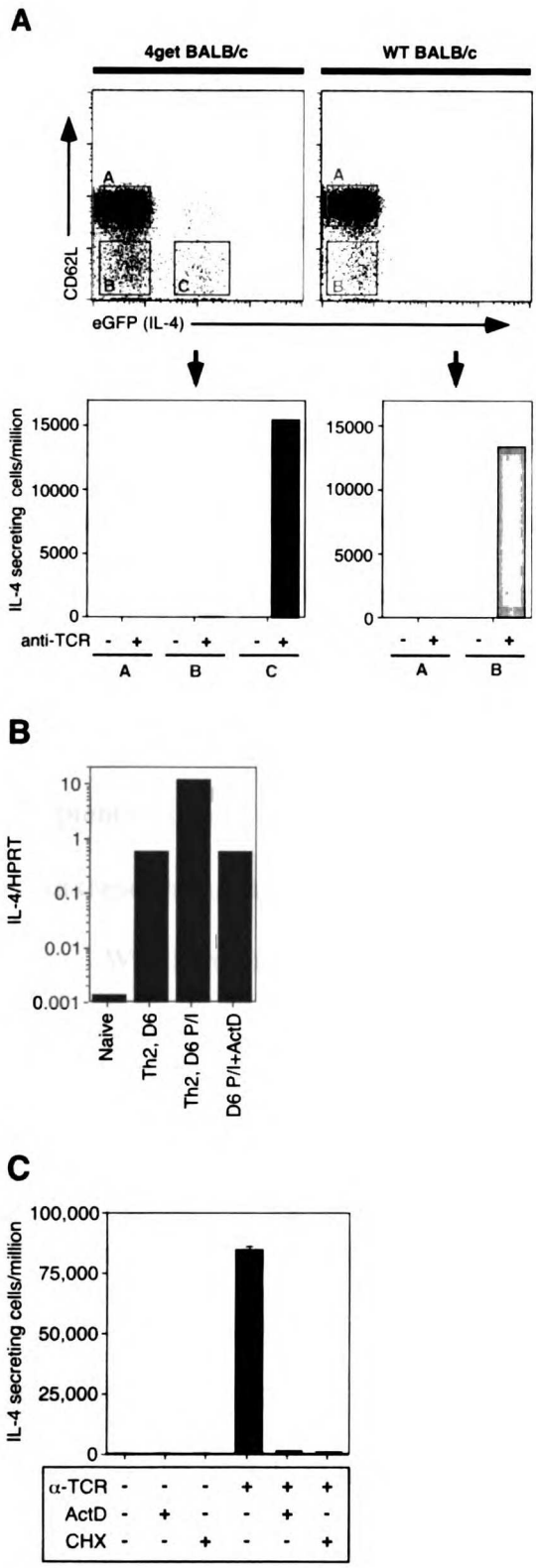
Figure 1. Preformed IL-4 mRNAs are necessary, but not sufficient, for rapid cytokine production by memory Th2 cells.

a. Peripheral lymph node CD4 T cells from 4get BALB/c mice (left panel) and WT BALB/c mice were examined for expression of CD62L and eGFP. The indicated populations were sorted to >99% purity and used in 4 hour IL-4 ELISPOT assays in the absence or presence of plate-bound anti-TCRβ antibody. IL-4 spots were counted and are represented as spot forming cells per million input cells. Data are representative of five independent experiments.

b. Naive, DO11.10 x $Ca^{-/-}$ transgenic T cells were stimulated under Th2 conditions with ovalbumin peptide and irradiated APCs for six days (Grogan et al., 2001). Some cells were restimulated with PMA and ionomycin in the absence or presence of 5 μg/ml actinomycin D. RNA was harvested and used for quantitative 5' nuclease fluorogenic RT-PCR (Grogan et al., 2001). mRNA abundance is expressed as the ratio of IL-4 to HPRT to account for differences in the amount of starting material in each sample. Data are representative of three experiments with comparable results.

c. Th2 cells stimulated *in vitro* for six days as described above were washed, counted, and used in 4 hour ELISPOT assays. Where indicated, plate-bound anti TCRβ, 5 μg/ml actinomycin D, and 100 μg/ml cycloheximide were included. Data represent means and standard deviations of duplicate wells, and are representative of two experiments.

Figure 1


A
4get BALB/c
WT BALB/c
CD62L
eGFP (IL-4)
A
B
C
IL-4 secreting cells/million
15000
10000
5000
0
anti-TCR - + - + - +
A B C
A B
B
IL-4/HPRT
10
1
0.1
0.01
0.001
Naive
Th2, D6
Th2, D6 P/I
D6 P/I+ActD
C
IL-4 secreting cells/million
100,000
75,000
50,000
25,000
0
α-TCR - - - + + +
ActD - + - - + -
CHX - - + - - +

Although the eGFP+ CD4 T cells capable of rapid IL-4 production identified in unimmunized 4get mice have a memory phenotype and presumably arose through stimulation by environmental antigens, nothing is known about their specificity or history. To address this issue using T cells of known specificity, we stimulated DO11.10 x 4get transgenic T cells with ovalbumin peptide under Th2 conditions *in vitro* for six days, and then purified the eGFP+ Th2 cells by sorting before intravenous transfer into normal, 4get BALB/c recipients. After resting the mice for five weeks, we identified the transferred cells using the clonotypic KJ1.26 antibody and examined the fraction which maintained expression of IL-4 message as assessed by eGFP fluorescence. Interestingly, more than two thirds of the transgenic memory T cells still expressed eGFP five weeks after transfer (Figure 2a). Naive, eGFP- DO11.10 x 4get T cells rested in normal mice for the same duration did not begin to express eGFP (Figure 2a), confirming that the recipient mice did not contain antigens capable of activating de novo IL-4 expression from these transgenic T cells.

We then sorted the eGFP+ and eGFP- memory DO11.10 4get Th2 cells and assayed their ability to produce IL-4 protein. Without TCR restimulation, we failed to detect a single IL-4 producing cell in either population (data not shown). Only two hours after restimulation, we observed that eGFP+ cells were 75-fold more potent at producing IL-4 protein than the T cells which had extinguished eGFP expression (Figure 2b). By four hours, a small but significant number of eGFP- cells produced IL-4, consistent with their original isolation as IL-4-expressing cells. However, at all timepoints tested, the memory cells which remained positive for eGFP expression during the five week experiment were far more potent producers of IL-4 protein (Figure 2b). Thus, the

maintenance of IL-4 transcripts in most memory Th2 cells endows them with temporal and quantitative advantages over memory cells that extinguish IL-4 transcription.

During restimulation of primed or memory Th2 cells, IL-4 mRNA transcription was induced 30-fold, but the number of cells that produced IL-4 protein increased between 300 and 1000 fold in several experiments (Fig 1 and data not shown). We therefore hypothesized that translation of IL-4 message into protein was restricted in the poised state, and that the large discordance between mRNA and protein induction following restimulation might be accounted for by a concomitant increase in translation efficiency. We therefore prepared extracts from poised and restimulated Th2 cells for polyribosome analysis to examine IL-4 translation at a biochemical level.

We were surprised to find that the polysome profiles of both poised and restimulated Th2 cells indicated very low levels of global translation, as evidenced by the abundant representation of free ribosomal subunits and paucity of polysome-bound mRNAs (Figure 3). To control for sample preparation, we prepared polysome extracts from Jurkat T cells and found that the translation apparatus in this human T cell line was far more activethan the primary mouse Th2 cells (Figure 3). Interestingly, treatment of Jurkat cells with thapsigargin, which induces endoplasmic reticulum (ER) stress and global translation inhibition by depletion of calcium stores, collapsed their polysome profile, which then resembled poised Th2 cells (Figure 3). Northern blot analysis of GAPDH mRNA from fractions of poised and restimulated Th2 cell gradients revealed that GAPDH abundance and distribution was insensitive to the global translation attenuation, and confirmed the integrity of the gradients. These data indicate that the translation apparatus in differentiated Th2 cells is restricted.

