Protein Vaccines Induce Uncommitted IL-2-Secreting Human and Mouse CD4 T Cells, Whereas Infections Induce More IFN-γ-Secreting Cells[1]

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Mouse and human CD4 T cells primed during an immune response may differentiate into effector phenotypes such as Th1 (secreting IFN-γ) or Th2 (secreting IL-4) that mediate effective immunity against different classes of pathogen. However, primed CD4 T cells can also remain uncommitted, secreting IL-2 and chemokines, but not IFN-γ or IL-4. We now show that human CD4 T cells primed by protein vaccines mostly secreted IL-2, but not IFN-γ, whereas in the same individuals most CD4 T cells initially primed by infection with live pathogens secreted IFN-γ. We further demonstrate that many tetanus-specific IL-2$^+$IFN-γ$^-$ cells are uncommitted and that a single IL-2$^+$IFN-γ$^-$ cell can differentiate into Th1 or Th2 phenotypes following in vitro stimulation under appropriate polarizing conditions. In contrast, influenza-specific IL-2$^+$IFN-γ$^-$ CD4 cells maintained a Th1-like phenotype even under Th2-polarizing conditions. Similarly, adoptively transferred OTII transgenic mouse T cells secreted mainly IL-2 after priming with OVA in alum, but were biased toward IFN-γ secretion when primed with the same OVA peptide presented as a pathogen Ag during live infection. Thus, protein subunit vaccines may prime a unique subset of differentiated, but uncommitted CD4 T cells that lack some of the functional properties of committed effectors induced by infection. This has implications for the design of more effective vaccines against pathogens requiring strong CD4 effector T cell responses. *The Journal of Immunology,* **2006, 176: 1465–1473.**

Many successful vaccines induce protective Ab responses, but certain diseases such as HIV/AIDS, tuberculosis, malaria, and cancer may need effector T cells for efficient control. Due to the complexity of T cell effector functions, it is therefore critical to understand the types of CD4 and CD8 T cell responses induced by various vaccines.

CD4 T cells provide help for activation and Ab secretion by B cells, help for generation and maintenance of CD8 T cell responses, and cytokines that recruit and activate other immune cells. Within the CD4 T cell population are differentiated effector phenotypes such as Th1 and Th2 that mediate effective immune responses against intracellular pathogens and helminth parasites, respectively. Additional effector phenotypes of CD4 T cells generated under varied antigenic stimulations have also been described (1–4).

Although naive CD4 T cells can differentiate into these strong effector phenotypes during immune responses, Ag stimulation may also lead to the production of primed, but uncommitted (precursor) mouse CD4 T cells (Thpp)[3] (4, 5). These T cells, identified in vitro and in vivo, produce IL-2 and several chemokines, but few of the characteristic cytokines of Th1 or Th2 cells (6). Although these primed, uncommitted T cells may not have strong effector functions, they may constitute an expanded pool of Ag-specific T cells that can attract other immune cells to the site of reaction and provide flexibility for future immune responses.

Similar cells may exist in humans. CD45RA$^-$ CCR7$^+$ central memory T cells produced mainly IL-2 when first activated, and this population included precursors of Th1 or Th2 cells (7). However, the linkage between expression of CCR7 and IL-2-secreting, uncommitted cells is controversial (8–11), as many effector CD4 T cells expressing IFN-γ or IL-4 also express CCR7. Within the CCR7$^+$ population, the CXCR5$^+$ subpopulation is enriched for cells that secrete neither IFN-γ nor IL-4 (9, 12). These CCR7$^+$ CXCR5$^+$ cells include uncommitted cells (12) and may be equivalent to mouse Thpp cells.

To investigate the type of antigenic stimulation that directs the differentiation of CD4 T cells toward either uncommitted precursors or effector (Th1-like) cells, we have now measured CD4 T cell responses against Ags that were initially presented to the immune system either as protein vaccines or during infections by live organisms. Comparing these responses within the same human subjects, we found that vaccination with protein Ags generally induced a CD4 T cell memory response biased toward the production of IL-2, whereas responses primed by infection with live organisms induced a Th1-like effector response biased toward IFN-γ secretion. We further demonstrate that a major subset of tetanus-specific CD4 T cells (secreting IL-2, but not IFN-γ) is

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[3] Abbreviations used in this paper: Thpp, primed precursor Th cell; KLH, keyhole limpet hemocyanin; PPD, purified protein derivative; rLM-OVA, recombinant *L. monocytogenes* expressing OVA; RSV, respiratory syncytial virus; WSN-OVAII, recombinant influenza expressing OVA.

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uncommitted and can be polarized into either Th1 or Th2 cells under appropriate culture conditions. In contrast, influenza-specific cells, even those secreting only IL-2, were more committed to the Th1 phenotype, even under Th2-polarizing conditions. We also used a mouse TCR transgenic model to show that the difference between these two responses was due to the way that the Ag was presented to the immune system, not to differences in the epitope or TCR. These findings have important implications for the design of vaccines against infectious agents, particularly pathogens for which a strong effector CD4 T cell response is required.

Materials and Methods

Sample collection and processing

Blood was obtained from 15 healthy donors under a protocol approved by the University of Rochester Medical Center Research Subjects Review Board. PBMC were isolated by Ficoll-Hypaque (Mediatech) density gradient centrifugation. $CD4^+$ T cell enrichment was performed using the Miltenyi CD4 negative selection kit, depleting $CD8^+$ T cells, NK cells, monocytes, dendritic cells, granulocytes, platelets, and erythroid cells (Miltenyi Biotec). The $CD4^+$ T cell purity was always >95% ($CD8^+$ <0.2%, $CD56^+$ <0.2%).

ELISPOT assay

Ninety-six-well plates with a polyvinylidene difluoride filter base (Multi Screen Immobilon-P; Millipore) were coated with anti-human CD19 (clone HIB19; BD Biosciences for B cell capture) and anti-human cytokine (IL-2, R&D Systems; IFN-γ (1D1-K), Mabtech) Abs (for ELISPOT detection). For the IL-4 plus IL-5 ELISPOT assay, the wells were coated with the anti-CD19 Ab, anti-human IL-4 (clone IL-4-I; Mabtech), and anti-human IL-5 (clone TRFK5; BD Biosciences). Following a 2-h incubation, the plates were washed with RPMI 1640 (Mediatech) plus 8% FBS (HyClone) and antibiotic-antimycotic (Invitrogen Life Technologies). To provide APC, 2–3 × 10^5 PBMC were added to each well, and the plates were incubated at 37°C for 90 min to allow spontaneous adherence of monocytes and anti-CD19-mediated adherence of B cells. The plates were then washed with medium to remove the nonadherent cells. This method improved the linearity of ELISPOT numbers with dilution of responding cells (A. Divekar and T. Mosmann, unpublished observations). $CD4^+$ T cell-enriched populations (3 × 10^5 and 1 × 10^5 cells/well) were added to the wells, with Ags, including tetanus toxoid (1:500; Cylex), 10 μg/ml diphtheria toxoid (List Biological Laboratories), 10 μg/ml hepatitis B Ag (Aldevron), inactivated mumps virus 1:50 (Cylex), 10 μg/ml mycobacterial product purified protein derivative (PPD; Statens Serum Institut), 5 μg/ml inactivated influenza virus H3N2 X31 (A/Aichi/68) (Charles River Spafas), and 10 μg/ml respiratory syncytial virus (RSV) protein F and G (gift from E. Walsh, Department of Medicine, University of Rochester, Rochester, NY). For some of the subjects, RSV responses were tested using inactivated RSV lysate (BioDesign). Plates were incubated at 37°C for 24 h and then washed with PBS-Tween 20 (0.1%), and biotinylated anti-human IL-2 (1:60) (R&D Systems), IFN-γ (clone 7B6-1; Mabtech), biotin anti-human IL-4 (clone IL-4-II; Mabtech), or IL-5 (JES1-5A10; BD Biosciences) was added, followed by streptavidin alkaline phosphatase (1:1000) (Jackson ImmunoResearch Laboratories), and the assay was developed with the Vector AP substrate kit (Vector Laboratories). Once spots were clearly visible, the plates were washed with water to stop the reaction. The spots were counted on the Immunospot Analyzer (CTL Analyzers LLC).

