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Quantitative events determine the differentiation and function of helper T cells

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In recent years, numerous qualitative discoveries have been made in immunology research. However, the effect of quantitative events, long recognized as the driving factors for determinism in developmental biology, that dictate the quality of the immune response elicited to an antigen in concert with microbial products still requires serious attention. Here we discuss how the often-neglected issue of quantification affects the specification, differentiation and commitment of helper T cells. As reductionist *in vitro* approaches have been instrumental in the elucidation of the factors determining the development of helper T cells, in this perspective we highlight the need for the standardization of protocols, also fundamental for the comparison of immune responses in mice and humans. Improving understanding of how these *in vitro* quantitative events translate to immune responses *in vivo*, which can be studied in mouse models, is of importance in obtaining information on immune responses in humans, thus empowering translational research.

Positively selected CD4$^+$ T cells emerging in the periphery are selected mainly to respond to invading pathogens, and the effector response is largely attributable to the production of different sets of cytokines by discrete helper T cell subsets[1]. Quantitative events are important for the specification, differentiation and commitment of helper T cells, including the dose and affinity of antigen, the dose and gradient of cytokines induced early during the immune response that contribute to this process, and the resulting gradient of helper T cell subset–defining transcription factors[2,3]. Collectively, the balance of these factors will determine the effector response elicited and in some cases maintained—for example, during chronic infection and allergy. Because of the explosion of qualitative molecular discoveries in the past 15 years in terms of previously unknown cytokines, expanding numbers of potential helper T cell subsets and the influence on helper T cell differentiation of many newly identified factors and pathways triggered in innate cells by pathogen-derived products, such quantitative events have been mostly neglected. *In vitro* studies of mouse and human systems have provided evidence that quantitative events have a major role in determining the extent of effector responses mediated by helper T cell subsets. Mouse studies have confirmed that quantitative events also drive effector responses elicited during infection or vaccination and minimize damage to the host. Careful analysis of how *in vitro* quantitative events translate into the *in vivo* situation in the mouse is important for predicting the effects of these events *in vivo* in humans. A major challenge will also be to understand the biological reason for the induction of different effector T cell responses by different quantitative cues.

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Effects of concentrations of transcription factors and cytokines

After encountering antigen, naive CD4$^+$ helper T cells differentiate into discrete effector cells, including but not limited to the T_H1, T_H2, T_H17 and T_H22 helper T cell subsets, each specialized to eradicate different classes of pathogens. Thus, CD4$^+$ T cells producing distinct patterns of cytokines develop during immune responses to invading pathogens. An appropriate response leads to the elimination of the pathogen. However, an incorrect or limited response can result in chronic infection[1]. Intracellular pathogens and their products trigger the production of interleukin 12 (IL-12) from macrophages and dendritic cells (DCs) to induce the development of T_H1-type cells that produce large amounts of interferon-γ (IFN-γ) and express the transcription factor T-bet[3,4], a response essential for protection from infection with pathogens, including mycobacteria and salmonella[1,5,6]. Helminth pathogens elicit the development of T_H2 cells that express the cytokines IL-4, IL-5 and IL-13 and the transcription factor GATA-3; this response can be dependent on IL-4 and is critical for pathogen control[1,3,4,7]. Extracellular bacteria and fungi seem to elicit protective T_H17 responses[8,9] through a balance of cytokines secreted into the environment, including transforming growth factor-β (TGF-β), IL-6, IL-1 and IL-23 (refs. 7,10,11) and the transcription factor RORγt[9]. T_H22 cells have been found in humans[12–14] but so far not in mice. A substantial proportion of T_H22 cells recognize lipid antigens presented by CD1a expressed on Langerhan's cells, some dermal DCs and macrophages[15]. Tumor necrosis factor and IL-6 favor the development of T_H22 cells from naive cells *in vitro*[12]. An overexuberant or dysregulated helper T cell response may result in immune-mediated damage to the host; this is kept in check by regulatory mechanisms, including those provided by CD4$^+$ regulatory T cells (T_{reg} cells) positive for the transcription factor Foxp3, which develop in the thymus[9] or in the periphery, where they are dependent on high concentrations of TGF-β and retinoic acid[16–18]. Hence, large amounts of TGF-β in the absence of inflammatory mediators will result in the development of Foxp3$^+$ T_{reg} cells, whereas lower concentrations of TGF-β in the presence of inflammatory mediators such as IL-6, IL-1 and IL-23 produced in the context of infection would mediate the development of T_H17 cells. If regulated, T_H17 cells may contribute to protective

