Polarization of effector CD4+ T cells can be influenced by both antigen-specific signals and by pathogen- or adjuvant-induced cytokines, with current models attributing a dominant role to the latter. Here we have examined the relationship between these factors in shaping cell-mediated immunity by using intravital imaging of CD4+ T cell interactions with dendritic cells (DCs) exposed to polarizing adjuvants. These studies revealed a close correspondence between strength of T cell receptor (TCR)-dependent signaling and T helper 1 (Th1) versus Th2 cell fate, with antigen concentration dominating over adjuvant in controlling T cell polarity. Consistent with this finding, at a fixed antigen concentration, adjuvants inducing Th1 cells operated by affecting DC costimulation that amplified TCR signaling. TCR signal strength controlled downstream cytokine receptor expression, linking the two components in a hierarchical fashion. These data reveal how quantitative integration of antigen display and costimulation regulates downstream checkpoints responsible for cytokine-mediated control of effector differentiation.

Although many of the reports linking cytokine milieu to effector fate choice have been conducted with cells from TCR transgenic animals and in vitro culture systems, a substantial body of in vivo evidence also supports the key role played by cytokines in CD4+ T cell polarization (Zhu et al., 2010). Mice deficient in or overexpressing specific cytokines show dramatic changes in the nature of the effector CD4+ T cells that emerge after immunization or infection (Finkelman et al., 2004). Likewise, infection with particular organisms drives polarized effector CD4+ responses and manipulation of the cytokine environment changes the character and efficacy of these pathogen-driven responses (Sacks and Noben-Trauth, 2002), providing in vivo support to a model in which it is the qualitative effects of these soluble mediators that play a dominant role in directing the nature of the cell-mediated immune response.

Despite the widespread acceptance of this qualitative (cytokine-defined) model, there are data showing that quantitative factors, especially the strength of antigen stimulation through the TCR, make important contributions to T cell polarity choice. Both in vitro and in vivo studies (Constant et al., 1995; Hosken et al., 1995; Milner et al., 2010; Yamane et al., 2005) have demonstrated that the extent of signaling through the TCR and associated costimulatory receptors can dictate the outcome of differentiation. A high dose of peptide or a strongly agonistic ligand favors development of Th1 (IFN-γ-producing) cells whereas stimulation with a low dose of peptide or a weakly agonistic ligand favors Th2 (IL-4-, IL-5-, and IL-13-producing) cells.

Because most studies evaluating the role of cytokines in vitro are done at single antigen or anti-TCR antibody concentrations, the quantitative component is generally removed from consideration, giving the appearance that cytokines dominate. In vivo, infections provide a particular degree of antigenic stimulation that is not usually subject to experimental manipulation, making it difficult to parse out the role of signaling strength in experiments that alter the cytokine environment in infected animals. Given that variations in both the cytokine milieu and extent of TCR signaling exist in vivo during infections or upon vaccination, we felt that it was important to ask how the cell interprets such complex stimuli and specifically whether one category of inputs is hierarchically dominant. To this end, we devised a model system in which both the cytokine milieu and the strength of antigen stimulation could be independently varied to explore how quantitative and qualitative aspects of signaling regulate CD4+ T cell differentiation. Dynamic 2-photon microscopy (2P-IVM) was used to directly assess T cell-DC interaction duration, synapse size, and calcium signaling. By varying both the adjuvant exposure used to activate DCs and control their cytokine
production and costimulatory capacity, as well as by carefully modulating the peptide-MHC class II (pMHC) ligand display encountered by the responding T cells, we obtained direct information about how these distinct factors influenced strength of signaling in vivo. Through this crossover experimental design, imaging-based measurements, and assessment of postpriming effector T cell phenotype, we found that strength of signal dominates over adjuvant and cytokines in dictating Th1 versus Th2 cell fate. Adjuvants influenced polarization through the effects of costimulation on TCR signaling, with the strength of the combined TCR and costimulatory stimulus in turn controlling cytokine receptor expression. These findings reveal that antigen-dependent events act as upstream regulators of secondary checkpoints leading to the type of cytokine control typically given priority in models of T cell differentiation.