Figure 2. Stable IL-4 mRNAs in memory Th2 cells correlate with a rapid recall response.

a. DO11.10 x 4get T cells were stimulated for 6 days under Th2 conditions *in vitro*. EGFP (IL-4)+ cells were then sorted to >99% purity and transferred into normal, 4get recipients (top panels). Five weeks later, donor T cells were identified with KJ1.26 antibody and assessed for maintenance of eGFP expression. Naive DO11.10 4get T cells (bottom panels) were transferred into normal 4get recipients as a negative control. FACS plots are 5% probability contours of CD8/B220-, KJ+ T cells. Data are representative of four experiments. Similar results were seen seven weeks after transfer.

b. eGFP+ and eGFP- transgenic T cells were sorted from lymph nodes of recipient mice five weeks after transfer and used in ELISPOT assays with plate-bound anti-TCRβ restimulation for the indicated durations. In control ELISPOT experiments, none of the cells produced IL-4 protein in the absence of restimulation (data not shown).

Figure 2

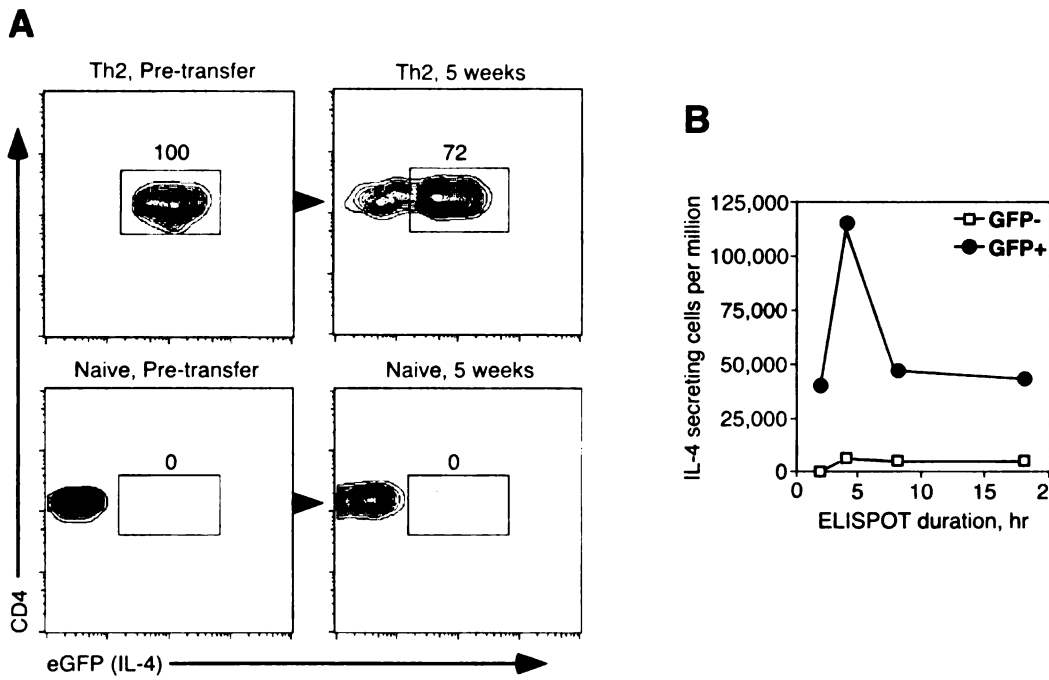
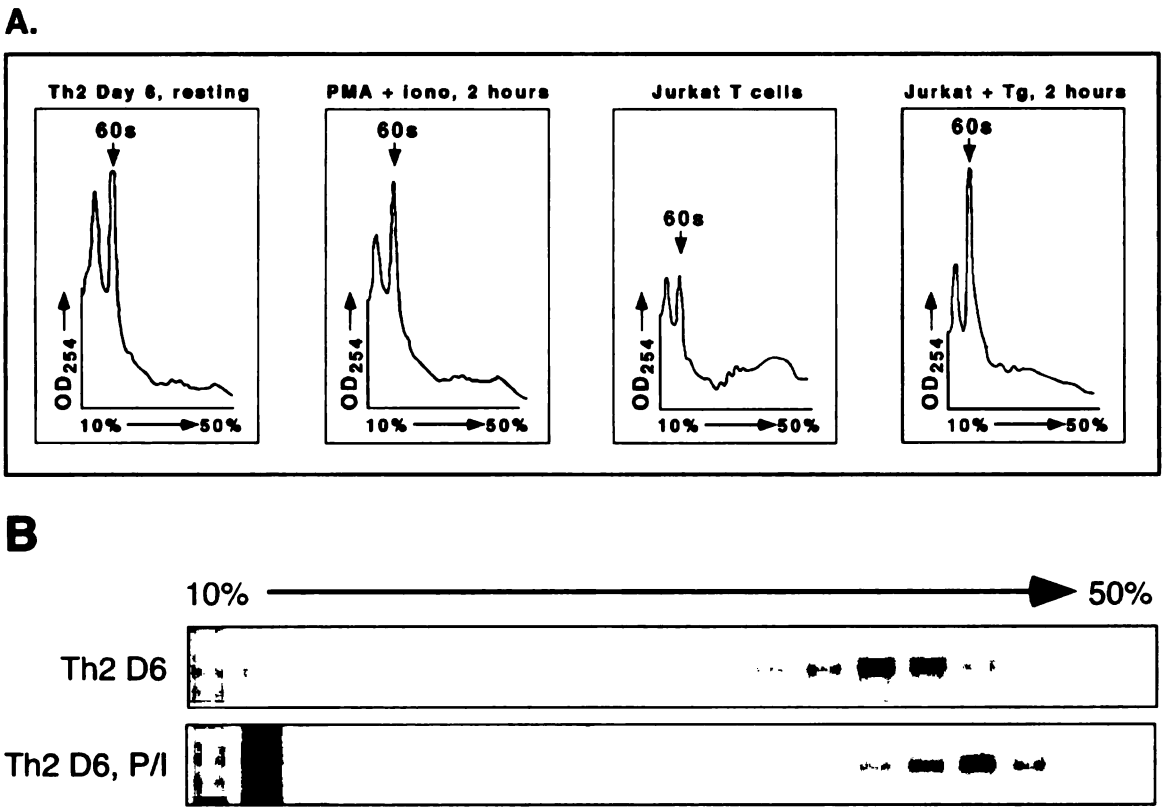

A
Th2, Pre-transfer
Th2, 5 weeks
100
72
Naive, Pre-transfer
Naive, 5 weeks
0
0
CD4
eGFP (IL-4)
B
IL-4 secreting cells per million
125,000
100,000
75,000
50,000
25,000
0
GFP-
GFP+
0
5
10
15
20
ELISPOT duration, hr

Figure 3. Polysome analysis of Th2 cells reveals restricted global translation

a. Extracts from Th2 cells, either poised or restimulated, were centrifuged over a continuous 10-50% sucrose gradient to resolve ribosome-bound mRNAs. RNA abundance was monitored by measuring the OD_{260} as the gradient was harvested. The position of the 60s free ribosomal subunit is indicated by arrows. Control Jurkat T cells or Jurkats treated with 400 nM thapsigargin for two hours, were processed similarly. Data are representative of five independent experiments.

b. 19 fractions were collected from poised and restimulated Th2 gradients, and RNA was isolated and separated on agarose gels for northern blot analysis with a probe to the coding sequence of GAPDH. Data are representative of three experiments.