Fluorospot assay

This assay was modified from the method described by Gazagne et al. (13). Cells and Ags were set up, as described above for the ELISPOT, except that anti-human CD19, anti-human IL-2 (BD Biosciences), and anti-human IFN-γ (Mabtech) coating Abs were included together in each well. After overnight incubation, the Fluorospot plates were washed and developed using a biotinylated anti-human IL-2 Ab (1:100) (BD Biosciences) and streptavidin-Cy3 (1 μg/ml) (Molecular Probes). IFN-γ spots were developed using FITC-conjugated anti-human IFN-γ Ab (1 μg/ml) (Mabtech), followed by an Alexa488-conjugated anti-FITC Ab (5 μg/ml) (Molecular Probes). The plates were dried, and individual wells were photographed under a fluorescent microscope.

Intracellular staining of human PBMC

Human PBMC were stimulated with medium alone, tetanus toxoid, or inactivated influenza virus with anti-human CD28 (BD Biosciences) costimulation for 6 h (last 4 h with Golgi Plug) (BD Biosciences). The cells were stained for surface markers CD4 and CD45RO and then fixed and permeabilized using Fix-Perm (BD Biosciences). Anti-human IL-2 APC and anti-human IFN-γ FITC were used to detect IL-2 and IFN-γ producers, respectively. Two million events were collected for all the samples on an LSRII flow cytometer (BD Biosciences), and data were analyzed using CellQuest (BD Biosciences).

IL-2 and IFN-γ single cell secretion assay

Human naive cells were removed by depleting PBMC using anti-CD45RA beads (Miltenyi Biotec), and IL-2- and IFN-γ-secreting cells were detected using the Miltenyi IL-2 enrichment and IFN-γ detection kit (Miltenyi Biotec). Briefly, CD45RA-depleted PBMC were stimulated with tetanus toxoid or inactivated influenza virus at 37°C for 16 h. The cells were harvested and then incubated with appropriate amounts of IL-2 and IFN-γ catch reagent for 5 min on ice. The cells were then resuspended in warm medium and incubated at 37°C for 45 min with intermittent mixing. The cells were washed, stained with anti-human IL-2 PE and anti-human IFN-γ FITC reagents, and incubated at 4–8°C for 10 min. The secreted cytokines (IL-2 and/or IFN γ) bound to the catch reagent were detected with anti-human IL-2 PE and anti-human IFN-γ FITC Abs. The cells were washed and enriched using anti-PE microbeads. Following two cycles of enrichment on magnetic columns, the cells were stained with anti-human CD4 CyChrome (clone RPA-T4; BD Biosciences), and $IL\text{-}2^+IFN\text{-}\gamma^-$ $CD4^+$ T cells were sorted on the FACSVantage (BD Biosciences) or the Epics Coulter (for single cell cloning experiments) or FACSAria (BD Biosciences) and stimulated under Th1- and Th2-polarizing conditions.

Th1 and Th2 culture conditions

Five purified IL-2-secreting CD4 T cells/well were cultured in the presence of tetanus toxoid (1:500) or inactivated influenza (10 μg/ml) in Th1 or Th2 conditions with irradiated (10,000 rad) autologous B cells (produced by culturing autologous B cells in the presence of CD40 ligand-transfected mouse fibroblasts and IL-4 (14)). Th1 conditions included 2 ng/ml recombinant human IL-12 (BD Biosciences) and anti-human IL-4 (2 μg/ml) (eBiosciences), whereas Th2 conditions included 40 ng/ml recombinant human IL-4 (Schering-Plough) and anti-human IL-12 (2 μg/ml). After 3 days, 1 ng/ml human rIL-2 (Chiron) was added to each well, and the cells were grown for another 4 days, followed by a second round of stimulation.

For single cell cloning experiments, a single $IL\text{-}2^+IFN\text{-}\gamma^-$ cell was deposited per well of a 96-well round-bottom tray. The cells were grown under nonpolarizing conditions: recombinant human IL-2 (1 ng/ml) and anti-human IL-12 (clone 20C2, 10 μg/ml) and anti-human IL-4 (eBiosciences; 10 μg/ml) for 5–6 days. Aliquots of each clone were then cultured for another 10 days in Th1 or Th2 conditions and tested for IFN-γ, IL-4, and IL-5 production.

TCR typing and sequencing

To confirm the clonality of the Th1 and Th2 aliquots of a single well, we performed PCR analysis of the Th1 and Th2 clone (15), followed by purification, cloning (TOPO cloning kit; Invitrogen Life Technologies), and sequencing (Northwoods DNA).

Mice and Ags

OTII/CD45.1 transgenic mice (recognizing the OVA peptide 323–339) were bred in our facility, and C57BL/6 mice were purchased from Jackson ImmunoResearch Laboratories. A total of 1 × 10^5 $CD4^+CD44^{low}$ $CD62L^{high}$ naive OTII transgenic T cells was transferred i.p. into C57BL/6 mice. Twenty-four hours later, the mice were immunized i.p. with PBS plus alum, PBS plus OVA (50 μg), OVA plus alum (50 μg), i.v. with 1 × 10^5 recombinant *Listeria monocytogenes* expressing OVA (rLM-OVA), or intranasally with a recombinant influenza virus expressing OVA (WSN-OVAII). The rLM-OVA strain of *L. monocytogenes* contains a chromosomally integrated cassette encoding truncated OVA (aa 143–378) (16). The OVA sequence is fused to the virulence gene (*hly*) promoter and signal sequence, which control expression and secretion of OVA. The influenza virus (WSN-OVAII) is a recombinant of the parent H1N1 A/WSN/33 influenza virus. The OVA epitope (323–339) is inserted into aa 42–59 of the neuraminidase, making the neuraminidase 7 aa longer.

Intracellular staining

Mice were sacrificed and spleens were harvested on day 11 postimmunization. Spleens were processed into a single cell suspension, red cells were lysed, and splenocytes were stimulated with 10 μM MHCII OVA peptide ISQAVHAAHAEINEAGR. After 12 h in the presence of monensin, the cells were stained with CD45.1 CyChrome (clone A20; BD Biosciences)

and CD4 APC (clone RM4-5; BD Biosciences), fixed with 2% paraformaldehyde, and stained with IFN-γ PE (clone XMG1.2; BD Biosciences) and IL-2 Alexa488 (clone JES6-5H4; BD Biosciences). Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Numbers of IL-2- or IFN-γ-expressing cells were measured in the $CD4^+CD45.1^+$ TCR transgenic T cell population.

Statistical analysis

Significance of individual Ag responses relative to the background was evaluated by the Mann-Whitney *U* test. The means of IL-2 and IFN-γ frequencies were also compared between the vaccine-primed and infection-primed groups. The means of triplicate background values (no Ag) were subtracted from individual Ag-stimulated IL-2 and IFN-γ responses for each subject. Linear regression analysis was used to study the relationship between the IL-2 and IFN-γ responses for each Ag group. Significance of correlation between the IL-2 and IFN-γ responses was examined by the *t* test. A regression model $Y = bX + e$ was fitted, where X is the mean IL-2 response and Y the mean IFN-γ response to a given Ag. All ANOVA comparisons of the means were based on the F test and tested the significance of difference in the means, but did not test the magnitude of the difference.