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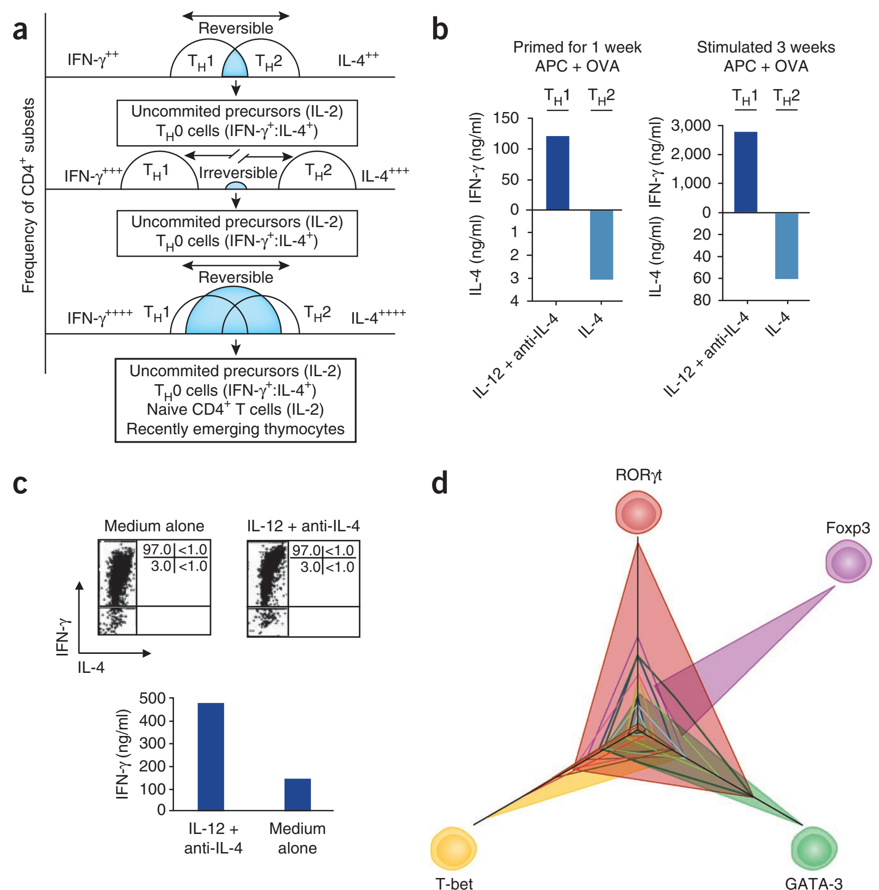

Figure 1 Plasticity and commitment of helper T cells. (**a**) Heterogeneity and reversal of helper T cell phenotypes, based on data obtained from flow cytometry analysis of the synthesis of intracellular cytokines in polarized T_H1 and T_H2 populations obtained from TCRαβ-transgenic CD4⁺ T cells stimulated *in vitro* with APCs plus ovalbumin in the presence of IL-12 plus monoclonal antibody to IL-4 (T_H1) or IL-4 (T_H2) for 1 week (top) or 3 weeks (middle); bottom, hypothetical helper T cell pool *in vivo* (from ref. 26). The number of superscripted plus symbols corresponds to the amount of cytokine. (**b**) Enzyme-linked immunosorbent assay of cytokines in supernatants of T_H1 and T_H2 cells stimulated for 48 h by the phorbol ester PMA and ionomycin, assessed after 1 week or 3 weeks of polarization *in vitro* (from ref. 26). (**c**) Flow cytometry of intracellular IFN-γ production (top) and enzyme-linked immunosorbent assay of secreted soluble IFN-γ production (bottom) by cells generated from a resting T_H1 clone obtained from TCRαβ-transgenic CD4⁺ T cells stimulated for 3 weeks *in vitro* with APCs plus ovalbumin in the presence of IL-12 plus monoclonal antibody to IL-4, then restimulated for 4–6 h PMA, with brefeldin A added for final 2 h (top) or 48 h (bottom) with PMA and ionomycin. Modified with permission from ref. 26. (**d**) Graded expression of transcription factors. Modified from ref. 20.

immune responses to pathogens, but if not, they can contribute to immunopathology[19] or inflammatory diseases[8–10].