Figure 3


A.
Th2 Day 6, resting
PMA + Iono, 2 hours
Jurkat T cells
Jurkat + Tg, 2 hours
60s
OD254
10%
50%
B
10%
50%
Th2 D6
Th2 D6, P/I

Based on the paucity of mRNAs bound to ribosomes in both poised and restimulated Th2 cells, it appeared that global translation was repressed in Th2 cells. It is intruiging to note that ionomycin and brefeldin A, which are commonly used to elicit maximal cytokine protein expression and trap the otherwise secreted protein in cells, respectively, are both potent inducers of ER stress and attenuation of translation initiation (Nakagawa et al., 2000). Both of these agents inhibit translation via phosphorylation of the α subunit of the initiation factor eIF2 by PERK, an ER-resident kinase that balances translation with the load of nascent client proteins (Harding et al., 1999). eIF2 delivers the methioninyl tRNAi to the 40s ribosomal subunit, and hydrolizes GTP to initiate translation. Phosphorylation of eIF2α on serine 51 prevents recycling of spent GDP for GTP, thus attenuating translation initiation. The fact that IL-4 can be robustly translated upon cognate stimulation in the presence of ionomycin and brefeldin A led us to hypothesize that cytokine production bypasses a global stress response which regulates the translation machinery of Th2 cells.

We sorted poised Th2 cells from *in vitro* cultures and examined the phosphorylation state of eIF2α serine 51 by immunofluorescence microscopy (Figure 4). While naive T cells contained undetectable levels of phospho-ser51, poised Th2 cells demonstrated elevated amounts of phosphorylated eIF2α (Figure 4). Interestingly, sorted memory ($CD62L^{lo}$) T cells, although poised for cytokine expression (Figure 1), contained levels of phosphorylated eIF2α similar to naive T cells, consistent with the transient nature of the integrated stress response (Harding et al., 2002). We next sorted Th2 effector cells generated *in vivo* from 4get BALB/c mice infected 120 hours earlier with *Leishmania major* (Stetson et al., 2002). Interestingly, these cells also demonstrated

increased levels of phosphorylated eIF2α, suggesting that regulation of global translation initiation occurs during *in vivo* differentiation of effector Th2 cells.

Four kinases, which respond to diverse stimuli, are capable of phosphorylating eIF2α on serine 51 (Harding et al., 2002). The ER-resident kinase PERK is maintained under normal conditions in an inactive state by the binding of the ER chaperone BiP to its lumenal domain (Bertolotti et al., 2000). Accumulation of client proteins in the stressed ER results in titration of BiP away from PERK, which then oligomerizes, trans-autophosphorylates, and becomes active. Similarly, GCN2 couples amino acid availabilty to translation rates via a His-tRNA synthase-like domain (Berlanga et al., 1999). The double stranded RNA-dependent kinase PKR attenuates translation in response to viral infection (Meurs et al., 1990). The heme-regulated kinase HRI is expressed primarily in reticulocytes and balances heme content with globin synthesis (Chen et al., 1991). These four kinases balance the activity of the translation apparatus to various physiological stresses, and thus comprise an important function of the integrated stress response (ISR) (Harding et al., 2002).

Figure 4

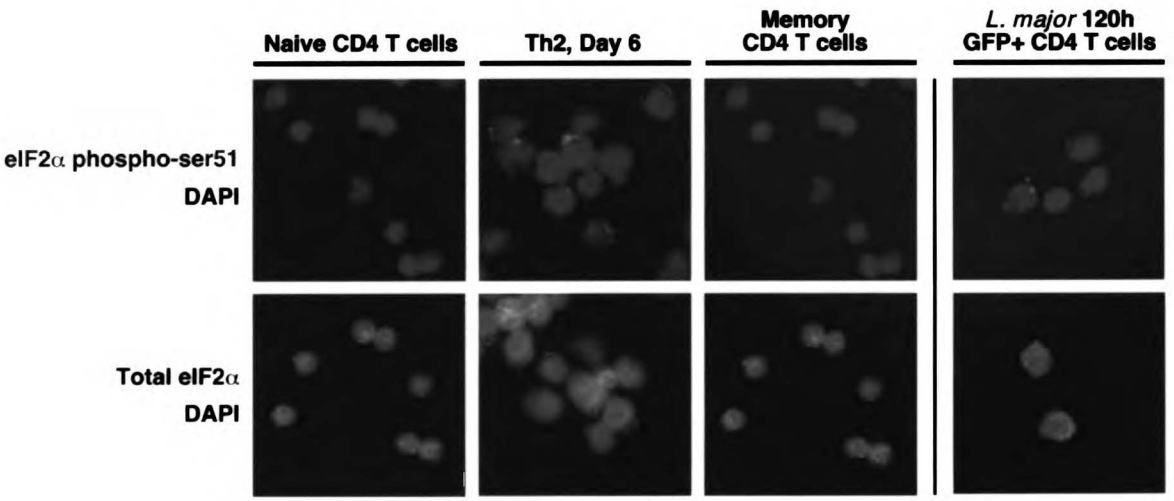


Figure 4. eIF2α ser51 is phosphorylated in effector Th2 cells.

DO11.10 x 4get GFP+ Th2 cells were sorted from day 6 in vitro cultures, and naive and memory phenotype cells were sorted from WT BALB/c mice as described in figure 1. *Ex vivo* effector Th2 cells were isolated from the draining lymph nodes of mice infected 120 hours earlier with *L. major* (Stetson et al., 2002). Cells were spun onto coverslips and stained with antisera to eIF2α phospho-ser51 (top row, red), or to total eIF2α (bottom row, green). Data are representative of three independent experiments.

Dominant negative forms of these kinases, encoded by catalytically inactive proteins still capable of oligomerization, are useful reagents for probing the function of each individual kinase (Berlanga et al., 1998; Donze et al., 1999; Harding et al., 1999; Sood et al., 2000). For example, cells transfected with a trans-dominant negative PERK are unable to attenuate translation in response to ER stress, but remain competent to respond to nutrient deprivation (Harding et al., 1999). We therefore generated retroviruses containing catalytically inactive PERK and GCN2 to examine a role for each of these kinases in eIF2α phosphorylation and translation regulation in Th2 cells. We also made a retrovirus encoding the dominant negative form of the ER protein kinase/endoribonuclease IRE1β (Urano et al., 2000), which is important for the transcriptional response to ER stress (Tirasophon et al., 1998; Wang et al., 1998). IRE1, like PERK, binds BiP via its lumenal domain and remains quiescent under steady state conditions (Bertolotti et al., 2000). Once IRE1 is activated by ER perturbation, its sole known function is to cleave the cytoplasmic mRNA of the transcription factor XBP-1 at two sites, resulting in removal of an intron and translation of XBP-1 protein (Calfon et al., 2002; Shen et al., 2001).

We confirmed expression of each protein in NIH3T3 fibroblasts infected with bicistronic retroviruses encoding Thy1.1 as a marker of infection (data not shown). Interestingly, we found that dominant negative (DN) PERK and DN GCN2 caused cell death in Th2 cells, such that no retrovirus+ cells remained four days after infection (Figure 5a). NIH 3T3 cells under normal growth conditions tolerated DN PERK as well as empty retrovirus, and DN GCN2 to a lesser extent (Figure 5b). DN IRE1β had no effect on Th2 cell survival (Figure 5b). Two days after retroviral infection, cells