RESULTS

CD4+ T Cells Undergoing Th1 as Compared to Th2 Cell Polarization Show Greater T Cell-DC Interaction Times

We began our studies by examining the ability of adjuvant treatment of DCs to promote Th1 versus Th2 effector cell development at a fixed moderate concentration of available TCR ligand. DCs were pulsed with 0.1 μM pigeon cytochrome C peptide (pPCC) in the presence of either lipopolysaccharide (LPS) (LPS-DC) or papain (papain-DC) and the DCs were adoptively transferred into CD45.1+ B10.A animals. After 18 hr, naive antigen-specific 5CC7 CD4+ T cells and wild-type (WT) polyclonal CD4+ T cells specific for pPCC were transferred into the same animals. Four days later, Th1 versus Th2 cell differentiation was assessed by measuring IFN-γ or IL-4 production, respectively, by restimulated recovered 5CC7 cells. LPS-treated DCs induced a strongly biased Th1 cell response, whereas interaction with papain-treated DCs led to a Th2 cell skewed response (Figures 1A–1C). We did not detect cells producing IL-17 under these conditions (data not shown) and note that the fraction of cells showing either a Th1 or Th2 cell effector state is in accord with other in vivo immunization findings (León et al., 2012; Tokoyoda et al., 2009; van Panhuys et al., 2008).

To determine whether adjuvant treatment altered in vivo trafficking or the uptake and display of ligand by these cells, thus affecting the intensity of the TCR signaling, DCs were pulsed with long chain biotinylated-pPCC (LC-pPCC) in the presence of LPS or papain and LC-pPCC binding to MHCII was assessed. Both LPS-DC and papain-DC displayed similar MHCII and LC-pPCC staining (Figures S1A–S1G available online). In addition, similar numbers of adjuvant-treated DCs accumulated in the draining LN (dLN), showing that the adjuvant pretreatment did not alter the migratory capacity of DCs (Figure S1H), and no significant difference was detected in MHCII or LC-pPCC staining on DCs recovered from the dLN (Figures S1I–S1L), indicating equivalent antigen presentation in vivo.

Although these data suggested that adjuvants did not have a direct effect on quantitative aspects of T cell activation via modification of ligand display, a possible effect on overall strength of TCR signaling was possible. To explore this issue in vivo, 2P-IVM was employed. The duration of T cell-DC interactions was measured and the effect of adjuvants evaluated in the context of previously defined sequential stages of T cell-DC interaction (Mempel et al., 2004) in which phase transition has been linked to the strength of TCR-related signaling (Henrickson et al., 2008). DCs were fluorescently labeled with distinct fluorophores were transferred and cellular interactions in the dLN immediately imaged (Movie S1). Because previous work has indicated that adjuvants induce differential chemokine production from DCs (Tang et al., 2010), we sought to determine whether adjuvant pretreatment would lead to preferential interaction of 5CC7 cells with LPS- or papain-treated DCs.
We observed that both WT and 5CC7 cells contacted LPS- and papain-treated DCs at similar rates, indicating that a differential chemoattractant potential was not conferred on the DCs by the distinct adjuvant pretreatments (Figures S2A–S2C). Polyclonal cells interacting with either LPS-DCs or papain-DCs exhibited brief browsing behavior as previously reported (Miller et al., 2004), whereas antigen-specific 5CC7 cells exhibited more prolonged interactions with the antigen-bearing DCs (Figures 1D and 1E). A proportion of the 5CC7 contacts involving LPS-treated DCs rapidly transitioned to phase 2-like interactions, with contacts lasting >1 hr (Mempel et al., 2004). In contrast, prolonged interactions characteristic of phase 2-like behavior were virtually absent among 5CC7 cells interacting with papain-DCs.

To assess whether such interaction differences were generally associated with adjuvant effects promoting Th1 versus Th2 cell development at the same TCR ligand density, we compared DCs treated with the Th1-cell-inducing adjuvant CpG oligodeoxynucleotides (CpG) with those treated with the Th2-cell-inducing adjuvant Schistosomal egg antigen (SEA) (Figures 1F–1H). Pretreatment of DCs with distinct adjuvants did not alter the chemoattractive potential of DCs; the 5CC7 cells showed equal rates of contact with the antigen-bearing DCs (Figures 1D and 1E). A proportion of the 5CC7 contacts involving LPS-treated DCs rapidly transitioned to phase 2-like interactions, with contacts lasting >1 hr (Mempel et al., 2004). In contrast, prolonged interactions characteristic of phase 2-like behavior were virtually absent among 5CC7 cells interacting with papain-DCs.