Results

Vaccination and infection history of subjects

To compare immune responses initially induced by either natural infection or vaccination with protein vaccines, we analyzed IFN-γ and IL-2 responses in normal individuals against several common infections or vaccines. Fifteen healthy subjects (10 females and 5 males) between the ages of 23 and 52 years were enrolled in this study (Table I). All of the subjects had been vaccinated against diphtheria, and all had been boosted against tetanus one or more times within the past 10 years. Three subjects had not received a hepatitis B vaccine; one individual was primed, but not boosted; and the rest had received the complete vaccination series as adults. Thus, most of the subjects had been primed against tetanus, diphtheria, and hepatitis B by protein subunit vaccines. In contrast, four other recall responses were initially primed mainly by live infections. Seven subjects had been vaccinated against influenza in the previous year, but none of the subjects had been vaccinated in early childhood. As the incidence of influenza infection is 5–20% per year, most or all would have been primed by infection before adult vaccination. Two of the fifteen individuals had been vaccinated with live bacillus Calmette-Guerin, and additional subjects may have been primed by subclinical infection with cross-reactive mycobacteria (17–19). Thirteen of the individuals received live, attenuated mumps vaccine in childhood, and the other two had a history of mumps infection. All of the RSV responses were primed by natural infection because no RSV vaccine is available. To focus on CD4 T cell responses, we depleted other cell types from PBMC (<0.2% of either $CD8^+$ or $CD56^+$ cells remaining) and then stimulated these cells with recall Ags in the presence of adherent PBMC as APC in vitro. Fig. 1 shows the detailed responses of one individual, and Fig. 2 summarizes the responses of all subjects.

IL-2 and IFN-γ responses to protein Ag immunizations

To analyze T cell responses that had initially been primed by protein Ags, the CD4 T cell-enriched population was stimulated with tetanus, diphtheria, and hepatitis B Ags in ELISPOT assays. Substantial IL-2 and IFN-γ responses were detected in several of the subjects (Figs. 1 and 2). IL-5 responses to tetanus were also detected occasionally, but at much lower levels than IL-2 or IFN-γ responses (mean 10 spots/million CD4 T cells, range 5–15). In six additional subjects, the average ratio of IL-2-secreting cells to IL-4/IL-5-secreting cells was 9.9 (data not shown). In 10 of the 15 subjects in the present study, more cells secreted IL-2 than IFN-γ in response to in vitro tetanus stimulation. Nine of the 15 subjects had significantly higher IL-2 than IFN-γ responses to in vitro hepatitis B stimulation. Although not all subjects responded to diphtheria toxoid, the majority of the responding subjects also had higher IL-2 than IFN-γ responses to this Ag. ANOVA analysis of the three protein Ags considered as a group showed that the net number of IL-2 producers was significantly greater than the number of IFN-γ producers ($p = 0.02$). Considering the three Ags individually, the tetanus and hepatitis B responses showed significantly more IL-2 than IFN-γ spots, while the diphtheria response, although showing the same trend, did not reach significance on its own. For the combined protein Ag vaccination group, the fitted regression model showed a significant correlation between IL-2 and IFN-γ levels ($p < 0.0001$) with a slope of 0.33 (95% confidence limits 0.3–0.6).

Cytokine responses primed by live immunization/infection

To investigate the cytokine responses that had initially been primed by live infection, CD4-enriched T cells were stimulated in vitro with Ag preparations from *Mycobacterium tuberculosis*, mumps virus, RSV, and influenza virus. Protein Ags or killed virus

Table I. *Patient demographics*

Subject No.	Age	Sex	Vaccination History					
			Tetanus	Diphtheria	Hepatitis B	Mumps	Bacillus Calmette-Guérin	Influenza
494	52	Female	Yes	Yes	1 of 3	Infection (no vaccination)	Not sure	2003
497	23	Female	1999 booster	>10 years	2001	1999	1994	2003
499	33	Male	>10 years	Yes	1993	1998	1982	No
501	24	Female	1994 booster	Infancy	1997/2001	Infancy	No	2003
503	33	Male	2003 booster	Yes	Yes	Infection (no vaccination)	No	No
505	37	Female	Yes	Infancy	No	Infancy	No	2003
508	25	Male	2000 booster	Infancy	2001	Infancy	No	No
509	29	Female	2003 booster	Infancy	1993	Infancy	No	2003
511	29	Male	2000 booster	Infancy	1991	2000	No	No
834	21	Female	2002 booster	Infancy	No	Infancy	No	No
835	25	Male	Infancy	Infancy	2003	Infancy	No	No
837	23	Female	Infancy	Infancy	1999	Infancy	No	No
838	27	Female	1998 booster	Infancy	No	Infancy	No	No
839	36	Female	2000 booster	Infancy	2003	Infancy	No	2005
840	46	Female	2004 booster	Infancy	2000	2000	No	2004

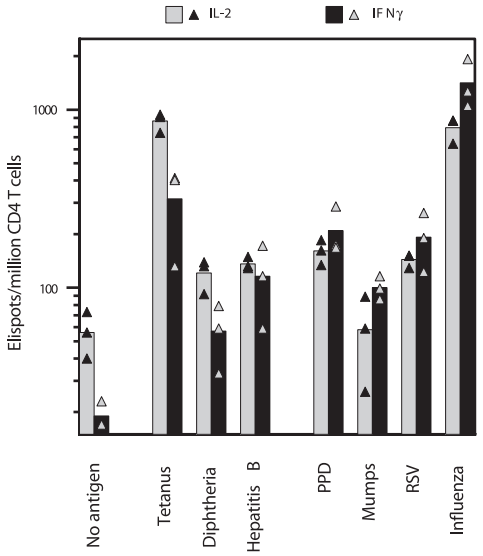


FIGURE 1. Secretion of IL-2 and IFN-γ by human CD4 T cells. The number of CD4 T cells secreting IL-2 and IFN γ in response to tetanus, diphtheria, hepatitis B, mumps, RSV, influenza, and PPD was measured by ELISPOT in one individual (subject 509). Triangles represent triplicate values; ▨, represent mean IL-2 responses; and ■, represent mean IFN-γ responses.

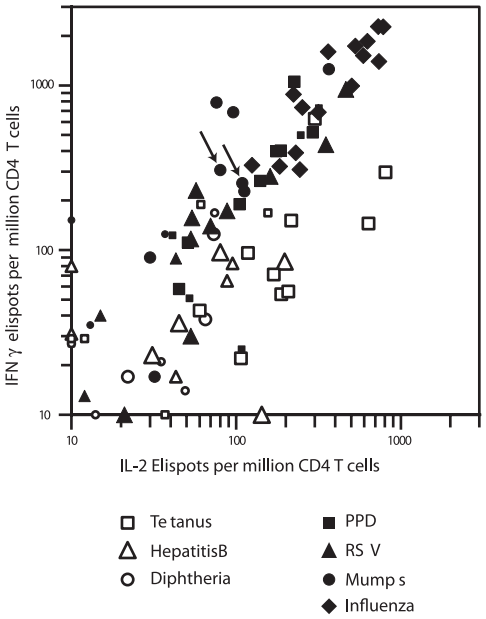


FIGURE 2. Human IL-2 and IFN-γ responses primed by proteins or infections. CD4-enriched PBMC from 15 individuals were stimulated with tetanus, diphtheria, hepatitis B, influenza, mumps, RSV, and PPD, and the IL-2 and IFN-γ responses were measured by ELISPOT. Open symbols show responses originally primed by immunizations with protein Ags, and closed symbols show responses primed by infection. The arrows indicate two subjects who had been naturally infected with mumps virus. Each point represents the average of triplicates for one individual. Background values have been subtracted, and responses <10 have been plotted at 10. Large symbols indicate that both IL-2 and IFN-γ were significantly above background, and small symbols indicate that only one of the responses was significant.

were used rather than live organisms, to minimize potential interference with Ag-presenting function or cytokine responses by pathogen defense mechanisms. Strong IFN-γ and IL-2 responses were induced by these Ags in most subjects. IL-4 and IL-5 responses to RSV and influenza Ags are normally undetectable (<10/million PBMC) in memory responses, and thus are at least 10-fold lower than the IL-2 and IFN-γ responses (20). For all four Ags against which the immune system was initially primed by infection, the net IFN-γ response was higher than the IL-2 response in the majority of the subjects for each Ag separately, and for the group of Ags considered together ($p < 0.0001$ by ANOVA analysis). Linear regression analysis showed a significant correlation between IL-2 and IFN-γ responses primed by infection ($p < 0.0001$), with a significantly higher proportion of IFN-γ-producing cells (slope of 2.25, 95% confidence limits 2–2.5). The IL-2 vs IFN-γ responses of the seven subjects vaccinated as adults against influenza were not significantly different from the responses of the eight nonvaccinated subjects ($p > 0.83$). Also, the responses against viral Ags were not significantly different from responses against the bacterial Ag PPD ($p > 0.117$). There was also no significant difference in the ratio of IL-2 to IFN-γ in responses primed by viruses vs bacteria ($p = 0.28$).