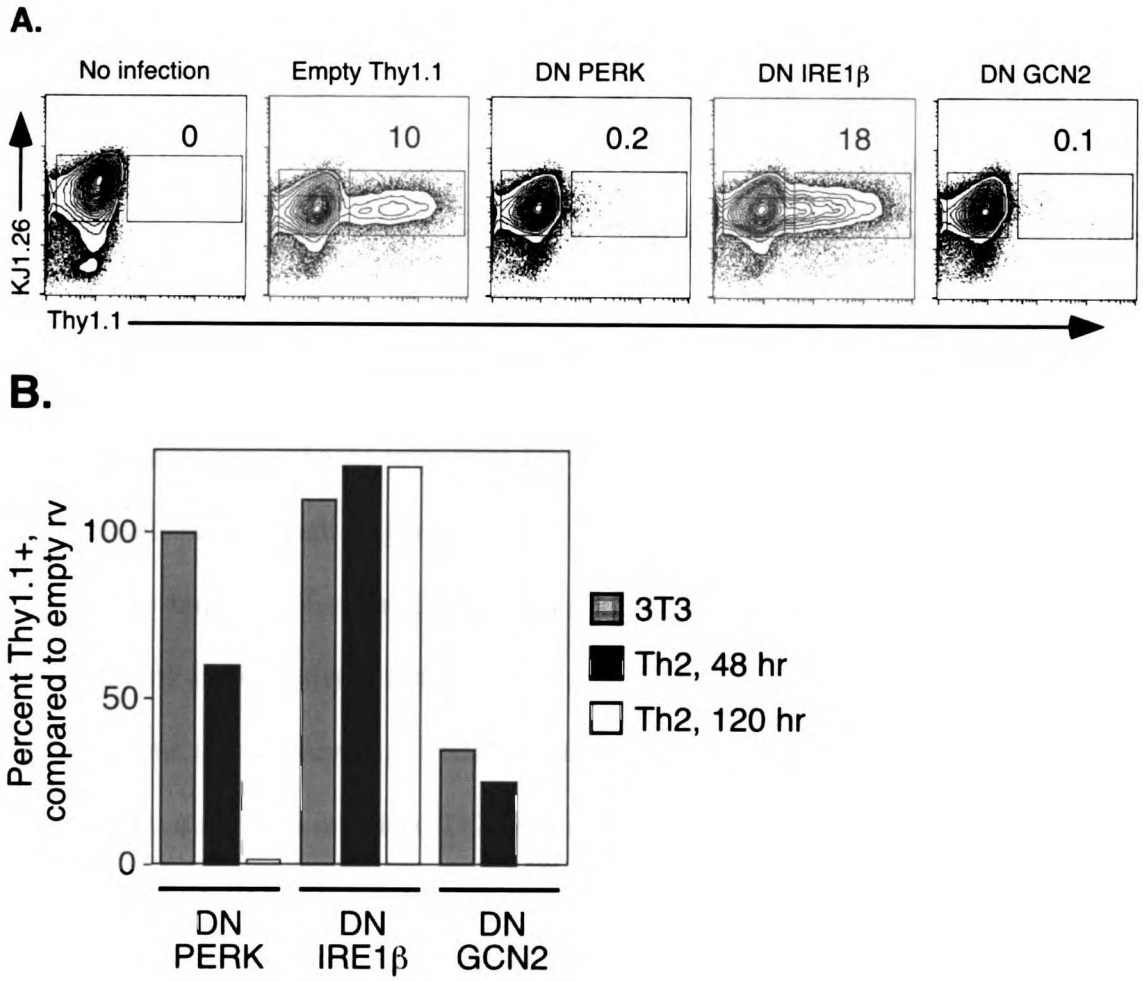
containing DN PERK and DN GCN2 were detectable (Figure 5b), suggesting that initial infection had been successful. Studies have shown that PERK-/- and GCN2-/- cells grow normally under steady state conditions, but rapidly undergo apoptosis under conditions of ER stress or nutrient deprivation, respectively (Harding et al., 2000; Zhang et al., 2002). These studies demonstrated a delicate balance between translation regulation and apoptosis in response to insults which perturb cellular homeostasis. Thus, the death of DN PERK+ and DN GCN2+ Th2 cells may be indicative of an important role for these kinases during Th2 differentiation. Further characterization of the role of PERK and GCN2, along with similar experiments using catalytically inactive forms of PKR and HRI, are underway and will be informative in linking global translation regulation to T cell effector function and survival.

Figure 5. A role for PERK and GCN2 in Th2 cell survival.

a. DO11.10 x Ca-/- T cells were infected 36 hours after stimulation with retroviruses containing DN PERK, DN IRE1β, or DN GCN2, or with empty Thy1.1 retrovirus as a control. FACS plots are of live, CD4+ T cells isolated 120 hours after infection (6.5 days after stimulation), and indicate the percentage of Thy1.1+ cells. Data are representative of three experiments with similar results.

b. NIH 3T3 cells were infected for 48 hours with the same retroviral supernatants used to infect Th2 cells. Th2 cells were analyzed for Thy1.1 expression 48 and 120 hours after infection. Graphs indicate the percentage of Thy1.1+ cells for each construct compared to empty Thy1.1 retrovirus at the indicated timepoints, and are representative of two experiments.

Figure 5


A.
No infection
Empty Thy1.1
DN PERK
DN IRE1β
DN GCN2
0
10
0.2
18
0.1
KJ1.26
Thy1.1
B.
Percent Thy1.1+, compared to empty rv
100
50
0
3T3
Th2, 48 hr
Th2, 120 hr
DN PERK
DN IRE1β
DN GCN2

We have shown that stable cytokine transcripts underlie the rapid IL-4 production by memory Th2 cells *in vitro* and *in vivo*. Using bicistronic knockin mice which report the presence of IL-4 transcripts, we found that over 90% of memory Th2 cells *in vivo* contain preformed IL-4 mRNAs (Figure 1). Although new transcription is required for effector function (Figure 1c), preformed IL-4 transcripts nucleate a more rapid and robust recall response (Figure 2). Translation of mRNAs in Th2 cells is carefully regulated (Figure 3), and is associated with active repression of translation initiation (Figure 4). Inactivation of two kinases important for translation regulation in response to cellular stress results in cell death (Figure 5).

One important issue not addressed here is the nature of the stable IL-4 mRNAs: are they long-lived in the absence of transcription *in vivo*, or is does basal transcription and turnover in memory Th2 cells result in a pool of steady-state transcripts? The abundant IL-4 mRNAs in poised Th2 cells show no evidence of decay when transcription is blocked for four hours (Figure 1c), suggesting that the mRNA molecules themselves can be long-lived. However, transcription from the accessible, poised IL-4 locus in differentiated Th2 cells may also be important (Avni et al., 2002).

Interestingly, dominant negative forms of the eIF2α kinases PERK and GCN2 caused cell death in Th2 cells. In preliminary experiments, however, we were unabe to rescue the cells with a single dose of the pan-caspase inhibitor z-VAD (data not shown.) Although the activity of PERK and GCN2 is redundant, the apoptotic programs balanced by these two kinases appear to be unique. It is intriguing that dominant negative IRE1 had no effect on Th2 cell survival, because both IRE1 and PERK are repressed by BiP (Bertolotti et al., 2000). The apparent dissociation of these two ER stress response may

be occuring for a few reasons. First, PERK may be independently activated by a unique signal in T cells. Second, PERK and IRE1 may have different response thresholds, such that PERK activity is induced and IRE1 is not. Third, the substrate(s) of IRE1, namely the XBP-1 mRNA, may be dispensable for T cell function. This seems likely because XBP-1-/- T cells have no defect in differentiation or cytokine production (Reimold et al., 2001).

Further work will be required to elucidate a role for each of these kinases in T cell differentiation and effector function, and to dissect the global and cytokine-specific regulation of translation in T cells. Our data support a model whereby activaton and differentiation of naive T cells is accompanied by induction of an integrated stress response. This response and its associated restriction of translation may serve two important functions. First, it streamlines the energetically costly machinery of protein biosynthesis during T cell differentiation. Second, it prevents inappropriate synthesis of potentially harmful effector molecules, instead poising their mRNAs for rapid induction. Upon cognate restimulation, translation of a limited set of mRNAs is released, linking antigen recognition to rapid effector function in the memory response.