The concept of stable, terminally differentiated helper T cells makes sense but probably represents the end point of differentiation of cells in response to chronic stimulation with a particular pathogen, such as *Toxoplasma gondii* for T_H1 cells, or *Schistosoma mansoni*, *Trichuris muris*, *Nippostrongylus brasiliensis* or an allergen for T_H2 cells[20]. Such terminally differentiated helper T cells probably represent a small proportion of the pool of memory effector T cells, which maintain the correct specificity and the appropriate cytokine profile. It is likely that these cells undergo further differentiation in the tissue, as suggested by studies demonstrating the presence of more IFN-γ-producing cells in the lungs than in spleen and lymph nodes after immunization with a peptide antigen plus lipopolysaccharide[21].

Follicular helper T cells (T_{FH} cells) are another distinguishable subset of helper T cells specialized in providing help for B cell responses. After priming by antigen-presenting DCs in the T cell zone of secondary lymphoid organs, a fraction of effector helper T cells migrate toward the B cell follicle, where they interact with antigen-presenting B cells. Studies suggest that although effector helper T cells develop locally in the lymph node, most of them exit the draining lymph node after priming[22,23]. Although T_{FH} cells can express T-bet and IFN-γ during infection with lymphocytic choriomeningitis virus[24], or GATA-3 and IL-4 during infection with *N. brasiliensis*[23], T_{FH} cells have lower expression of these T_H1 and T_H2 hallmark transcription factors and cytokines, because of the action of the transcriptional repressor Bcl-6 (refs. 23–25), than do non-T_{FH} classical T_H1 and T_H2 cells. Hence, there is a strong possibility, during certain infections or allergic responses, that although the initiation of T_H1 and T_H2 differentiation is initiated in the lymph node, these subsets leave the lymph node as effector cytokines start to be produced and undergo further differentiation and commitment in the infected or allergen-challenged tissue to produce much higher concentrations of their hallmark cytokines to elicit cell-mediated immune responses to eradicate the pathogen[23,24]. Conversely, T_{FH} cells produce much smaller amounts of helper T cell hallmark cytokines once in association with B cell follicles, although the amounts are sufficient to direct immunoglobulin class switching[23,24].

It is likely that terminally differentiated T_H1 and T_H2 cells exist in a broad spectrum of more plastic cells and that such responses remain 'locked' only during responses to chronic stimulation, such as chronic infection or allergic inflammation (T_H2 type), in which maximal and repeated stimuli 'instruct' cells in a particular direction[1]. This was modeled in studies from the mid-1990s demonstrating that CD4⁺ T cells primed once or twice are not stable when subsequently stimulated under different conditions and show much more plasticity than do helper T cells restimulated multiple times under polarizing conditions[26] (**Fig. 1a**). Furthermore, the helper T cells polarized for 1 week produce concentrations of cytokines at least one log lower than those produced after restimulation of helper T cells that had been polarized for many weeks[26] (**Fig. 1b**). This can also be seen in T_H1 clones that produce the most IFN-γ when restimulated with antigen and antigen-presenting cells (APCs) in the presence of IL-12, which maintain abundant IFN-γ production in differentiated T_H1 cells[26] (**Fig. 1c**). However, it is of importance to note that the frequency of IFN-γ-producing T_H1 cells may not reflect the actual amount of IFN-γ protein secreted by each cell[26] (**Fig. 1c**). In keeping with this model, the original definition of helper T cell subsets was based on stable cytokine patterns produced by several mouse clones established from mice chronically immunized or infected with parasites *in vivo* and then cloned and maintained *in vitro*, and these cells provided the basis for the T_H1 and T_H2 designations[27]. These end-differentiated helper

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T cells probably express the largest amounts of their distinct subset-specifying transcription factors[3,4,7,9,20,28] whose expression is governed by an array of extrinsic and intrinsic signals and that will determine and maintain the greatest production of the stereotypic cytokines (or regulatory molecules, in the case of inducible T_{reg} cells)[29]. At an earlier stage in differentiation, extrinsic or intrinsic factors may result in lower concentrations of transcription factors, which allows their coexpression in different relative amounts; this may consequently result in graded mixtures of cytokines and thus effector properties, as has been discussed before[20,26,30] (**Fig. 1a**,**d**). However, higher concentrations of one particular transcription factor and high concentrations of the cytokines and factors produced by one particular subset have been shown to exert inhibitory effects on the other, which could explain the dominance of one subset in a particular chronic infection or response, such as T_H1 responses to intracellular pathogens and T_H2 responses to extracellular parasites or allergens[1,4,7]. It is likely that most immune responses, which do not constitute chronic antigenic stimulation in concert with pathogen-derived products, reflect smaller and balanced amounts of transcription factors and effector molecules, allowing more flexibility, as has been suggested before[20,26,30,31] (**Fig. 1a**,**d**). Formal demonstration, however, will require quantification of mRNA and protein *in vivo* in single cells, which is limited at present by technology.