Thus, the CD4 T cell responses primed initially by vaccination with protein Ags generally showed an excess of IL-2- over IFN-γ-secreting cells, whereas the responses initially primed by live infections showed the opposite trend. This difference was confirmed by comparing the slopes of the linear regressions of IFN-γ against IL-2 for the protein Ag group and the live infection group by F test ($p < 0.0001$).

Detection of simultaneous production of IFN-γ and IL-2 by Fluorospots and intracellular staining

Two-color Fluorospots were performed to confirm that protein Ags and infections induced biases toward IL-2 or IFN-γ single producers, respectively. Tetanus toxoid predominantly induced single IL-2 producers (red) and few IFN-γ producers (green), whereas influenza induced more cells producing IFN-γ (green), or both IFN-γ and IL-2 (yellow) (Fig. 3*A*). Similar results were obtained with the other subjects. Further confirmation was obtained with intracellular cytokine staining of PBMC (Fig. 3*B*) after stimulation with tetanus or influenza Ags. Note that both techniques revealed a significant population of IFN-γ^+ IL-2^- anti-influenza cells in addition to Th1-like IFN-γ^+IL-2^+ cells, consistent with the excess of IFN-γ- over IL-2-secreting cells demonstrated by ELISPOT in anti-mumps, RSV, influenza, and PPD responses (Fig. 2). Therefore, the Fluorospot and intracellular cytokine staining confirm the ELISPOT results, showing that immune responses primed by protein Ags induced predominantly IL-2^+IFN-γ^- CD4 T cells, whereas responses primed by live infections gave more IL-2^+IFN-γ^+ and IL-2^-IFN-γ^+ cells.

Human CD4$^+$ memory T cells secreting only IL-2 in response to tetanus toxoid can proliferate and differentiate into either Th1 or Th2 cells

Human CD4 T cells producing IL-2, but not IFN-γ on restimulation with recall Ags could be Th1 cells that did not secrete IFN-γ on that particular occasion (21, 22), pre-Th1 cells (12), naive cells, or primed but uncommitted Thpp precursor cells (4, 5, 7). Naive T cells are unlikely to contribute to anti-tetanus IL-2 responses because the frequency of Ag-specific naive CD4 T cells would be too low to measure in our assays. To assess the contribution to the IL-2^+IFN-γ^- populations of Th1 cells temporarily secreting only IL-2 (21, 22), we stimulated PBMC with influenza or tetanus Ags, stained for IL-2 and IFN-γ secretion using the Miltenyi cytokine capture assay, and sorted IL-2^+IFN-γ^- and IL-$2^{+/-}$IFN-γ^+ populations. These were then restimulated, and the frequency of IFN-γ

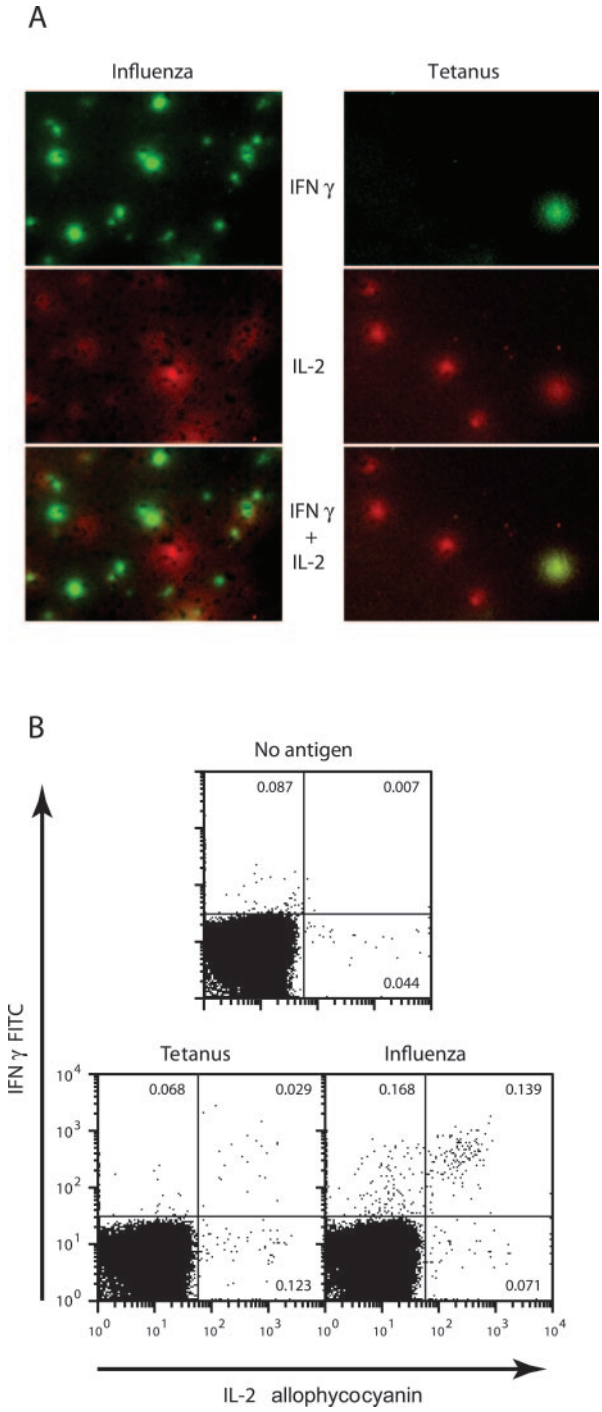


FIGURE 3. Detection of IL-2$^+$IFN-γ^- T cells by Fluorospot and intracellular cytokine staining. *A*, Fluorospot image of tetanus and influenza responses from CD4-enriched PBMC from one individual. Cells secreting predominantly IL-2, IFN-γ, or both IL-2 and IFN-γ produced red, green, and yellow spots, respectively. *B*, PBMC were stimulated with tetanus or influenza Ags, fixed, and stained to reveal intracellular IL-2 and IFN-γ. The plots shown are gated on CD4$^+$ cells.