Experimental Methods

Mice and flow cytometry. 4get, DO11.10 x 4get, and DO11.10 x $Ca^{-/-}$ mice have been described previously (Mohrs et al., 2001; Stetson et al., 2002). Female mice were used between 5 and 10 weeks of age, and were maintained in accordance with institutional guidelines in the UCSF specific pathogen free facility. Lymphocytes were isolated and stained for FACS analysis and sorting as described previously (Stetson et al., 2002), using antibodies to the following cell surface molecules: PE-KJ1.26, Cy5-PE-CD8, Cy5-PE-anti-CD45R/B220, PE-CD4, APC-CD4 (all from Caltag), APC-CD62L (BD PharMingen). Cells were analyzed and sorted to >99% purity using a MoFlo high speed cell sorter (Cytomation, Fort Collins, CO). For adoptive transfers, eGFP+ DO11.10 x 4get Th2 cells were sorted from day 6 cultures, washed, and resuspended in PBS. $3x10^6$-$6x10^6$ cells were injected into the tail vein of recipient 4get BALB/c mice, which were then rested for 5-7 weeks before analysis of eGFP expression in peripheral lymph node donor T cells.

ELISPOT assays. Cells were sorted or harvested from culture, washed, and distributed into wells of ELISPOT plates (Immulon IV HBX, Dynex Technologies, Chantilly, VA), coated with either 11b11 anti-IL-4 antibody alone, or 11b11 with 10 μg/ml H57 anti-TCRβ. cells were incubated at 37^0C for the indicated times, typically 4 hours, before washing and incubating with biotinylated BVD6 secondary antibody overnight at 4^0C. Plates were washed and incubated with streptavidin-alkaline phosphatase (Jackson Immunoresearch, West Grove, PA). Washed plates were then overlaid with a 0.6% agarose alkaline buffer solution containing 1 mg/ml 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP, Sigma) and developed in the dark for 1 hour at room temperature and

then for four hours at 4^0C. Spots from triplicate assays for each sample were scored using a dissection microscope.

Quantitative RT-PCR. Cells treated as indicated were harvested into RNAzol (Biotecx, Houston, TX), and quantitative RT PCR was performed using primer/probe sets specific for IL-4 and HPRT as described (Grogan et al., 2001). Cytokine signal in each population was normalized to HPRT levels to account for differences in starting cDNA amounts.

Polyribosome analysis. DO11.10 x $Ca^{-/-}$ T cells stimulated under Th2 conditions for six days *in vitro* were centrifuged over Histopaque 1083 (Sigma) to remove dead antigen presenting cells. After recovery from the interface, poised cells were incubated with 0.1 mg/ml cycloheximide (CHX, Sigma) for 10 minutes at 37° C, pelleted, washed with ice-cold PBS/CHX, and resuspended in polysome extraction buffer: 30 mM HEPES (pH7.4), 15 mM MgCl2, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/ml CHX, 1 mg/ml heparin, 500 U/ml RNAsin (Promega), 1x Complete EDTA-free protease inhibitor cocktail (Roche). Restimulated cells were treated with PMA/ionomycin for 2 hours or with 10 μg/ml plate-bound I-A^d/ova (Stetson et al., 2002) and anti-CD28 (37N1.1, 5 μg/ml, BD PharMingen) for 4-6 hours before harvesting into extraction buffer. Samples were incubated on ice for 30 minutes with occasional vortexing. Nuclei and debris were removed by centrifuging twice for 10 minutes at 9000 rpm and transferring cleared supernatants into fresh tubes. 450 μl of each extract was layered onto a 12.5 ml 10-50% sucrose gradient prepared in SW40 centrifuge tubes (Beckman) using the salts described above, but not detergent, RNAsin or protease inhibitors. Alternatively, larger gradients were prepared in SW28 centrifuge tubes by tripling the recipe; these accomodated 1.4 ml of extracts. Gradients

were spun in a Beckman ultracentrifuge for 100 minutes at 40,000 rpm (small gradients) or for 180 minutes at 28,000 rpm (large gradients). Fractions were isolated by pumping 60% sucrose into the bottom of the tube and collection of the displaced volumes into acid phenol:chloroform, with constant monitoring of the OD_{260}. Extracted RNA was precipitated with an equal volume of isopropanol, washed, resuspended in distilled water, quantitated, and precipitated again to remove residual salts. Resuspended RNA samples were separated on 1.2% formaldehyde gels and transferred to Hybond N+ membranes (Amersham) by wicking before northern blot analysis using ^{32}P-labeled probes to the coding sequence of GAPDH.

Immunofluorescence. DO11.10 x 4get Day 6 Th2 cells were sorted to > 99% purity and spun onto coverslips before fixing for 10 minutes with 3% paraformaldehyde in PBS. Cells were permeabilized with 0.1% Triton X-100 in 5% fetal calf serum (FCS)/PBS for 4 minutes at room temperature, washed, and blocked overnight with 5%FCS/PBS at 4°C. Duplicate cover slips were incubated with predetermined optimal concentrations of rabbit antisera to total eIF2 α (Santa Cruz) or to phosphorylated eIF2α serine 51 (Cell Signaling Technologies, Lake Placid, NY) for 2 hours at room temperature before washing and incubating with Cy5-labeled goat anti-rabbit secondary antibody (Jackson Immunoresearch) for 1.5 hr. Washed coverslips were then counterstained for five minutes with a 10 μg/ml solution of 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Roche/Boehringer-Mannheim, Indianapolis, IN) in PBS. Slides were mounted in Vectashield (Vector Laboratories). Digital images in the DAPI and Cy5 channels were collected using the Marianas digital microscopy workstation (Intelligent Imaging

Innovations, Denver, CO) equipped with Slidebook software. Fluorescent backround was set against isotype controls for each antibody staining.

Retroviral infections. Expression vectors for catalytically inactive forms of PERK and IRE1β were a gift from Dr. David Ron, and the DN GCN2 construct was a gift from Dr. Ron Wek. cDNAs were cloned by restriction digest and ligation into the first cistron of a variant of MSCV 2.2 (a gift of Gary Nolan) containing Thy1.1 driven by an IRES element. Plasmids were transfected into PHOENIX packaging cells (Shaw et al., 1997) with pCL-Eco using the Effectene lipid reagent (Qiagen). Supernatants were added at a 1:1 ratio to DO11.10 x $Ca^{-/-}$ Th2 cells 36 hours after activation. Retrovirus+ cells were identified by Thy1.1 expression at the indicated timepoints. A similar strategy was used to infect NIH3T3 cells.

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Chapter V:

Conclusions and future directions

Abstract

We have shown that cytokine transcription and translation are regulated by independent checkpoints during lymphocyte differentiation. In chapter II, we found that differentiation of naive T cells into IL-4 expressing Th2 cells was not predictive of the outcome of *L. major* infection in susceptible versus resistant mice. In Chapter III, we showed that NK T and NK cells, unlike conventional T cells, activate lineage-specific cytokine transcription - but not translation - during development and before pathogen encounter. In Chapter IV we found that memory Th2 cells contain stable IL-4 transcripts, and translate IL-4 protein only following cognate stimulation. This chapter will discuss a model in which preformed cytokine mRNAs endow effector lymphocyte populations with the ability to respond rapidly to activation, and outlines future experiments to further characterize the contributions of global and cytokine-specific translation regulation to lymphocyte effector function.

Preformed cytokine mRNAs underlie rapid cytokine responses

We have shown that transcription and translation of cytokine mRNAs can be independently regulated in lymphocytes, and that stable, constitutive cytokine mRNAs contribute to the rapid cytokine production of NK T cells, NK cells, and Th2 cells. Interestingly, NK T and NK cells activate cytokine transcription during development in the thymus and bone marrow, respectively, before pathogen recognition in the periphery. Thus, the rapid response of these cells appears hard-wired and only requires receptor-mediated stimulation to link recognition of antigen to elaboration of a defined cytokine program. In contrast, Th2 cells acquire stable IL-4 mRNAs during differentiation from naive, pluriptent precursors. These data support a model whereby two broad types of lymphocytes are born during development: cells which accumulate their effector capacity before infection and arm the periphery with stereotyped rapid responses, and conventional cells which delay the acquisition of rapid effector capacity until infection dictates their differentiation (Figure 1). It seems likely that the same mechanisms which maintain transcription -but not translation - of cytokines in the former population are reiterated during the differentiation of the latter.