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To determine whether adjuvant treatment affected cell interaction dynamics at later time points, also suggesting differences in TCR-associated signaling, we imaged the cohort of 5CC7 cells recruited to the LN 0–2 hr after transfer by blocking further recruitment with anti-CD62L (Mempel et al., 2004). At 12 hr posttransfer, a significant proportion of 5CC7 cells interacted stably with LPS-DCs for the majority of the imaging time, whereas only a small proportion of 5CC7 cells interacting with papain-DCs did so (Figures 2A, 2C, 2E, 2G, S3A, and S3B; Movie S2). At 22 hr after transfer, the majority of 5CC7 cells interacting with papain-DCs showed phase 3 behavior, exhibiting increased migration speeds and a mean interaction time similar to that of polyclonal cells (Figures 2B, 2D, and 2F; Movie S2). In contrast, many long-term interactions were still evident between 5CC7 cells and LPS-DCs (Figures 2B and 2G). Together, these results indicate that incubation of DCs with a Th1-cell-inducing adjuvant leads to stable long-lived interactions with antigen-specific T cells, whereas DCs exposed to a Th2-cell-inducing adjuvant show few prolonged interactions with specific T cells and the lymphocytes rapidly progress to phase 3 dynamic behavior.

To further determine whether qualitative mediators such as chemokines or cytokines released by DCs into the local milieu contribute to the differences in interaction times that we observed, antigen-loaded adjuvant-stimulated LPS-DCs and papain-DCs were labeled with distinct dyes and cotransferred into the same recipient. At 18 hr posttransfer, labeled naïve Dcs.
DCs after 2 hr of imaging (Figure 3F). These data argue that the T cells were found to be preferentially associated with LPS-exposure of DCs alters a T cell’s quantitative perception of the preceding dynamic imaging studies suggested that adjuvant- 

modulation of TCR-dependent signal strength alters T cell polarization outcome and overrides adjuvant effects. The observations described above revealed that Th1- and Th2-cell-polarizing adjuvants influenced the strength and duration of early antigen-induced signals in a manner correlating with past evidence that strong signaling promotes Th1 cell responses whereas weaker stimulation favors Th2 cell development. However, it remained unclear whether these quantitative differences causally determined T cell fate or were simple correlations, with qualitative signals from cytokines induced by adjuvants having a more important role in guiding differentiation. Therefore, we examined whether manipulating the strength of signal by varying the concentration of peptide used to pulse DCs would override putative dominant qualitative cytokine signaling mechanisms induced by adjuvant treatment. DCs were pulsed with a range of peptide concentrations (10.0–0.01 μM) in the presence of either LPS or papain and used to prime 5CC7 cells in vivo. After priming with the DCs exposed to different peptide concentrations, dLNs were collected and the relative Th1 versus Th2 cell polarization analyzed using 2P-IVM. In these conditions, the T cell-DC interface was imaged for 2 hr. As a further measure of TCR-dependent interactions, we analyzed the dimensions of the T cell-DC interface (immunological synapse) (Klauschen et al., 2009). At 2 hr posttransfer, LNs were fixed, serially sectioned, and imaged, and whole LNs were digitally reconstructed (Figure S4I; Movie S5). From these images, the mean number of CD4+ T cell interactions per DC (C) and frequency distribution of interactions (D).

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differentiation of 5CC7 cells was assessed. A cytokine-dominant qualitative model would predict that changing pPCC amounts on adjuvant-treated DCs should result in the same bias of Th cell polarization but with more or fewer cells adopting the expected fate, whereas a quantitative model involving TCR-dominated signaling would predict a change in Th cell fate outcome as peptide concentration was altered.

To assess whether variable loading with synthetic peptides had any effect on the DCs other than in terms of pMHC display, we examined the surface activation phenotype of these cells as well as their production of IL-6, IL-10, and IL-12 (Figures S5A–S5D). These various measures should sensitively report the presence of PAMPs in the peptide preparations that might influence how the DCs behave in vivo. We found no significant differences in these various measures after peptide treatment, providing strong evidence that any changes in T cell fate influenced by varying the peptide loading of the DCs reflect the influence of pMHC density.

After activation with DCs pulsed with 10.0 μM pPCC, a Th1-cell-biased response predominated even after DC treatment with the Th2-cell-evoking adjuvant papain (Figures 5A–5C). However, as the concentration of pPCC was decreased, the proportion and numbers of IL-4-producing cells increased coincident with a decrease in IFN-γ-producing cells, such that at 0.01 μM pPCC, a Th2 cell response was predominantly evoked regardless of the adjuvant used to treat the DCs (Figures 5D and S5A). These findings suggest that the strength of proximal signaling induced in the T cell by the DC dominates over any qualitative effects imparted by adjuvants. Consistent with this, 5CC7 cells showed significantly decreased interaction times and Ca2+ fluxes (Figures 5E–5H and S5B–S5D) when recognizing LPS-DCs pulsed with a low concentration of pPCC (Lo-LPS: 0.01 μM) as compared with papain-DCs pulsed with high concentrations of pPCC (Hi-pap: 10.0 μM). Thus, the degree of early proximal T cell signaling was more tightly correlated with Th cell differentiation outcome than was the adjuvant used to treat the DCs and quantitative features of T cell-DC interactions appeared to be dominant over qualitative signals arising from adjuvant exposure of the DCs.