TCR and costimulator strength influence helper T cells

CD4+ T cells respond to T cell antigen receptor (TCR) recognition of complexes of foreign peptide and major histocompatibility complex class II on APCs, which is a major early checkpoint in the induction of helper T cell responses, together with signals from APCs elicited in response to pathogen-derived products[32,33]. Vaccine adjuvants, such as agonists of Toll-like receptors 9 and 4 can skew TCR repertoire use by increasing TCR-selection thresholds and enhancing antigen-specific clonal expansion[33]. Furthermore, it has been recognized for many years that the differentiation of helper T cell subsets that produce distinct cytokine patterns can be determined by many factors, with a dominant role for cytokines induced in cells of the innate immune response by particular pathogens and their products in the microenvironment[34]. Other variables also influence the development of the helper T cell subsets that produce distinct effector cytokines, including the strength of T cell stimulation, which is determined by the dose of antigen, costimulatory interactions and the affinity and overall avidity for the signals produced by stimulation with major histocompatibility complex class II–peptide–TCR[2,35–40]. Serial triggering of many TCRs by a few complexes of peptide and major histocompatibility complex[41], T lymphocyte costimulation mediated by the reorganization of membrane microdomains[42] and the quantitative contributions of CD4 and CD8 to TCR serial triggering[43] may also contribute to the development of helper T cell subsets. Furthermore, it has been suggested that not only the strength but also the duration of the helper T cell TCR–APC contact represents a crucial parameter that influences the response to polarizing cytokines and the acquisition of T cell effector functions[2,3,35–38,40,44–48]. Examples of costimulators that affect the development of helper T cell subsets are CD28 and ICOS. Whereas costimulation with ICOS biases cells toward T_H17 development, costimulation via CD28 impedes the development of T_H17 cells[49]. In mice, ICOS seems to direct the sizes of the T_H17 and T_{FH} pools through upregulation of the transcription factor c-Maf and subsequently of IL-21 (ref. 50). As these factors have also been shown to affect IL-10 production in various helper T subsets[51], it is likely that the actions of ICOS and CD28 as well as of IL-21 and c-Maf may be context specific, as it has been shown before that the strength of the TCR signal determines the costimulatory requirements for the T_H1 and T_H2 differentiation of CD4+ T cells[52].

Influence of antigen dose on helper T cells

It is well recognized, largely from *in vitro* studies, that the strength of the signal provided by the antigen dose can regulate the differentiation of T_H1 and T_H2 cells. Specifically, the high antigen dose presented by DCs favors the development of T_H1 responses, whereas a low antigen dose favors the development of T_H2 responses[2,3,35–37,40]. Although high doses of antigen, with sustained TCR signaling and phosphorylation of the mitogen-activated protein kinase Erk, result in T_H1 cells that produce IFN-γ via an IL-12-independent mechanism, low doses of antigen, with transient activation of Erk1 and Erk2, favor T_H2 responses and IL-4 secretion[2,35,36,53,54]. The suppression of T_H2 responses by TCR stimulation resulting from high receptor occupancy seems to be due in part to more phosphorylation of Erk that in turn suppresses *Gata3* transcription[54] and results in a T_H1 response. Furthermore, in addition to the fact that a low concentration of agonist peptides favors T_H2 development, priming with high concentrations of partial agonists also favors T_H2 responses independently of exogenous IL-4 (refs. 35,54,55). The association of weak signaling with T_H2 responses is further supported by studies demonstrating that hypomorphic mutations in genes encoding TCR signaling molecules can lead to severe atopic or T_H2-linked disease[56–62] and by the T_H2-promoting activity of schistosome egg antigen through attenuation of the antigen-presenting potency of DCs[63]. Importantly, cells bearing TCRs that are weakly stimulated by their cognate antigen 'preferentially' adopt a T_H2 phenotype when primed in the absence of competition from cells with receptors of higher affinity[64]. It is worth noting, however, that *in vivo* allergic responses resulting from T_H2 sensitization occur only if inhaled allergens are encountered in the context of microbial products such as lipopolysaccharide[65,66].