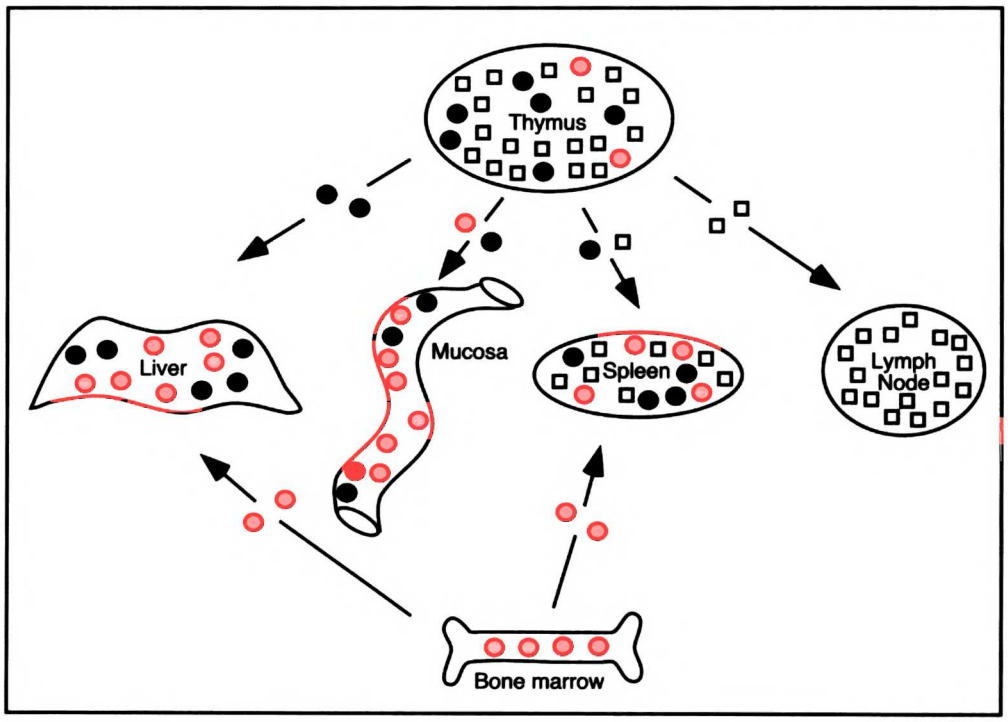
Figure 1. A model for the coordination of tissue effector lymphocytes and conventional T cells.

a. In the thymus and bone marrow, populations of lymphocytes developmentally activate cytokine transcription and remodel the respective cytokine genes for IL-4 expression (blue circles), IFN-γ expression (yellow circles), or both IL-4 and IFN-γ (green circles). Unlike conventional T cells (clear boxes), which do not activate cytokine expression during development and are exported to lymphoid organs, tissue effector lymphocytes arm the periphery loaded with lineage-specific cytokine mRNAs and provide the first line of defense against an invading pathogen.

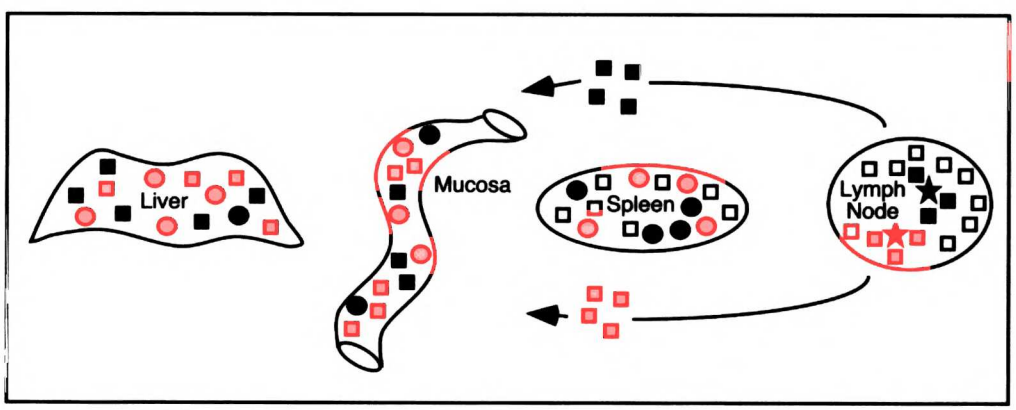
b. Upon pathogen infection, dendritic cells (grey stars) migrate from the infection site to lymph nodes and influence conventional T cell differentiation into IL-4 (blue boxes) or IFN-γ (yellow boxes) expressing effector cells. These cells then migrate to the site of infection to provide antigen-specific defense and memory. We hypothesize that one feature of the rapid recall response of conventional T cells is the presence of preformed cytokine mRNAs.

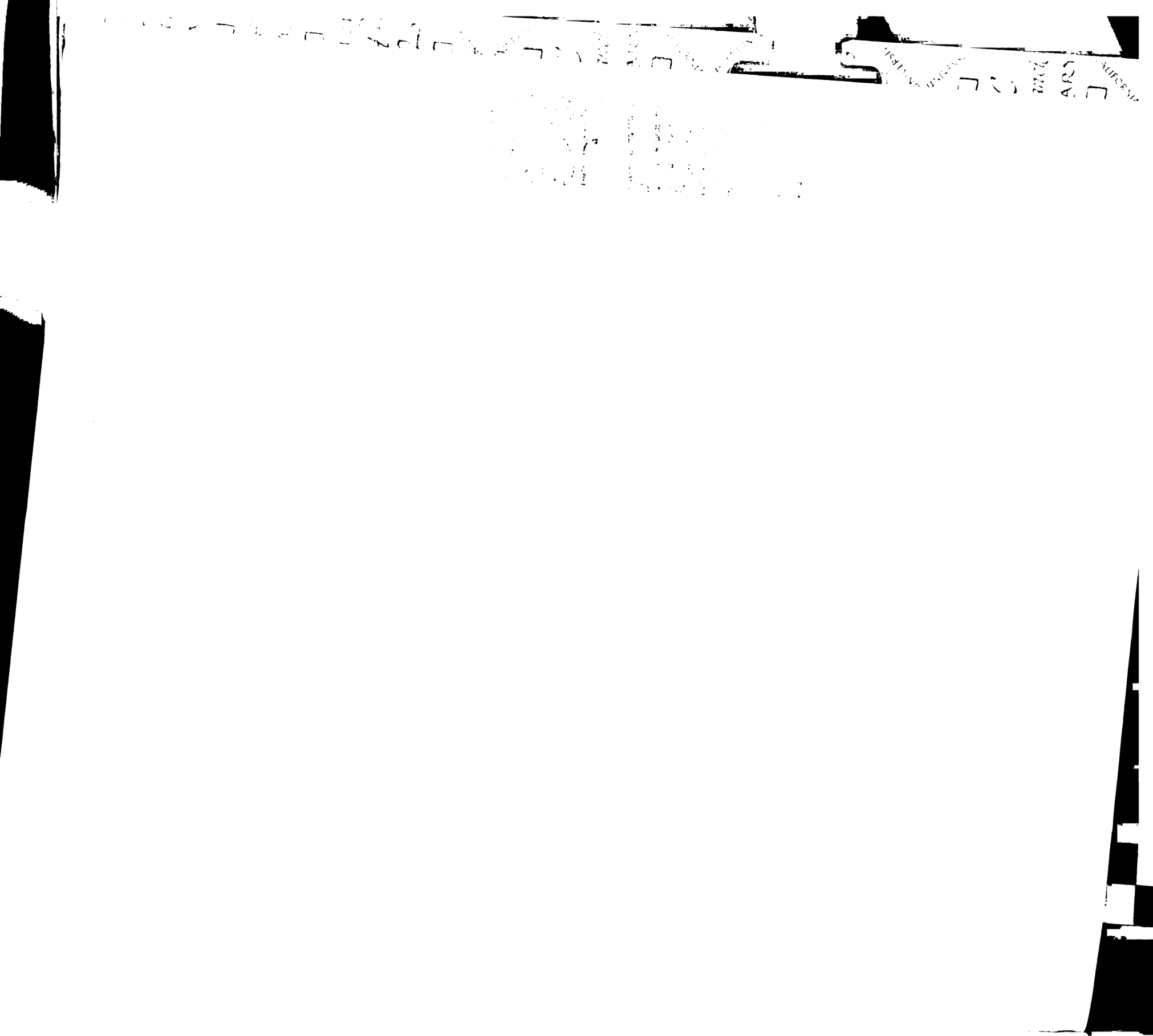
Figure 1

A.