**Adjuvants Influence Signal Strength through Effects on Costimulatory Molecule Expression**

To probe how elevated proximal TCR-related signaling could occur using LPS- or CpG-treated DCs presenting similar amounts of pMHC TCR ligand in comparison to papain- or SEA-treated DCs, we extended our phenotypic analysis of DCs in vitro and ex vivo. There were no significant differences between the DC treatment groups in terms of physical...
characteristics or in expression of MHCII, intercellular adhesion molecule-1 (ICAM-1), and inducible costimulatory molecule ligand (ICOSL) or presentation of LC-pPCC (Figures 6 A, 6C, and S6A–S6F). One major difference that correlated with outcome did emerge, however; DCs treated with Th1-cell-inducing adjuvants had higher expression of the CD28 ligands CD80 and CD86 than did those exposed to Th2-cell-inducing adjuvants (Figures 6B, S6E, and S6F). CD80 signaling can potentiate Ca\(^{2+}\) flux upon TCR activation (Nurieva et al., 2007) and mediate tighter T cell-DC interactions (Lim et al., 2012), potentially explaining the increased TCR-associated signaling observed after treatment of DCs with Th1 cells as compared with Th2-cell-inducing adjuvants at a constant pMHC density.

To assess this possibility, we utilized antibody blockade of CD80 signaling during in vivo activation of 5CC7 cells with LPS-DCs or papain-DCs. Although affecting only one of several costimulatory ligands on the DCs upregulated by LPS treatment, antibody treatment led to a significant decrease in IFN-\(\gamma\) production and a corresponding increase in IL-4 production by the recovered 5CC7 cells (Figures 6 D and 6E). 2P-IVM imaging showed that treatment with aCD80 resulted in a decrease both in T cell interaction times with the LPS-DCs and in the associated T cell Ca\(^{2+}\) flux (Figures 6 F–6I), yielding a pattern more similar to that observed under Th2-cell-inducing conditions. These

Figure 5. The Magnitude of Antigen-Dependent Signal Strength Dominates over Qualitative Effects of Adjuvants on DCs

(A) Assessment of 5CC7 cell differentiation after priming with LPS- or papain-treated DCs loaded with 10–0.01 \(\mu\)M pPCC, at day 4 after adoptive transfer.

(B) Quantification of percent of 5CC7 cells expressing IL-4 or IFN-\(\gamma\).

(C) Number of 5CC7 cells expressing IL-4 or IFN-\(\gamma\).

(D) Ratio of %IFN-\(\gamma\)/%IL-4 producers.

(E) Cellular interaction times 0–1 hr posttransfer of 5CC7 cells with Lo-LPS- or Hi-papain-treated DCs.

(F) Mean interaction times for individual experiments.

(G) Integrated Ca\(^{2+}\) flux areas were calculated for individual cellular tracks.

(H) Mean integrated Ca\(^{2+}\) flux areas for individual experiments.

Data are representative of four experiments (n = 4) (A–D), pooled from four experiments (E and G), or representative of four experiments (F and H). Mean ± SEM. *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\). 1-way ANOVA with Tukey’s posttesting.
findings explain how proximal TCR-related signals can differ with DCs exposed to distinct adjuvants even when peptide-MHC display is equalized and indicate that adjuvants can influence T cell polarization through dominant effects on proximal strength of signal involving TCR cooperation with CD28.

**TCR-Associated Signal Strength Regulates Downstream Cytokine Signaling Checkpoints**

To reconcile our results with previous data showing a crucial role for adjuvant-elicited cytokines in directing effector T cell development, we theorized that TCR signaling may operate upstream of cytokine-mediated checkpoints; that is, signal strength controls T cell responsiveness to polarizing cytokines, which would then drive the molecular events involved in differentiation. IL-12p70 is a critical myeloid-cell-derived cytokine involved in Th1 cell polarization (Zhu et al., 2010), which has been shown to be delivered via the immunological synapse (Pulecio et al., 2010). The complete IL-12 receptor is not expressed on naive CD4+ T cells (Desai et al., 1992), so we tested whether upregulation of the relevant IL-12Rβ2 chain was associated with DC signal strength and the length of interaction with the antigen-bearing DCs. DCs were pulsed with varying concentrations of pPCC and used to activate 5CC7 cells in vitro. After activation, increased IL-12Rβ2 expression was found to correlate directly with the concentration of pPCC presented (Figure 7A). Activation was required for the induction of IL-12Rβ2 expression and both the frequency of positive cells and mean fluorescent intensity (MFI) of IL-12Rβ2 expression showed a direct correlation with the dose of antigenic peptide (Figures 7B and 7C).