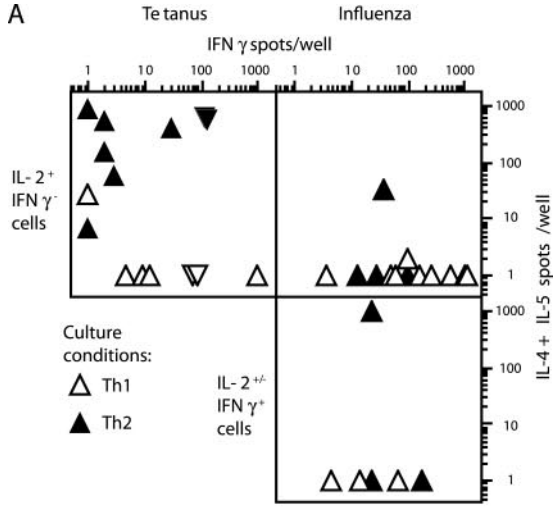
producers was measured by ELISPOT analysis. Among the influenza-specific cells, 13.6% of the major IFN-γ^+ population and 7.9% of the minor IL-2$^+$IFN-γ^- population secreted IFN-γ after a second Ag stimulation, implying that many of the influenza-specific cells that initially secreted only IL-2 were really Th1 cells. Although the frequency of 13.6% is low, even within CD4 T cell clones only a minority of cells express cytokines on restimulation, and the extensive manipulation of the populations (stimulation, staining, sorting, and restimulation) may also have reduced the responsiveness of the final cell population. In contrast to the influenza-specific cell results, of the major population of IL-2$^+$IFN-γ^- tetanus-specific cells, only 1.9% secreted IFN-γ on restimulation, suggesting that most of this population were not Th1 cells.

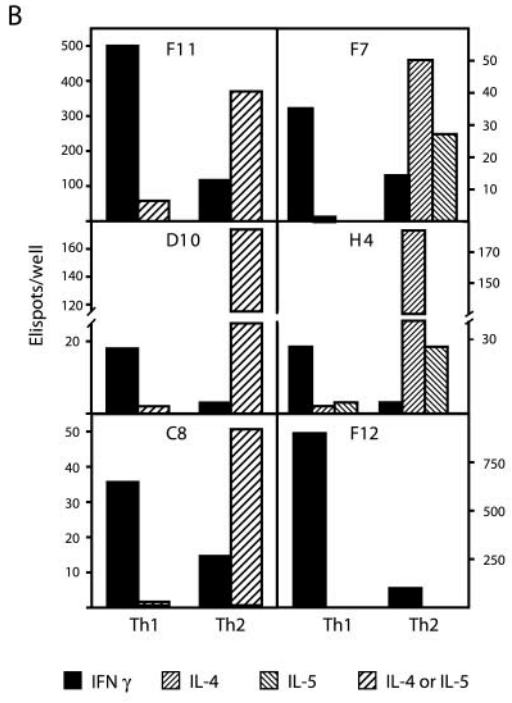
To distinguish between committed and uncommitted IL-2-secreting tetanus-specific CD4 T cells, we depleted naive cells from PBMC, and then stimulated the remaining cells with tetanus toxoid. Cells secreting IL-2 and/or IFN-γ were stained using the cytokine capture technique, and tetanus- and influenza-specific IL-2$^+$IFN-γ^- cells were sorted and small aliquots were cultured with autologous B cells and tetanus toxoid or inactivated influenza virus in Th1- or Th2-polarizing conditions. After two rounds of stimulation, the cells were tested for IFN-γ or IL-4 plus IL-5 production in an ELISPOT assay. As only 30–35% of the tetanus-specific aliquots responded, it is likely that most of the responding wells represented the progeny of single cells. Fig. 4*A* shows that some tetanus-specific CD4 T cells that initially secreted IL-2, but not IFN-γ, could be polarized toward the predominant secretion of either IFN γ or IL-4/IL-5 under appropriate culture conditions (Fig. 4*A*, *left panel*). In contrast, a majority of the influenza-specific IL-2$^+$IFN-γ^- and IL-2$^{+/-}$IFN-γ^+ cultures maintained a Th1-like profile under either Th1- or Th2-polarizing conditions (Fig. 4*A*, *right panel*).

To confirm that single tetanus-specific IL-2-secreting cells could be uncommitted precursors, we performed single cell cloning of these cells. A single IL-2$^+$IFN-γ^- CD4 T cell was put in each well of a 96-well tray and cultured for 4–5 days in the presence of tetanus toxoid and autologous B cells. Each well showing T cell growth was then split into Th1 or Th2 conditions. After two further rounds of stimulation, the cells were tested for IL-4 and IFN-γ production. As shown in Fig. 4*B*, five of the six individual tetanus-specific IL-2$^+$IFN-γ^- cells were bipotential and could differentiate into Th1- or Th2-biased cells under appropriate culture conditions, whereas clone F12 remained committed to a Th1-like pattern regardless of culture conditions. To confirm clonality, one pair of Th1 and Th2 aliquots (clone F11) was analyzed for TCRβ usage by PCR, followed by sequencing. Both Th1 and Th2 aliquots of this clone expressed Vβ13/Dβ1/Jβ2–3, with identical junctional diversity (Fig. 4*C*). Two discrepancies in the Vβ region may have been due to sequence alterations introduced during the PCR step. These results confirm that both Th1- and Th2-biased cells arose from a single initial T cell. Thus, at least some of the human IL-2$^+$IFN-γ^- CD4 T cells induced by protein vaccination have a flexible differentiation phenotype, suggesting that these cells are equivalent to the primed, but uncommitted mouse CD4 Thpp cells described previously (4).

Mouse IL-2 and IFN-γ responses against OVA in the context of protein vaccination or live bacterial/viral infection

We next took advantage of a mouse experimental model to compare the immune responses against a single antigenic epitope, the ISQAVHAAHAEINEAGR epitope of OVA (pOVA) (23), after priming either by OVA protein in adjuvant, or by the same epitope expressed by a pathogen during active infection. We tracked the response against this epitope by using cells from the TCR transgenic mouse OTII, in which all CD4 T cells express a TCR recognizing pOVA. Naive OTII cells (from CD45.1 mice) were transferred to normal naive C57BL/6 (CD45.2) recipients, and the mice were then immunized with alum alone, or with alum and OVA. Two additional groups were infected after OTII transfer with either the WSN-OVAII influenza virus or the rLM-OVA strain of *L.*





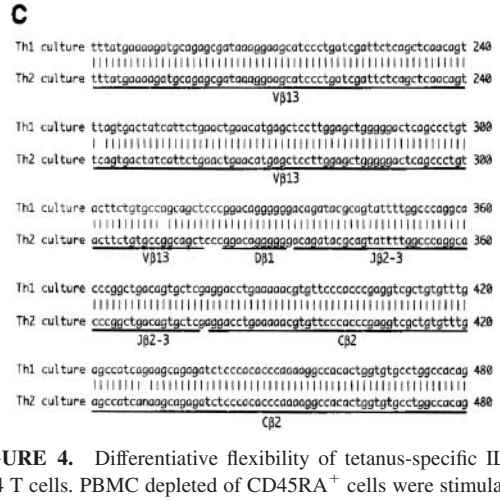


FIGURE 4. Differentiative flexibility of tetanus-specific IL-2^+IFN-γ^- CD4 T cells. PBMC depleted of $CD45RA^+$ cells were stimulated for 16 h with tetanus toxoid, and IL-2- and IFN-γ-secreting cells were stained with the IL-2 and IFN-γ capture assay. IL-2 producers were enriched, as described in *Materials and Methods*, and IL-2^+IFN-γ^- cells were sorted. *A*, Tetanus-specific IL-2^+IFN-γ^- cells (5 cells/well) and influenza-specific IL-2^+IFN-γ^- and IL-$2^{+/-}$IFN-γ^+ cells were cultured under Th1 or Th2 conditions. The cells were then tested in IFN-γ and IL-4 plus IL-5 ELISPOT assays. △, Depict cells grown under Th1 conditions; ▲, cells grown under Th2 conditions. Results shown are from two different subjects for the tetanus-specific cells (up and down triangles) and one subject for influenza-specific cells. *B*, Single cell cloning: single IL-2^+IFN-γ^- cells were deposited per well of a 96-well tray. The cells were grown in neutral conditions with autologous CD40L-stimulated B cells + Ag for 4–5 days and then split into Th1 and Th2 conditions and restimulated with tetanus toxoid. Two weeks later, the cells were analyzed in an ELISPOT assay for IFN-γ and IL-4 and/or IL-5 production. H4 and F7 were from one subject, and the other four clones were from a second subject. *C*, The Th1 and Th2 aliquots of clone F11 were analyzed for Vβ expression by RT-PCR, and the PCR products were sequenced. The V, D, J, and junctional sequences from the Th1 and Th2 cultures showed >96% identity.