At high peptide concentrations, T cells from TCR-transgenic mice generally differentiate into T_H1 cells[2,35,36,40]. This has also been demonstrated in human studies in which autologous naive T cells cultured with DCs previously stimulated for 8 h with lipopolysaccharide and subsequently pulsed with high doses of toxic shock syndrome toxin 1 (TSST-1) were induced to develop into T_H1 cells with abundant production of IFN-γ[38]. In contrast, DCs similarly stimulated but pulsed with low doses of antigen favored T_H2 responses[38], much as has been demonstrated with CD4+ T cells from TCR-transgenic mice[2,36,40]. DCs pulsed with lipopolysaccharide for longer periods of time (24 or 48 h), however, produced IL-12 only transiently, and when subsequently pulsed with high doses of TSST-1, they failed to induce the development of T_H1 cells[38]. These findings were suggested to represent changes in priming conditions in the lymph nodes, which indicated another mechanism for the regulation of effector and memory T cells. However, it is also worth reconsidering the idea that T cells may leave the lymph nodes as they start to produce IFN-γ[21] and that encounters with signals in the tissues enhance and maintain the production of large amounts of IFN-γ to fight infection at the site of microbial entry. Although downstream TCR signals may influence the development of T_H1 or T_H2 cells, the antigen dose and the interaction of the integrin LFA-1 with its ligand ICAM regulate the expression of the ligand for the costimulatory molecule CD40 (CD40L) and subsequent helper T cell cytokine production via effects on the DC, including the induction of IL-12, to favor the development of T_H1 responses[40]. CD40-CD40L crosstalk has also been shown to integrate strong antigenic signals and microbial stimuli to induce the development of IL-17-producing CD4+ T cells[37], as well as T_H1 cells

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producing IFN-γ, and the development of experimental autoimmune encephalomyelitis[37]. It is thus evident that weak antigenic signals in concert with small amounts of microbial stimuli skew T_H2 responses, and strong antigenic signals in concert with microbial stimuli promote the development of T_H17 and T_H1 responses.

TCR signaling strength and Foxp3+ T_{reg} cells

The differentiation of effector helper T cell subsets results from the positive signals discussed above, each additionally reinforcing its own response not only via cross-regulation with effector cytokines and transcription factors but also by the inhibition of Foxp3+ T_{reg} cells[67,68]. Indeed the 'conversion' of truly naive CD4+ T cells into T_{reg} cells expressing Foxp3 *in vivo* has been achieved by targeting of peptide-agonist ligands in minute doses to DCs with suboptimal DC activation and little to no costimulation[69]. This 'conversion' is enhanced by the addition of TGF-β, which prevents extensive proliferation, whereas cells expressing a dominant negative form of the receptor TGF-βRII proliferate extensively even when antigen is delivered in non-immunogenic conditions, and these cells do not convert to Foxp3-expressing cells. The development of Foxp3+ T_{reg} cells from truly naive CD4+ T cells has also been shown to occur *in vitro*[70,71], where the extent of TCR-CD28 triggering of the CD4+ T cells determines the TGF-β-mediated induction or maintenance of Foxp3 in induced T_{reg} cells. A high threshold of TCR triggering inhibits the development of TGF-β-driven Foxp3+ T_{reg} cells[70–72], as does the presence of CD44+ memory T cells[67,68] and inflammatory mediators[9]. The addition of rapamycin, which lowers signal strength downstream of TCR activation and costimulation, can overcome this and allow the differentiation of Foxp3+ T_{reg} cells[70,73,74]. Thus, the amount of Foxp3 expression, crucial for T_{reg} cell function[75], is strongly influenced in the periphery by the relative amounts of TCR and costimulatory signals and inflammatory mediators, which compete with TGF-β signaling.

Repeated high-dose antigen: anergy and IL-10

The cytokine IL-10 is produced by many different immune cells and may limit immune responses to inhibit the overproduction of inflammatory cytokines and immune pathology[51]. Repeated immunization with high doses of soluble peptide or protein antigens or superantigens result in anergy and/or tolerance and the suppression of autoimmune diseases[76–78]. Suppression is mediated by IL-10 produced by these anergic helper T cells[76,78]. Analysis of the differentiation pathway taken by CD4+ T cells over the course of tolerance induction has shown that peptide-induced IL-10-producing T cells are of T_H1 origin and that IL-10-producing T cells complete the negative feedback loop of pathogenic T_H1 responses in autoimmunity[76]. IL-10-producing T_H1 cells that can suppress innate responses have been reported during chronic infection with intracellular pathogens and may contribute to chronic disease. For example, T_H1 cells producing IL-10 have been isolated from lungs of patients with tuberculosis and other chronic diseases, both in mouse and man, which suggests that abundant inflammation and antigenic stimulation may determine whether T_H1 cells produce IL-10 or not[79,80]. Indeed, this has now been verified in *in vitro* cultures in which IL-12 and high-dose antigenic stimulation result in T_H1 cells producing the highest concentrations of IL-10 and IFN-γ[81]. Although this is also possible to demonstrate *in vivo*, the reductionist and robust system allows delineation of the molecular mechanisms that lead to IL-10 production in various helper T cell subsets[51,81].