Thymus
Liver
Mucosa
Spleen
Lymph Node
Bone marrow

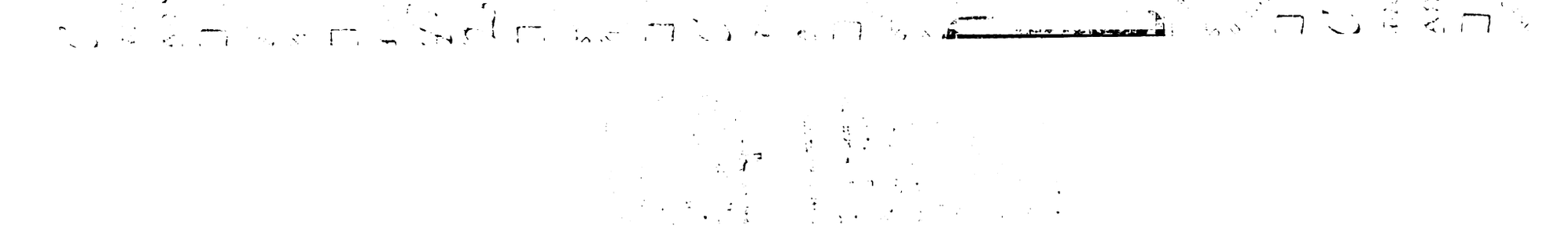
B.


Liver
Mucosa
Spleen
Lymph Node



The fact that NK T and NK cells activate and maintain lineage-specific cytokine transcription is intriguing because it implicates a process mediated by evolutionarily fixed, developmental cues rather than pathogen recognition. Further, NK cells are an arm of innate immunity because they do not require recombinase activating genes (RAGs) for their development and function. Thus, the cues which activate the program and the transcription factors which maintain the poised IFN-γ transcripts in NK cells may be informative in understanding the analogous process in NK T cells and Th1 cells. Common pathways utilized by these cells are emerging in the literature: for instance, NK and Th1 cells require the transcription factor T-bet for IFN-γ production, but it is dispensable in CD8 T cells (Szabo et al., 2002).

The regulation of IL-4 and IFN-γ translation in several cell types suggests that other cytokines and effector molecules might be maintained in a poised, untranslated state. Tumor necrosis factor α (TNFα) is perhaps the best characterized example (Dumitru et al., 2000). The 3' untranslated region of TNFα is important in control of translation, because mice bearing deletion in the AU-rich 3' untranslated element (ARE) of TNFα develop autoimmune pathology associated with misregulation of TNF biosynthesis (Kontoyiannis et al., 1999). In the case of IL-4, however, the ARE appears to be dispensable for its gross regulation, because splicing and ployadenylation of the bicistronic 4get mRNA removes the ARE (Mohrs et al., 2001). Further, the ARE's of TNFα and IL-6 have been shown to bind different proteins and regulate biosynthesis of each cytokine at different levels (Neininger et al., 2002). Therefore, using an unbiased approach to identify mRNAs that are regulated at the level of translation in T cells will be useful in approaching this checkpoint from a more global perspective.



Mining transcription and translation profiles of Th2 cells

Polysome analysis of Th2 cells illuminated two important features. First, there is very little global translation occurring in poised Th2 cells, likely because of specific inhibition of translation initiation (Chapter IV). Second, cognate restimulation of Th2 cells does not result in the mobilization of the global translation apparatus, but rather selects a limited set of mRNAs for translation - including cytokines. Thus, T cell receptor signaling bypasses a global stress response. This idea is supported by the fact that ionomycin and brefeldin A, two potent inducers of ER stress and inhibitors of translation, are used routinely during restimulation for intracellular cytokine staining.

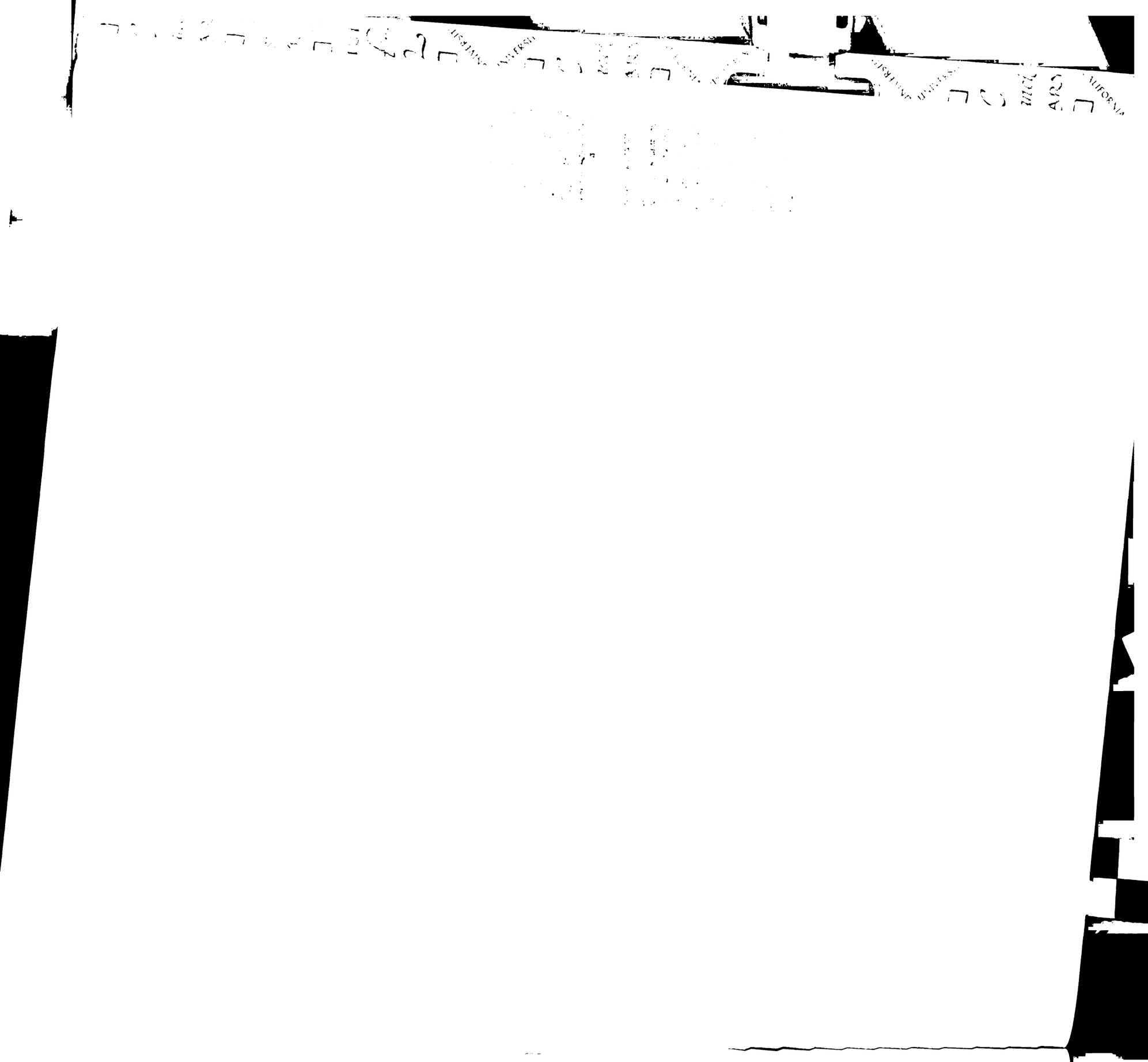
Analysis of the gene expression profiles of lymphocytes by microarrays has been insightful in understanding the diverse transcriptional programs which underly their differentiation (Shaffer et al., 2001; Teague et al., 1999). However, while these arrays offer a dazzling wealth of data, they do not segregate between translated and untranslated mRNAs. Approximately 75% of cytoplasmic RNA segregates in the untranslated portion of poised Th2 cells (Chapter IV, figure 3). Thus, a powerful tool for understanding the relative contributions of transcription versus translation to the global gene expression program of Th2 cells will be a combination of polysome and microarray analysis. This technique has been applied with success to fibroblasts and human T cells (Garcia-Sanz et al., 1998; Johannes et al., 1999; Mikulits et al., 2000; Zong et al., 1999).