Because our in vivo results indicated that Th1 cell development was associated with long-term T cell-DC interactions, we examined whether there was also a temporal component to the upregulation of IL-12Rβ2. IL-12Rβ2 expression was induced only after >6 hr in culture with high concentrations of pPCC (Figure 7D). To determine whether polarizing cytokines materially affected IL-12Rβ2 expression, we compared normal 5CC7 cells with Il4−/C0−/C0−Ifng−/C0−5CC7 cells and found that there was no alteration in IL-12Rβ2 expression in the absence of these key mediators (Figure 7D). To investigate the physiological significance of these in vitro findings, we again used antigen-loaded, adjuvant-exposed DCs to activate naive 5CC7 cells in vivo. After in vivo activation, CD4+ T cell interaction with LPS-DCs resulted in higher IL-12Rβ2 expression in comparison to T cell exposure to papain-DCs (Figures 7E and S7A–S7D). Additionally, 5CC7 cells activated by LPS-DCs had increased amounts of the phosphorylated transcription factor STAT4 in comparison to those activated by papain-DCs (Figures 7F and 7G). Together, these findings indicate that the strength of antigen-dependent signals, as influenced by pMHC concentration and costimulation and the corresponding duration of T cell-DC interactions, control downstream cytokine response checkpoints that ultimately direct effector polarization.

**DISCUSSION**

The differentiation of naive CD4+ T cells into distinct effector subsets plays a major role in governing the outcome of the adaptive immune response. Both qualitative characteristics of the cytokine milieu and quantitative signals imparted through...
TCR-mediated stimulation can influence the differentiation of specific T helper subsets (Yamane and Paul, 2013). Our data demonstrate that the differentiation of naive CD4+ T cells into Th1 or Th2 cell effectors involves a series of checkpoints that begins with a quantitative T cell assessment of antigen and costimulatory signals, which in turn secondarily control the responsiveness of the T cell to polarizing cytokines. Weak signals are sufficient to activate CD4+ T cells and induce Th2 cell differentiation after brief interactions, potentially through an endogenous Th2-cell-differentiation program (Zhu et al., 2012). Conversely, Th1 cell differentiation requires strong signaling and a transition to long-term interactions. Integration of strong signals induces a divergent program of early activation gene expression, including upregulation of IL-12Rβ2, which allows further tuning of the immune response by the cytokine environment. Taken as a whole, these findings reveal how adjuvants not only alter the ability of APCs to produce qualitative signals (Medzhitov and Janeway, 1997) affecting CD4+ T cell differentiation, but play a critical and indeed dominant role in the latter process through effects on costimulatory molecule expression that synergizes with antigen to control signals associated with TCR engagement.

This relationship between signal strength and effector fate can be observed in animals with a polyclonal T cell repertoire and absent artificially applied adjuvants, arguing against the present findings being unique to the experimental system we employed. Consistent with our findings showing that low signal strength promotes Th2 cell induction, expression of hypomorphic variants of the TCR-proximal signaling adaptor LAT leads to spontaneous induction of Th2-cell-associated autoimmunity (Mingueneau et al., 2009). Likewise, ZAP70−/− mice exhibit defective T cell Ca2+ mobilization along with elevated IgG1 and IgE, iso- types characteristic of a Th2 cell response (Siggs et al., 2007). Proteins further downstream in the signaling cascade such as PKCδ and Wiskott Aldrich Syndrome protein (WASP) play opposing roles in the maintenance of the IS (Sims et al., 2007), with PKCδ contributing to termination of stable synaptic

![Figure 7. Signal Strength Determines the Ability of CD4+ T Cells to Respond to Polarizing Cytokines](image)

5CC7 cells were activated in vitro using P13.9 artificial antigen-presenting cells preincubated with the concentrations of pPCC indicated. At 24 hr postactivation, IL-12Rβ2 expression by the T cells was determined by flow cytometry.

(A) Representative plots of IL-12Rβ2 expression by stimulated (black lines) or naive (gray lines) 5CC7 cells.

(B) Percent of activated (CD69+) and nonactivated (CD69−) 5CC7 cells expressing IL-12Rβ2.

(C) Comparison of IL-12Rβ2 MFI for activated versus nonactivated 5CC7 cells.

(D) WT 5CC7 cells were stimulated in vitro with P13.9 cells preincubated with either 0.01 μM or 10 μM pPCC and IL-12Rβ2 expression determined.