monocytogenes (16). Immune responses were assessed 11 days later by stimulating spleen cells in vitro with pOVA, and analyzing IL-2 and IFN-γ synthesis by intracellular staining. OTII cells were identified by the expression of CD45.1. The number of OTII cells per spleen secreting cytokines takes into account both the proliferation of OTII cells in response to the different Ags, and the differentiation of naive cells into cytokine-secreting effectors. The OTII transgenic cells in the mice immunized only with alum showed small IL-2 and IFN-γ responses. Although even naive OTII CD4 T cells should be able to synthesize IL-2 in response to Ag stimulation, the low numbers may have been due both to the lack of expansion of the OTII population after transfer in the absence of stimulation, and to the lower responsiveness (compared with memory or effector cells) of naive cells responding to the peptide within 12 h. We have previously shown that normal and TCR-transgenic keyhole limpet hemocyanin (KLH)- or OVA-specific T cells responded to protein immunization by differentiating into IL-2-secreting, Thpp-like cells that could further differentiate into either Th1 or Th2 cells (4). In agreement with these results, immunization with OVA induced significant increases in the numbers of OTII cells secreting IL-2, but not IFN-γ, in most mice (Fig. 5). In contrast, most of the mice infected with the WSN-OVAII showed increased numbers of IL-2^+IFN-γ^+ and IL-2^-IFN-γ^+OTII cells. A similar bias toward IFN-γ production occurred in mice infected with rLM-OVA, although the number of responding mice was lower. Thus, T cells primed by immunization with a protein vaccine generated qualitatively different immune responses compared with mice immunized as a consequence of infection, even in experiments in which the T cell repertoire and Ag epitope were kept constant.

Discussion

In this study, we show that priming with protein Ags generates a unique phenotype of uncommitted human CD4 T cells, which produce IL-2, but not IFN-γ, and demonstrate flexibility to differentiate into Th1 or Th2 effector-like cells. In contrast, infections induced responses characterized by a higher ratio of IFN-γ-producing cells, with few cells secreting only IL-2. Even the IL-2^+IFN-γ^- cells induced by influenza had less flexibility and maintained a Th1-like profile under Th2-polarizing conditions. To reach these conclusions, it is necessary to consider the immunization history for these responses. Tetanus and diphtheria rarely occur as primary infections in our target group. As these pathogens induce severe symptoms, it is unlikely that the subjects would have been previously exposed to the toxins during subclinical infection. All subjects were vaccinated with protein subunit vaccines for tetanus and diphtheria. None of the subjects reported a history of hepatitis B infection, and 12 of the 15 subjects had been vaccinated. Therefore, the IL-2-dominated CD4 T cell responses of most of our

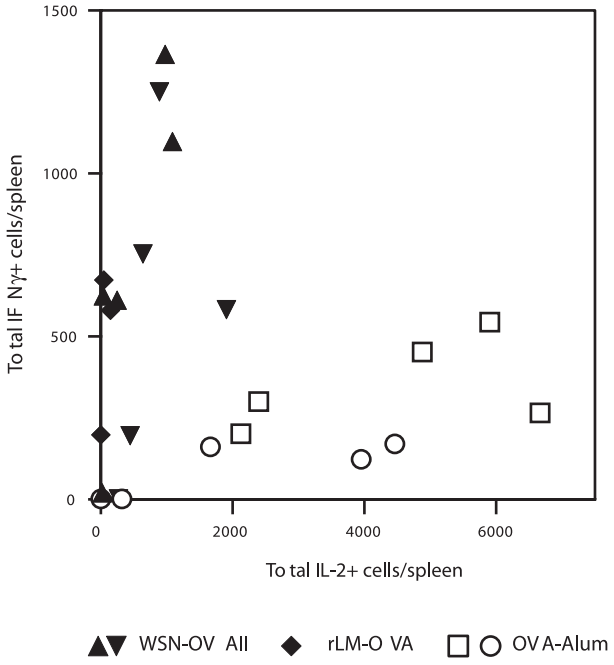


FIGURE 5. Cytokine responses in mice immunized with OVA either in alum or expressed by live organisms. OVA-specific TCR transgenic OTII spleen cells (CD45.1) were injected into normal C57BL/6 mice, which were then immunized with alum only, alum plus OVA, the recombinant influenza strain WSN-OVAII, or the recombinant *L. monocytogenes* strain rLM-OVA. Eleven days later, spleen cells were stimulated in vitro with OVA peptide, and stained for intracellular IL-2 and IFN-γ, as well as CD4 and CD45.1 (donor specific). Each point represents the number of OTII cells producing IL-2 vs IFN-γ in one mouse spleen. The percentage of OTII cells secreting cytokines in responding mice varied between 1 and 6%. Background values (no Ag in vitro) have been subtracted. In spleen cells from control mice immunized with PBS + alum, in vitro responses to pOVA were <350 cells/spleen for either IL-2 or IFN-γ. Results from two experiments are shown for alum-OVA and WSN-OVAII, and one experiment for rLM-OVA.

subjects against tetanus toxoid, diphtheria toxoid, and hepatitis B Ag were probably primed initially by protein Ags, not by infection.

Conversely, there is a strong likelihood that the responses we detected against RSV protein Ags, inactivated influenza and mumps preparations, and mycobacterial Ag PPD were initially primed in our subjects by live infections. RSV infections occur in a majority of infants during the first year, and by the age of 2 years, >90% of the population are immune (24, 25). Further reinforcement of these responses may occur by repeated infections in subsequent years. Influenza also infects the great majority of children by the age of 3 years (26, 27), and the subjects in our study were not vaccinated against influenza as children. Thus, initial priming against influenza occurred by infection, and the anti-influenza response may have been boosted by additional infections, and in some subjects, by adult vaccination. Interestingly, an IFN-γ-dominated response to mumps virus was present in subjects primed either by natural infection (arrows in Fig. 2) or by vaccination with a live, attenuated strain, suggesting that even mild infection can induce a committed effector response. Responses against PPD in our subjects were induced either by vaccination with bacillus Calmette-Guerin live vaccine, or presumably by subclinical infection with environmental mycobacteria-expressing Ags that cross-react with PPD (17–19). Thus, we conclude that in most or all of the subjects, the IFN-γ-dominated CD4 T cell responses against RSV proteins, inactivated influenza and mumps virus, and mycobacterial Ag PPD were initially primed by live infections.

Although CD4 T cells secreting IL-2, but not IFN-γ, could be primed but uncommitted Thpp cells, even in a fully differentiated Th1 cell population some cells may express only IL-2, or only IFN-γ, after Ag stimulation (21, 22). Thus, the CD4 T cells secreting IFN-γ, but not IL-2, in response to RSV proteins, inactivated influenza and mumps virus, and mycobacterial Ag PPD (Figs. 2 and 3) may have been Th1 cells that did not synthesize IL-2. Although some of the tetanus-specific IL-2^+IFN-γ^- cells we have identified may have been "lazy Th1" cells of the reciprocal type, there are three reasons that such cells are unlikely to account for most of the tetanus-, hepatitis B-, or diphtheria-specific IL-2-secreting cells. First, in both mouse and human systems, only a minority of Th1 cells secrete IL-2, but not IFN-γ (4) (A. Divekar and T. Mosmann, unpublished observations), and so the low number of IFN-γ^+ cells observed in the protein-primed responses implies an even lower number of "lazy Th1" cells. Second, restimulation of IL-2^+IFN-γ^- tetanus-specific cells resulted in very few cells that could secrete IFN-γ, whereas a higher percentage of the equivalent population of influenza-specific CD4 T cells secreted IFN-γ on restimulation. Third, IL-2^+IFN-γ^- CD4 cells responding to tetanus (human; Fig. 4) or KLH (mouse (4)) showed considerable flexibility of differentiation, whereas influenza-specific cells secreting IL-2, but not IFN-γ, were less flexible and maintained a Th1-like profile even under Th2-polarizing conditions (Fig. 4*B*). Thus, the responses primed by RSV proteins, inactivated influenza and mumps virus, and mycobacterial Ag PPD may include some Th1 cells that temporarily secrete only IL-2, but the IL-2^+IFN-γ^- cells primed by protein Ags probably include mostly primed, uncommitted non-Th1 cells.