We are currently accumulating RNA from polysome gradients of poised and restimulated transgenic Th2 cells to examine the global changes in translated mRNAs resulting from T cell receptor stimulation. Polysome-bound mRNAs pooled from the late fractions of gradients will be compared directly between the two samples on a microarray

consisting of 17,000 oligonucleotides representing unique expressed sequence tags in the mouse genome. This data will be related to the transcription profile of the same cells, which will allow us to assign a value of translation efficiency to each mRNA. We hypothesize that while the transcript profile of poised Th2 cells will be diverse, only a minority of transcripts will be translated efficiently. After TCR restimulation, a unique subset of mRNAs will shift to the translated portion. Identifying these mRNAs will be an informative first step in understanding the mechanism of translation regulation conferred by the T cell receptor. Analysis of polysome-bound mRNAs in restimulated Th2 cells treated with actinomycin D will be a more stringent test for mRNAs regulated solely at the level of translation.

Stressed out T cells?

The correlation between globally repressed translation and phosphorylation of eIF2α serine 51 in poised Th2 cells suggests that an integrated stress response is active in these cells (Harding et al., 2002). We found that the activity of both GCN2 and PERK were required for Th2 cell survival during *in vitro* culture (Chapter IV, Figure 5), suggesting that multiple pathways converge to regulate translation during Th2 differentiation. It is easy to imagine that both nutrient deprivation and ER stress are attendant processes which accompany T cell differentiation. Glucose uptake by T cells, which feeds directly into macromolecule synthesis, is carefully regulated by CD28 costimulation (Frauwirth et al., 2002). Receptor mediated signaling causes extensive flux of calcium stores from the ER (Feske et al., 2001), and the demand of generating new ER components during rapid cell division places an energetic load on that compartment (Chapman et al., 1998). Thus, translation regulation by kinases which sense the nutrient



environment and status of the ER may be important for limiting the translation machinery during differentiation, thus minimizing energy waste. Further work is underway to determine the relative contributions of these pathways, and also to examine a role for the other two known eIF2α kinases, PKR and HRI.

Phosphorylation of eIF2α and the resultant attenuation of translation does not completely shut down a cell. Rather, the selection of mRNAs which are translated becomes streamlined to maintain cell viability and function at a minimal energetic cost (Harding et al., 2002). In fact, translation of certain mRNAs is induced by orders of magnitude when the translation apparatus becomes limiting (Sachs et al., 1997). Many of these mRNAs share features in their 5' untranslated region, including multiple open reading frames (uORFS) upstream of the canonical translation start site. The best characterized of this class of mRNAs is yeast GCN4, whose translation is repressed under normal growth conditions in a uORF-dependent manner, but is induced over 200-fold by eIF2α phosphorylation (Gaba et al., 2001). It is intriguing to note that the transcription factor RFLAT-1, whose mRNA is constitutive in T cells but becomes translated abruptly three days after activation, contains multiple uORFs in its 5' UTR (Nikolcheva et al., 2002).

One way to test the role of each kinase is the examination of knockout mice, which have been made for all four kinases. Interestingly, all four knockout strains are viable. PERK-/- mice develop diabetes as a result of apoptosis of insulin-producing pancreatic β cells (Harding et al., 2001), and have skeletal dysplasias associated with dysfunctional osteoblasts (Zhang et al., 2002a). GCN2-/- mice develop normally but fecundity of offspring is severely reduced when pregnant mothers are reared on nutrient-

poor diets (Zhang et al., 2002b). PKR-/- mice are normal (Abraham et al., 1999) but demonstrate increased susceptibility to infection with certain dsRNA-containing viruses (Stojdl et al., 2000). HRI-/- mice become anemic and are unable to limit globin synthesis in red blood cell precursors during iron deficiency (Han et al., 2001). The phenotypes of the knockouts for these kinases highlight their important role in sensing specfic insults to the homeostasis of the cellular environment. T cell phenotypes for these knockouts remain largely uninvestigated. One study showed that IFN-γ translation is limited by binding of PKR to its own 5' UTR (Ben-Asouli et al., 2002), although PKR-/- mice do not demonstrate gross overproduction of IFN-γ protein (Abraham et al., 1999). Remarkably, there is a high level of eIF2α phosphorylation in thymi of normal mice which is completely ablated in the absence of PERK (Harding et al., 2001). A T cell developmental phenotype has not yet been reported, and the cell types affected in the thymi of PERK-/- mice remain unkown. Examination of T cell development and differentiation in these mice will be informative in revealing an *in vivo* role for the integrated stress response in lymphocyte function.

Visualizing the biosynthesis of IL-4 in real time

The studies described herein suggest that translation of cytokines constitutes a major regulatory checkpoint which regulates effector function. Thus, strategies to visualize IL-4 protein in real time will be informative in exmaining the kinetics, location, and regulation of its production *in vivo*. Since early reports demonstrated that T cells secrete cytokines in a polarized fashion towards the site of TCR ligation (Kupfer and Singer, 1989; Kupfer et al., 1986; 1987; 1994; Poo et al., 1988), surprisingly few studies have characterized the kinetics or mechanism of polarized secretion. Perhaps one reason

is the difficulty in detecting the cytokine during the rapid process of synthesis and secretion. Because translation is an important part of this process, we are exploring the utility of novel tagged forms of IL-4 that may allow their direct visualization. Widely used strategies such as GFP- or LacZ tagging would not be useful because GFP requires several hours for its fluorescence to mature and LacZ is non-functional when delivered to the secretory pathway. Therefore, we are testing a new class of biarsenical labeling compounds for their ability to bind and illuminate IL-4 molecules tagged at their c-terminus with a tetracysteine motif (Griffin et al., 1998). There are two main advantages of this system. First, the biarsenical compounds bind with extremely high affinity to the engineered tag, with an empirical on-rate of just 2-3 seconds (Adams et al., 2002). Second, the compounds are cell-permeable and largely non-fluorescent before they bind the tetracysteine motif (Griffin et al., 1998), which would allow cells to be pre-loaded with the fluorophore beforeinitiation of IL-4 translation. Further, certain biarsenicals can be directly adapted to electron microscopy studies, allowing for the generation of fluorescence and ultrastructural data from the same cell (Adams et al., 2002). We are currently testing the biologic activity of tetracysteine tagged IL-4 and its ability to be labeled by biarsenicals using an insect cell expression system. If successful, this strategy will become a very useful reagent for probing questions regarding the cell biology of IL-4 synthesis, transport and secretion *in vitro* and *in vivo*.

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