Fifty years of CD4+ T cell responses

We are tempted to speculate that some of the findings described in this perspective may represent what earlier studies reported as 'high-zone tolerance'[82–84]. In support of this, it has been suggested that repeated challenge of beekeepers with a high dose of bee venom renders them tolerant via IL-10 action[85,86]. Other findings discussed may explain some of the historical reports of 'low-zone tolerance'[82–84], although these earlier studies reported mainly antibody tolerance, which could, given the present knowledge of Foxp3+ T_{reg} cells, have resulted from active suppression of T cell help by induced T_{reg} cells. Antibody tolerance to a high or low dose of bacterial flagellin (a cyanogen bromide digest) was shown in 1972 to be accompanied by heightened delayed hypersensitivity[87], and this inverse relationship between humoral and cell-mediated immunity has since been equated to the reciprocal regulation of T_H1 and T_H2 responses[27,88,89]. However, given the further heterogeneity of CD4+ T cells described so far, it is likely that these varying responses that occur at different doses and during repeated stimulation with digested bacterial flagellin may represent a broader spectrum of responses in addition to T_H1 and T_H2 responses, including T_H17 and other effector responses, and that regulation by Foxp3+ T_{reg} cells at one end of the spectrum and anergic IL-10-producing T cells at the other end of the spectrum account for unresponsiveness.

Quantification of a helper T cell cytokine response *in vivo*

Investigating the regulation of effector functions of helper T cells *in vivo* in terms of their specification, differentiation and potential commitment will help elucidate the factors that control infections in response to particular pathogens or result in pathogenesis. This will also help identify pathways for therapeutic intervention in inflammatory diseases such as asthma and autoimmune diseases, in which helper T cells and innate cells become 'locked' in a particular direction.

The measurement of such responses *in vivo* has the added complication of differences in T cell migration after antigenic challenge that are possibly affected by a spectrum of T cells with different antigen reactivities and affinities[33] together with microbial signals. In addition, the influence of the tissues into which T cells migrate in response to chemokine gradients set up during different stages of the immune response should be considered. Thus, the total amount and effect of a helper T cell hallmark cytokine produced *in vivo* may reflect the number of T cells recruited to an infected tissue and the amount of the effector cytokine that each T cell makes. The small difference in the percentage of IFN-γ-secreting T_H1 cells and the mean fluorescent intensity measured by flow cytometry in the lungs of *Mycobacterium tuberculosis*–infected IL-10-deficient mice translates to a logarithmically greater amount than that in similarly infected wild-type control mice, when the greater number of CD4+ T cells in the lungs is taken into account (**Fig. 2**). This is reflected by the amount of IFN-γ detected in supernatants from antigen-restimulated lung cells. However, these data rely on antigen restimulation of cells, which may not reflect what actually occurs *in vivo*. However, for these data, similarly larger amounts of IFN-γ protein[90] and IFN-γ mRNA (A.O.G., unpublished data) can be detected in the serum and blood, respectively (**Fig. 2**), which reflects the size of the response in the lung that is required for a substantial decrease in mycobacterial load[90].

In other *in vivo* settings, however, responses in tissues may not be reflected in the serum and/or blood. In addition, other cytokines may be difficult to absolutely quantify, as many cytokines act at short range and are rapidly degraded post-transcriptionally[91]. Although cytokines can be measured at the mRNA level in tissue[22,23], it cannot be assumed that this reflects the amount of protein *in situ*. Reporters for particular cytokines and even combinations of cytokines may help in *in vivo* studies of mice but may not reflect the native mRNA or protein. Furthermore, the different amounts of cytokines and transcription factors may again be