Previous studies have shown the existence of IL-2^+IFN-γ^- CD4 T cells in tetanus- and hepatitis-immunized subjects (1, 2). These cells could be similar to the mouse primed, but uncommitted CD4 T cells secreting IL-2, but not IFN-γ described previously (4, 5) and also to the human CD45RA$^-$, CCR7$^+$ central memory population (7). Recent studies (9, 12) suggest that only the CXCR5$^+$ cells within the CCR7$^+$ population are uncommitted IL-2-secreting cells. Although some IL-2^+IFN-γ^- tetanus-specific T cells in our studies did express CXCR5, many expressed neither CXCR5 nor CCR7 (data not shown). Thus, these chemokine receptor surface markers may define complex patterns of CD4 T cells according to their circulation and migration properties, but the relationship of these subsets to the cytokine phenotypes that we have described remains unclear.

The human findings were confirmed in a mouse experimental model in which the T cell specificities and priming signals could be more precisely controlled, by monitoring the response of low numbers of TCR transgenic mouse T cells in normal mice. The results confirmed previous studies showing that priming of endogenous or TCR-transgenic T cells with protein Ags such as KLH and OVA induced mainly uncommitted, IL-2-secreting Thpp-like cells (28) that were uncommitted and could differentiate into either Th1 or Th2 cells (4), whereas priming in the context of infection induced stronger Th1-like responses (29–31). Thus, the type of differentiation of CD4 T cells specific for a single epitope is dependent on the context of Ag exposure, not the affinities or frequencies of the epitope or TCR.

Why are these IL-2^+IFN-γ^- memory CD4 T cells important? The expansion of a pool of Ag-specific cells may allow faster subsequent responses due to increased numbers and less stringent activation requirements, compared with naive cells. This expanded Ag-specific population could also provide flexibility in future responses by differentiating into either Th1 or Th2 effectors, as appropriate. As mouse Thpp cells secrete substantial amounts of chemokines (6), the primed uncommitted cells may also initiate

further responses by attracting other cells into the site of Ag reaction. The flexibility to differentiate into either Th1 or Th2 types may also be useful during the contraction phase of the immune response, as inflammation generated in a Th1 response may be contained by the secretion of Th2 cytokines.

Protein vaccines such as tetanus, diphtheria, and hepatitis B are successful vaccines that provide protective immunity by inducing neutralizing Ab responses, with >95% seroconversion rates. In addition to the major IL-2^+IFN-γ^- T cell populations we have described in this study, these vaccines induce lower levels of CD4 cells secreting the Th2 cytokines IL-4 and IL-5, and these Th2-like cells may provide the main B cell help for Ab responses induced by these vaccines. Although the ability of primed uncommitted cells to help B cell responses is currently unknown, the IL-2^+IFN-γ^- cells might also contribute to help for Ab production because of their higher frequency and their production of the B cell helper factor IL-2. Polysaccharide Ags can induce T cell-independent anti-polysaccharide Ab responses, but conjugation of polysaccharide Ags with carrier proteins such as diphtheria or tetanus is thought to enhance vaccination efficacy by providing T cell help, for example in children immunized with the Hib capsular polysaccharide (32, 33). These results raise the possibility that in addition to B cell help provided by IL-4/IL-5-producing CD4 T cells, the generation of IL-2^+IFN-γ^- CD4 memory T cells may also contribute to help for Ab responses. If CXCR5 is expressed on at least some primed, uncommitted CD4 T cells, this would also be consistent with a role for these cells in B cell help, as CXCR5 and CCR7 enhance homing to lymph nodes, and CXCR5 may direct homing of T cells within lymph nodes to the germinal centers (34–36), thus facilitating interaction with B cells.

Although the substantial population of Thpp-like cells clearly does not interfere with the ability of tetanus, hepatitis B, and diphtheria vaccines to induce protective Ab responses, potential disadvantages of the induction of the IL-2^+IFN-γ^- phenotype by other vaccines include the lack of strong T cell effector functions that would be contributed by the potent cytokines of effector (Th1, Th2, etc.) T cells. Differentiation of mouse Thpp cells into effectors requires 3–5 days (37), which could be a significant delay during infection by rapidly spreading pathogens such as influenza. Although the generation and maintenance of many CD8 T cell responses require CD4 T cell help, it is not known whether IL-2^+IFN-γ^- cells can provide this help. Thus, there is concern that the IL-2^+IFN-γ^- responses most likely to be induced by subunit vaccines may not provide the right immediate effector functions against pathogens that require rapid Th1- and CD8-dominated responses. These potentially include vaccines against tuberculosis, HIV, *Leishmania*, cancer, and possibly malaria.

Why do protein subunit vaccines induce CD4 T cell responses dominated by IL-2^+IFN-γ^- cells? The extra signals provided during actual infection include microbial products that provide immune stimulation through several receptors of the innate immune system, notably the TLRs (38, 39), as well as potentially higher levels of inflammatory signals induced by infection. Although limited Ag stimulation may preferentially induce the IL-2^+IFN-γ^- phenotype (40, 41), these cells are also found in responses that have been primed and boosted (tetanus in Fig. 2) (7) (X. Wang and T. Mosmann, unpublished observations). Thus, for many future vaccines that require the induction of strong effector T cell responses, it may be important to add stronger adjuvants that can supply the TLR and/or inflammatory signals that are required to push CD4 T cell differentiation beyond the primed, uncommitted precursors, into fully differentiated effector T cells.

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Disclosures

The authors have no financial conflict of interest.