(E–G) 5CC7 cells were activated in vivo with LPS- or papain-treated DCs and compared to control 5CC7 cells from non-dLNs. Ex vivo expression of IL-12Rβ2 (E) and pSTAT4 (F and G) by CD69+ 5CC7 CD4+ T cells was then determined at 24 hr posttransfer.

Data are representative of two experiments (n = 4) (A–C) or representative of three experiments (n = 4) (D–G). Means are plotted ±SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 as determined by 1-way ANOVA with Tukey’s posttesting.
interaction with DCs and WASp acting as a negative regulator of the PKC-ε-mediated symmetry breaking process. In accordance with our data indicating the importance of a temporal element in CD4+ T cell differentiation, activation conditions favoring PKC-ε signaling and shorter interactions preferentially induce Th2 cell differentiation (Cannons et al., 2004; Corn et al., 2005; Hilliard et al., 2002; Marsland et al., 2004; Medoff et al., 2006), whereas those that enhance IS stabilization and Ca2+ signaling result in preferential Th1 cell differentiation (Noble et al., 2000). Further, defects in WASp are associated with reduced Th1 cell differentiation (Taylor et al., 2010; Trifari et al., 2006) and development of Th2-cell-associated autoimmune disease (Ozcan et al., 2008) and molecules associated with enhanced Ca2+ signaling and IS formation are required for efficient Th1 cell differentiation or the suppression of spontaneous Th2-cell-associated disease (Oh-Hora et al., 2008; Tahvanainen et al., 2009; Varga et al., 2010).

Studies of the TCR itself provide further evidence for a primary role of signal strength in controlling T cell fate. Single naive polyclonal CD4+ T cells can produce an array of effector cells and the pattern of effector cells generated after in vivo activation with cognate antigen correlated with the TCR-pMHCII dwell time or the amount of pMHCII (Tubo et al., 2013). Depletion of CD4+ T cells with a high affinity for pMHCII from a polyclonal population of cells left lower-affinity T cells that preferentially differentiated into IL-4-producing Th2 cells (Milner et al., 2010). Single cell cloning showed that low-affinity cells had fewer preferred complementarity determining region-3 (CDR3) motifs, consistent with findings that the outgrowth of CD4+ T cells under Th2 cell conditions favored cells with elongated TCRα CDR3 motifs that potentially impeded TCR triggering (Boyton et al., 2002).

Finally, two strains of transgenic mice created with two different TCRs specific for the same self-antigen (gastric ATPase) show biased Th1- versus Th2-cell-associated disease associated with differences in availability of self-antigen-MHC class II ligands rather than intrinsic affinity for the ligands (Levin et al., 2008). Our present analysis that emphasizes the dominant and upstream role of antigen-dependent strength of signaling in controlling effector polarization provides a coherent, integrated explanation for these many distinct observations.

Although our data and these cited studies provide a consistent picture of regulation of Th1-Th2 cell fate choice, we have not yet fully addressed whether the same model applies equally well to other Th cell subsets such as Th17 cells or inducible regulatory T (iTreg) cells. However, there are also clear indications in the literature that strength of TCR signaling influences differentiation along these pathways as well (Fazilleau et al., 2009; Gottschalk et al., 2010). What remains to be determined is the overall scaling of signal input with fate determination for the entire range of Th cell subsets, the relationship of signal strength to induction of other cytokine receptors besides IL-12Rβ2, how polarizing adjuvants or PAMPS contribute to modulation of antigen-specific signaling through other costimulatory molecules as we report here for CD80 and CD86, and whether there are conditions in which cytokines or metabolites (Arpaia et al., 2013; Furusawa et al., 2013) achieve dominance over the antigen-associated signals. A last point involves understanding how the diversity of TCR affinities and the variation in the amount of antigen presented (self or foreign) shape the overall quality of the emerging response under infectious or steady-state conditions.

A particularly relevant question is how there could be a consistent difference in antigen stimulation strength between helminth infections (which typically promote Th2 cell responses) and bacterial, viral, or unicellular parasitic infections (which often promote Th1 cell responses) (Finkelman et al., 2004; Zhu et al., 2010). It is unlikely that the affinity of the TCRs recognizing peptide ligands derived from the two different classes of pathogens (Th1-cell-inducing versus Th2-cell-inducing) is on average different. However, as cited above for the gastritis model (Levin et al., 2008) and shown here via a reductionist system, variation in the amount of pMHC ligand displayed on a DC in concert with the extent of CD80 and CD86 expression can have dramatic effects on T cell effector choice. In the case of worms, they are too large to be phagocytized and they do not reside in intracellular compartments, whereas the Th1-cell-inducing pathogens can be taken up and often require intracellular residence. There is thus a high likelihood of extensive antigen delivery directly to presenting cells in the latter case, whereas for extracellular worms, only smaller amounts of antigen can access the presentation pathway through shed material acquired via endocytic uptake. When this is combined with evolution of the worms to limit detection by sensors controlling the DC activation pathways that promote maturation of the antigen processing machinery (Inaba et al., 2000) and upregulation of CD80 and CD86 (Lee and Kim, 2007), it becomes understandable how a broad parsing of the strength of T cell stimulation can occur for these different types of infectious agents and why the immune system would have evolved to link development of the necessary class of CD4+ T cell effector response to such differences in the T cell stimulatory environment.