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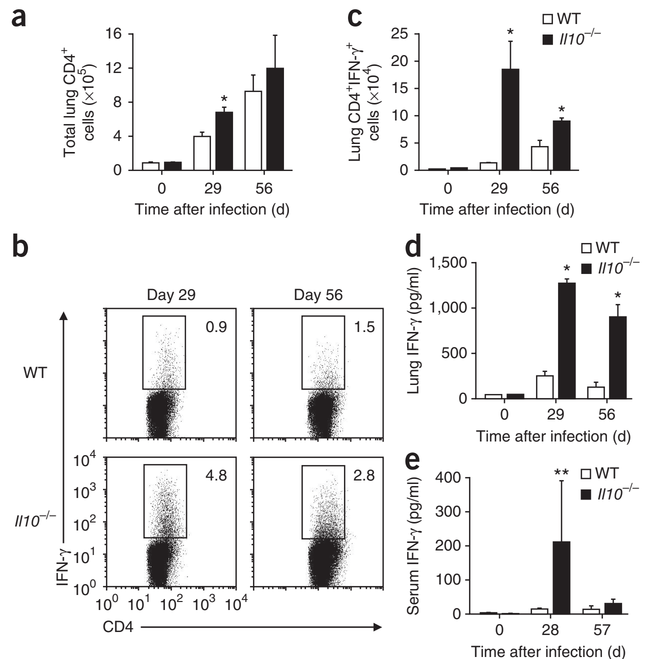

Figure 2 Several factors determine the absolute amount of helper T cell cytokines produced in response to infection. The greater abundance of CD4+IFN-γ+ T cells in the lungs of *Il10*−/− mice infected with aerogenic *M. tuberculosis* strain H37Rv (**a**–**c**) leads to more IFN-γ protein in supernatants of lung cell suspensions restimulated *ex vivo* with purified protein derivative (**d**) and more IFN-γ protein in the serum (**e**). WT, wild-type. *$P < 0.05$ and **$P < 0.01$ (unpaired Student's *t*-test). Modified with permission from ref. 90.

difficult to quantify, particularly as T cells in different tissues probably represent different stages of differentiation and/or commitment, with the most committed representing a very small number of cells with the highest expression of their hallmark transcription factors and cytokines. In humans, these cells may be impossible to quantify because of inaccessibility of tissues and limited numbers of cells. Although the study of blood transcriptomes may be helpful in understanding the composite immune response elicited during vaccination and/or disease pathogenesis[92,93], this will not elucidate the discrete cellular and molecular mechanisms that contribute to this global response; thus, there is a need for complimentary and alternative approaches.

Standardization of reductionist *in vitro* T cell systems

As has been elegantly discussed before[20,30], epigenetic modifications create gradients of factors that determine the differentiation and potential commitment of helper T cells in a manner similar to that proposed for determinism and flexibility in developmental biology[94]. Although it is essential to continue studying cytokine regulation and function *in vivo* to see how this affects health and disease, reductionist *in vitro* systems with polarized helper T cells have been useful in determining epigenetic changes and details of cytokine gene regulation and commitment. This is well illustrated by classical studies of the locus encoding IL-4, IL-13 and IL-5 in T_H2 cells[95], along with subsequent findings obtained with T_H1, T_H2 and T_H17 cells[20]. It is likely that the end-point polarized and committed T_H2 cells observed in the aforementioned reductionist studies[95] represent T cells positive for CRT_H2 (chemoattractant receptor–homologous molecule expressed on T_H2 cells), studied *ex vivo* immediately after isolation[96], which can be obtained only in extremely low numbers that preclude in-depth molecular work at this stage.

Thus, defined *in vitro* reductionist cultures are useful for further delineation of the molecular mechanisms underlying cytokine gene regulation in more recently described mouse CD4+ T cell subsets; such findings can then be used to investigate the regulation and function of these cells *in vivo*. Because in humans *in vitro* and *ex vivo* studies are usually the only experimental approach possible, careful comparison of *in vitro* reductionist studies and *in vivo* studies of mice with *in vitro* studies of human cells may help in predicting what happens in humans *in vivo*. For example, a high antigen dose leading to T_H1 responses, low poorly immunogenic doses of antigen resulting in the conversion of naive CD4+ T cells into Foxp3+ T_{reg} cells and other such findings should be used to obtain information about humans. One caveat in comparing human and mouse *in vitro* studies is that the sources of these T cells are often different, as mouse T cells are mostly isolated from spleen, whereas human T cells are obtained from peripheral blood.