References

1. De Rosa, S. C., F. X. Lu, J. Yu, S. P. Perfetto, J. Falloon, S. Moser, T. G. Evans, R. Koup, C. J. Miller, and M. Roederer. 2004. Vaccination in humans generates broad T cell cytokine responses. *J. Immunol.* 173: 5372–5380.
2. Harari, A., F. Vallelian, P. R. Meylan, and G. Pantaleo. 2005. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. *J. Immunol.* 174: 1037–1045.
3. Harari, A., F. Vallelian, and G. Pantaleo. 2004. Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load. *Eur. J. Immunol.* 34: 3525–3533.
4. Wang, X., and T. R. Mosmann. 2001. In vivo priming of CD4 T cells that produce interleukin (IL)-2 but not IL-4 or interferon (IFN)-γ, and can subsequently differentiate into IL-4- or IFN-γ-secreting cells. *J. Exp. Med.* 194: 1069–1080.
5. Sad, S., and T. R. Mosmann. 1994. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. *J. Immunol.* 153: 3514–3522.
6. Yang, L., and T. Mosmann. 2004. Synthesis of several chemokines but few cytokines by primed uncommitted precursor CD4 T cells suggests that these cells recruit other immune cells without exerting direct effector functions. *Eur. J. Immunol.* 34: 1617–1626.
7. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
8. Debes, G. F., U. E. Hopken, and A. Hamann. 2002. In vivo differentiated cytokine-producing $CD4^+$ T cells express functional CCR7. *J. Immunol.* 168: 5441–5447.
9. Kim, C. H., L. Rott, E. J. Kunkel, M. C. Genovese, D. P. Andrew, L. Wu, and E. C. Butcher. 2001. Rules of chemokine receptor association with T cell polarization in vivo. *J. Clin. Invest.* 108: 1331–1339.
10. Nanki, T., and P. E. Lipsky. 2000. Lack of correlation between chemokine receptor and T(h)1/T(h)2 cytokine expression by individual memory T cells. *Int. Immunol.* 12: 1659–1667.
11. Unsoeld, H., S. Krautwald, D. Voehringer, U. Kunzendorf, and H. Pircher. 2002. Cutting edge: $CCR7^+$ and $CCR7^-$ memory T cells do not differ in immediate effector cell function. *J. Immunol.* 169: 638–641.
12. Rivino, L., M. Messi, D. Jarrossay, A. Lanzavecchia, F. Sallusto, and J. Geginat. 2004. Chemokine receptor expression identifies pre-T helper (Th)1, pre-Th2, and nonpolarized cells among human $CD4^+$ central memory T cells. *J. Exp. Med.* 200: 725–735.
13. Gazagne, A., E. Claret, J. Wijdenes, H. Yssel, F. Bousquet, E. Levy, P. Vielh, F. Scotte, T. L. Goupil, W. H. Fridman, and E. Tartour. 2003. A Fluorospot assay to detect single T lymphocytes simultaneously producing multiple cytokines. *J. Immunol. Methods* 283: 91–98.
14. Schultze, J. L., S. Michalak, M. J. Seamon, G. Dranoff, K. Jung, J. Daley, J. C. Delgado, J. G. Gribben, and L. M. Nadler. 1997. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *J. Clin. Invest.* 100: 2757–2765.
15. Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober. 1999. *Current Protocols in Immunology*. John Wiley and Sons, New York.
16. Foulds, K. E., L. A. Zenewicz, D. J. Shedlock, J. Jiang, A. E. Troy, and H. Shen. 2002. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J. Immunol.* 168: 1528–1532.
17. Mustafa, A. S., K. E. Lundin, R. H. Meloen, and F. Oftung. 2000. Cross-reactive epitopes and HLA-restriction elements in human T cell recognition of the *Mycobacterium leprae* 18-kD heat shock protein. *Clin. Exp. Immunol.* 120: 85–92.
18. Oftung, F., E. Borka, G. Kvalheim, and A. S. Mustafa. 1998. Mycobacterial cross-reactivity of *M. tuberculosis* reactive T cell clones from naturally converted PPD positive healthy subjects. *FEMS Immunol. Med. Microbiol.* 20: 231–238.
19. Oftung, F., E. Borka, and A. S. Mustafa. 1998. *Mycobacterium tuberculosis* reactive T cell clones from naturally converted PPD-positive healthy subjects: recognition of the *M. tuberculosis* 16-kDa antigen. *FEMS Immunol. Med. Microbiol.* 20: 319–325.
20. Lee, F. E., E. E. Walsh, A. R. Falsey, N. Liu, D. Liu, A. Divekar, J. E. Snyder-Cappione, and T. R. Mosmann. 2005. The balance between influenza- and RSV-specific CD4 T cells secreting IL-10 or IFNγ in young and healthy-elderly subjects. *Mech. Ageing Dev.* 126: 1223–1229.
21. Bucy, R. P., A. Panoskaltsis-Mortari, G. Q. Huang, J. Li, L. Karr, M. Ross, J. H. Russell, K. M. Murphy, and C. T. Weaver. 1994. Heterogeneity of single cell cytokine gene expression in clonal T cell populations. *J. Exp. Med.* 180: 1251–1262.

22. Karulin, A. Y., M. D. Hesse, M. Tary-Lehmann, and P. V. Lehmann. 2000. Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. *J. Immunol.* 164: 1862–1872.
23. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
24. Iwane, M. K., K. M. Edwards, P. G. Szilagyi, F. J. Walker, M. R. Griffin, G. A. Weinberg, C. Coulen, K. A. Poehling, L. P. Shone, S. Balter, et al. 2004. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. *Pediatrics* 113: 1758–1764.
25. Simoes, E. A. 1999. Respiratory syncytial virus infection. *Lancet* 354: 847–852.
26. Long, C. E., C. B. Hall, C. K. Cunningham, L. B. Weiner, K. P. Alger, M. Gouveia, C. B. Colella, K. C. Schnabel, and W. H. Barker. 1997. Influenza surveillance in community-dwelling elderly compared with children. *Arch. Fam. Med.* 6: 459–465.
27. Mullooly, J. P., and W. H. Barker. 1982. Impact of type A influenza on children: a retrospective study. *Am. J. Public Health* 72: 1008–1016.
28. Chapman, T. J., M. R. Castrucci, R. C. Padrick, L. M. Bradley, and D. J. Topham. 2005. Antigen-specific and non-specific $CD4^+$ T cell recruitment and proliferation during influenza infection. *Virology* 340: 296–306.
29. La Gruta, N. L., S. J. Turner, and P. C. Doherty. 2004. Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific $CD8^+$ T cell responses: correlation of cytokine profile and TCR avidity. *J. Immunol.* 172: 5553–5560.
30. Soussi, N., G. Milon, J. H. Colle, E. Mougneau, N. Glaichenhaus, and P. L. Goossens. 2000. *Listeria monocytogenes* as a short-lived delivery system for the induction of type 1 cell-mediated immunity against the p36/LACK antigen of *Leishmania major*. *Infect. Immun.* 68: 1498–1506.
31. Yeung, V. P., R. S. Gieni, D. T. Umetsu, and R. H. DeKruyff. 1998. Heat-killed *Listeria monocytogenes* as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. *J. Immunol.* 161: 4146–4152.
32. Falugi, F., R. Petracca, M. Mariani, E. Luzzi, S. Mancianti, V. Carinci, M. L. Melli, O. Finco, A. Wack, A. Di Tommaso, et al. 2001. Rationally designed strings of promiscuous $CD4^+$ T cell epitopes provide help to *Haemophilus influenzae* type b oligosaccharide: a model for new conjugate vaccines. *Eur. J. Immunol.* 31: 3816–3824.
33. McVernon, J., N. A. Mitchison, and E. R. Moxon. 2004. T helper cells and efficacy of *Haemophilus influenzae* type b conjugate vaccination. *Lancet Infect. Dis.* 4: 40–43.
34. Hardtke, S., L. Ohl, and R. Forster. 2005. Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help. *Blood* 106: 1924–1931.
35. Kim, C. H., L. S. Rott, I. Clark-Lewis, D. J. Campbell, L. Wu, and E. C. Butcher. 2001. Subspecialization of $CXCR5^+$ T cells: B helper activity is focused in a germinal center-localized subset of $CXCR5^+$ T cells. *J. Exp. Med.* 193: 1373–1381.
36. Moser, B., P. Schaerli, and P. Loetscher. 2002. $CXCR5^+$ T cells: follicular homing takes center stage in T-helper-cell responses. *Trends Immunol.* 23: 250–254.
37. Akai, P. S., and T. R. Mosmann. 1999. Primed and replicating but uncommitted T helper precursor cells show kinetics of differentiation and commitment similar to those of naive T helper cells. *Microbes Infect.* 1: 51–58.
38. Jiang, Z. H., and R. R. Koganty. 2003. Synthetic vaccines: the role of adjuvants in immune targeting. *Curr. Med. Chem.* 10: 1423–1439.
39. Latz, E., J. Franko, D. T. Golenbock, and J. R. Schreiber. 2004. *Haemophilus influenzae* type b-outer membrane protein complex glycoconjugate vaccine induces cytokine production by engaging human Toll-like receptor 2 (TLR2) and requires the presence of TLR2 for optimal immunogenicity. *J. Immunol.* 172: 2431–2438.
40. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 8: 89–95.
41. Iezzi, G., E. Scotet, D. Scheidegger, and A. Lanzavecchia. 1999. The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *Eur. J. Immunol.* 29: 4092–4101.