Many biological systems use differences in the magnitude or duration of signaling to control the qualitative state of the cell (Chen et al., 2001; Purvis and Lahav, 2013). Here we show that the immune system employs a cascading checkpoint mechanism to translate quantitative differences in early antigen and costimulatory T cell signaling into qualitative regulation of CD4+ effector T cell differentiation by the cytokines whose role in this process is so well recognized. These findings have important implications for vaccine design in terms of how adjuvants actually mediate their effects and how the balance of antigen amount and choice of adjuvant affect the direct and indirect roles of CD4+ cells in mediating postvaccination host defense. They also may provide insight into the dominance of different states of adaptive immune polarity during autoimmune processes.

**EXPERIMENTAL PROCEDURES**

**Mice**

B10.A CD45.2-/-, B10.A CD45.2-/- SCC7 TCR-transgenic Rag2-/-, and B10.A CD45.2-/- SCC7 TCR-transgenic Rag2-/- Ly5.1+Ly5.2+ (Hu-Li et al., 2001) x Ifng-/- mice were obtained from Taconic Laboratories through a special NIAID contract. All mice were maintained in SPF conditions at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility. All procedures were approved by the NIAID Animal Care and Use Committee (NIA).

**Peptides**

Pigeon cytochrome C (pPCC): KAERDLAYLQATAK was from American Peptide Company and long chain biotinylated pigeon cytochrome
Adoptive Cell Transfer

CD11c+ DCs were purified by positive immunomagnetic cell sorting for CD11c (Miltenyi Biotec) as described in product literature from spleens of B10.A CD45.2+ donor mice. Polyclonal CD4+ T cells from LNs of B10.A CD45.2+ and from TCR transgenic SC7 B10.A CD45.2+ mice were purified by negative immunomagnetic cell sorting (Miltenyi Biotec). DCs were incubated in vitro in CRPMI (RPMI 1640 supplemented with 10% FCS, 2-ME, glutamine, penicillin, streptomycin, and sodium pyruvate [Lonza] with the adjuvants LPS (1.0 μg/ml, Invivogen), papain (100 μg/ml, Calbiochem), CpG (25 μg/ml, Invivogen), or SEA (40 μg/ml) for 4 hr at 37°C in the presence of various concentrations of peptide as indicated. CD11c+ DCs were transferred by s.c. injection into the right rear footpad at 1 x 10^6/recipient, and CD4+ T cells were transferred by i.v. injection at 2 x 10^6/recipient at 18 hr posttransfer of CD11c+ DCs. Where indicated mice were injected i.v. with 100 μg anti-CD62L blocking antibody (MEL-14) at 2 hr after CD4+ T cell transfer. For CD80 blockade studies, mice were injected with either 200 μg anti-CD80 blocking Ab (16.10A1) or 200 μg isotype control Ab (Ab298Allm) 30 min prior to CD4+ T cell transfer.

Ex Vivo CD4+ T Cell Restimulation

At day 4 after CD4+ T cell transfer, intracellular cytokine production was determined after preparation of cell suspensions from dLNs. Cells were restimulated in CRPMI with PMA (100 ng/ml, Sigma) and ionomycin (1 μg/ml, Sigma) in the presence of monensin (2 μM, Sigma) at 37°C for 4 hr. Cells were surface stained with anti-CD4 (GK1.5, Biologend) and anti-CD45.2 (104, Biologend) and live cell staining was performed with LIVE DEAD fixable violet dead cell stain (Invitrogen). Cells were fixed and permeabilized with BD Cytofix Cytoperm kit (BD PharMingen) according to the manufacturer’s instructions. Intracellular cytokine staining was performed with anti-IFN-γ (XM31.2, Biologend) and anti-IL-4 (11B11, Biologend). Flow cytometric data were collected on an LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

2P Intravital Imaging and Calcium Analysis

Isoflurane was used to anesthetize mice prior to exposure of popliteal LN (Baxter; 2.5% for induction, 1.25% for maintenance) and the surgically exposed LN was kept at 36°C. For CD4+ T cell transfer, intracellular calcium analysis was conducted on either (1) a Bio-Rad/Zeiss Radiance 2100MP equipped with a Chameleon laser (Coherent) tuned to 800 nm, configured with a Nikon water-dipping lens (NA 1.0, Zeiss) using Zen 2010 acquisition software. Imaging was conducted in enclosed environmental chambers in which anesthetized mice were warmed by heated air and the surgically exposed LN was kept at 36°C–37°C with warmed PBS.