For *in vitro* reductionist approaches to be used meaningfully, it is essential that certain standards be considered. For *in vitro* differentiation studies, highly purified naive CD4+ T cells are essential; this is not achieved solely through the use of bead selection but requires purification by flow cytometry for rigorous removal of potential contaminating effector T cells[68], Foxp3+ T_{reg} cells or contaminating non-CD4+ T cells, including APCs. This is essential for the simplest APC-independent stimulation with antibody to CD3 and antibody to CD28 as well as for APC-dependent antigen-driven responses. In humans, antigen-driven differentiation of helper T cells is hard to achieve because of the polyclonal nature of the TCR repertoire of the naive T cells. However, superantigens such as TSST-1 (ref. 38) may be used as an alternative. Given the findings described so far in this perspective, the appropriate dose of antibody to CD3, antibody to CD28 or other costimulatory antibodies and dose of antigen must be defined. In addition, whether T cells are removed from TCR stimulus and/or cultures are divided and when this is done after initial stimulation[38,45,70,74] is important. The type and density of APC and how each APC has been stimulated is also critical to define. The strength of signal, antigen dose and costimulation may determine not only the extent of activation of the T cell but also the reciprocal activation of the APC by activated T cells[40]. In studies of specification and differentiation it is also worth considering the length of culture and how many rounds of stimulation are used to distinguish early and terminal effector T cells[26,30,97]. The density of cells for *in vitro* cultures should be described, particularly in terms of autocrine factors and paracrine factors derived from the APC or the T cell itself. Differences in cell density may explain discrepancies in the literature. For example, at lower cell densities, paracrine or autocrine factors will be less prevalent and this could affect the differentiation of helper T cells or Foxp3+ inducible T_{reg} cells. The choice of culture medium is often critical, as illustrated by T_H17 differentiation, which is affected by factors in the medium that activate the modifying transcription factor AhR[98]. Furthermore, the exact concentrations of cytokines added to cultures and the combinations in which they are added may explain the difference between the absolute amounts of the hallmark cytokines and transcription factors expressed by IL-17-producing CD4+ T cells and their degree of epigenetic changes, which in concert contribute to their ability to induce pathology *in vivo*[98–100].

It is clear that reductionist approaches *in vitro* are essential for the definition of the molecular pathways of helper T cell differentiation; however, it is critical that these assays be standardized and, where possible, be related to *in vivo* findings. This is important to point out, because a range of T cells with different affinities and avidities are often stimulated with different amounts of TCR costimulation with or without cytokines,

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and these conditions are commonly poorly defined. Alternatively, such T cells are stimulated with APCs in concert with different doses of antigen together with different concentrations of ligands for pattern-recognition receptors. As the amount of endogenous cytokines generated and costimulators induced will have a considerable influence on the quality of helper T cells that differentiate in culture, it is fundamental that the ratio of cells, size of wells and volume used for such *in vitro* cultures are well defined and standardized. This will allow laboratory-to-laboratory comparisons as well as comparisons between humans and mice. Although it is important that the molecular pathways and factors that determine the differentiation and function of helper T cells are recapitulated *in vivo* in physiological systems, it should be kept in mind that it is through *in vitro* studies that the molecular basis for the differentiation of effector helper T cells were defined. Strict quantification and standardization are fundamental to obtain widely generalizable data in both mouse and human systems, which can then be tested *in vivo*. In addition, as discussed, quantitative events will determine the specification, differentiation and commitment of helper T cells that have a major role in determining the extent of effector responses mediated by helper T subsets essential for effective responses during infection or vaccination with minimum damage to the host. Standardization of *in vitro* protocols used with helper T cells and T_{reg} cells is also important, as it is the first step for the development of cell-based immunotherapies.

The development of *in vitro* systems that more closely resemble the complexity of T cell priming *in vivo* may help to align human systems with the *in vivo* scenario. For example, the creation of artificial lymph nodes that incorporate stromal cells or other cells that may contribute, directly or indirectly, would advance the study of T cell activation and differentiation. Such systems, however, will also need to be carefully designed and interpreted to incorporate quantitative as well as qualitative events that may contribute to immune responses *in vivo*. In addition, there is a need for more robust measurements of T cell activation and better quantitative measurements of signal transduction, linked to the different stages of specification, differentiation and commitment of helper T cells *in vivo,* for solving the problem of cellular heterogeneity. However, this still requires *in vitro* systems because of the limitations of the technology at present. New tools that would advance the knowledge of quantitative as well as qualitative events *in vivo* include quantitative multiplex analysis of cytokines and quantitative measurement of transcription factor expression and epigenetic modifications at the single-cell level. There also is a need for *in vivo* imaging of trafficking cells with simultaneous quantitative measurement of cellular activation and differentiation. Such analyses would need to take into account the fact that many cytokines act at short range and are rapidly degraded post-transcriptionally[91]; how this would be assessed *in vivo* is as yet difficult to envisage. An additional complexity is that events must be measured *in vivo* over time in different tissues and lymphoid organs to reflect the different stages of the immune response that dictate protection against pathogens or pathogenesis during infection or inflammatory disease.

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COMPETING FINANCIAL INTERESTS

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