To visualize cells, CD11c+ DCs were labeled with either 100 μM CMTPX (Invitrogen) or 1.25 μM CMFDA (5-chloromethylfluorescein diacetate, Invitrogen) for 20 min at 37°C in Hanks buffered salt solution (HBSS). CD4+ T cells were stained with either 125 μM CMFDA or 1.25 μM CMTXPI (Invitrogen) for 20 min at 37°C in HBSS. For Ca2+ flux assessment, cells were costained with 1.25 μM CMTPX and 2.5 μM Fluo-4 (Invitrogen) for 20 min at 37°C in HBSS. After staining with CMTPX, cells were then further incubated in CRPMI for 30 min at 37°C. All dyes were supplied by Molecular Probes. Calcium flux analysis was conducted in a relative fashion by determining the basal ratio of Fluor-4:CMTPX intensity for individual cells after tracking with Imaris Imageworks. At subsequent time points, the ratio of Fluor-4 intensity to CMTPX intensity was determined and related to the basal ratio to determine the relative amount of calcium flux present in specific cells. This method was internally validated in each experiment by analysis of CD4+ T cells not interacting with DCs, where the ratio of Fluor-4:CMTPX had a mean of ~1.0.

Static two-photon imaging was conducted after PFA fixation of whole LNs, which were subsequently frozen in OCT compound (Tissue Tek) and serially sectioned into 100 μm sections. CD4+ T cell-DC interfaces were calculated after the digital reconstruction of whole LNs by Imaris Imageworks. Channel-specific 3D objects were generated and a pixel colocalization gating strategy was employed to determine the specific voxel size of cell-to-cell interactions.

Analysis of Peptide-Loaded, Adjuvant-Treated DCs

CD11c+ DCs were prepared as above for adoptive cell transfer in the presence of adjuvant with either 0.1 μm pPC0 or 10.0 μm LC-pPCC as indicated. DCs were then either analyzed immediately by flow cytometry for in vitro analysis or adoptively transferred into the popliteal LN and recovered at 24 hr posttransfer for ex vivo analysis. The DC phenotype was determined after staining with MHCII (MS/114,15,2), CD80 (16-10A1), CD86 (GL-1), ICAM-1 (Y1/1,7,4), and ICOSL (HK53). LC-pPCC binding was analyzed after staining with Streptavidin-PE (BD Pharmingen) or Streptavidin-Qdot 605 (Invitrogen), as per Huang et al. (2013). ELISA was conducted for the detection of IL-12p70 (Biolegend, ELISA Max Delux), and Cytometric Bead Array (BD, Multiplexed Bead Array) was conducted for the detection of IL-6 and IL-12 production at 4 hr or 24 hr poststimulation in the presence or absence of pPCC or LPS as per the manufacturer’s instructions. Flow cytometry was conducted on an LSRIII (BD) and analyzed with FlowJo software (TreeStar).

CD4+ T Cell Activation Analysis

WT or Il4−/−/Il12−/− SC7+ CD4 T cells were activated by in vitro culture in CRPMI with P13.9 fibroblasts stably expressing MHCII, CD80, and ICAM-1 (Ding et al., 1993) that had previously been treated with 25 μg/ml mitomycin C, in the presence of 0.005–10.0 μM pPCC. Alternately, WT SC7+ CD4+ T cells were activated in vivo with CD11c+ DCs treated with either LPS or papain in the presence of 0.1 μM pPCC (as per adoptive cell transfer methodology). After activation for 0–24 hr as indicated, CD4+ T cells were harvested and stained for CD4 (GK1.5), CD45.2 (104), CD86 (H1.2F3), IL-12R (11D2), IL-12Rβ2 (114), and IL-4R (mIL4R-M1). Flow cytometry was conducted on an LSRIII (BD) and analyzed with FlowJo software (TreeStar).

Statistical Methods

One-way ANOVA with Tukey’s posttesting were used for the statistical analysis of multiple groups. Student’s t-test (two-tailed) were used for the statistical analysis of differences between two groups.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.06.003.

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REFERENCES


