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# In Vivo Generated Th1 Cells Can Migrate to B Cell Follicles to Support B Cell Responses<sup>1</sup>

Karen M. Smith,<sup>2</sup> James M. Brewer, Catherine M. Rush, Jillian Riley, and Paul Garside

The description of Th1 and Th2 T cell subsets rationalized the inverse correlation between humoral and cell-mediated immunity. Although Th1 cells were described to support cell-mediated immune responses, their role in supporting certain B cell responses was firmly established. However, there is now a prevailing preconception that provision of B cell help is entirely the domain of Th2 cells and that Th1 cells lack this capacity. Previous studies demonstrated that immunization using aluminum hydroxide adjuvants induces Ag-specific Th2 responses, whereas incorporation of IL-12 with aluminum hydroxide produces a Th1 inducing adjuvant. By immunizing TCR transgenic recipient mice in this fashion, we have generated Ag-specific, traceable Th1 and Th2 cells in vivo and assessed their follicular migration and ability to support B cell responses. In this study we have shown that in vivo polarized Th1 and Th2 cells clonally expand to similar levels and migrate into B cell follicles in which they support B cell responses to a similar degree. Critically, we present direct evidence that in vivo polarized, IFN- $\gamma$  secreting Th1 cells migrate into B cell follicles where they can interact with Ag-specific B cells. *The Journal of Immunology*, 2004, 173: 1640–1646.

Early studies described an inverse correlation between cell-mediated and humoral immune responses (1). The reciprocal nature of this relationship was further clarified with the description of mutually antagonistic subsets of T cells, defined by their cytokine profiles (Th1 and Th2) (2). Although Th1 cells were described to support cell-mediated immune responses, their role in supporting certain B cell responses was firmly established (3–11). In particular, the prototypical Th1 cytokine, IFN- $\gamma$ , was shown to be obligatory for the production of Ab subclasses, such as IgG2a in the mouse, involved in opsonization and phagocytosis (4, 9, 12, 13). This premise was recently challenged by the observation that Th1 cells fail to express the appropriate chemokine receptors required for follicular migration and consequently are unable to migrate into follicles and help B cells (14). These studies may preclude a role for Th1 cells in providing help to B cells unless they do this in an anatomical location other than the follicle. However, there is an increasing amount of evidence to suggest that Th1 cells may support B cell responses (15–17). Unfortunately, many of these studies were performed with in vitro polarized cells that may not accurately reflect the in vivo situation. It has previously been shown that immunization with Ag prepared in aluminum hydroxide (Alum)<sup>3</sup> generates specific Th2 responses, whereas immunization with Alum plus IL-12 generates a Th1 response. By immunizing TCR transgenic (tg) recipient mice in this fashion, we have generated Ag-specific, traceable Th1 and Th2 cells in vivo and assessed their (18, 19) follicular migration and ability to support B cell responses. In this study we have shown that in vivo

polarized Th1 and Th2 cells clonally expand to similar levels and migrate into B cell follicles in which they support B cell clonal expansion and Ab production to a similar degree. Critically, we present direct evidence that in vivo polarized, IFN- $\gamma$  secreting Th1 cells migrate into B cell follicles to interact with Ag-specific B cells.

## Materials and Methods

### Animals

IgH<sup>b</sup> BALB/c (H-2<sup>d/d</sup>, IgM<sup>b</sup>) mice were bred in house (Central Research Facilities, University of Glasgow, Glasgow, U.K.). Mice homozygous for the chicken OVA peptide<sub>323–339</sub>/I-A<sup>d</sup>-specific DO11.10 TCR transgenes (detected using the clonotypic mAb KJ1.26) on the BALB/c background (20) were used as donors. Similarly, mice heterozygous for the anti-hen egg lysozyme (HEL) IgM<sup>a</sup> and IgD<sup>a</sup> transgenes on the BALB/c background (MD4) (21) were screened by flow cytometry, and positive animals used as donors. IgH<sup>b</sup> mice 6- to 12-wk-old were used as recipients. All animals were specified pathogen-free and were maintained under standard animal house conditions in accordance with local and home office regulations.

### Preparation of cell suspensions for adoptive transfer

Peripheral lymph nodes (PLN) (axillary, brachial, inguinal, cervical), mesenteric lymph nodes, and spleens from MD4 BALB/c and DO11.10 BALB/c mice were pooled and forced through Nitex (Cadisch Precision Meshes, London, U.K.) using a syringe plunger. The suspensions were washed in sterile RPMI 1640 (Invitrogen Life Technologies, Paisley, U.K.). Cells were washed by adding 1 ml of FACS buffer (see below) before the suspensions were centrifuged at 450  $\times$  g for 5 min and the supernatant discarded. The percentage of IgM<sup>a+</sup>B220<sup>+</sup> MD4 B cells or KJ1.26<sup>+</sup>CD4<sup>+</sup> DO11.10 T cells in these preparations was determined by flow cytometric analysis as described. Cell suspensions containing 1–6  $\times$  10<sup>6</sup> tg T cells and 1–6  $\times$  10<sup>6</sup> tg B cells in 100  $\mu$ l were mixed and 200  $\mu$ l injected i.v. into unirradiated, age-matched IgH<sup>b</sup> BALB/c recipients as previously described (15, 22, 23). For CFSE labeling, cell suspensions were prepared as previously described, washed twice in HBSS (Sigma-Aldrich, Dorset, U.K.), then resuspended at a concentration of 5  $\times$  10<sup>7</sup> lymphocytes/ml. Cells were then incubated with 5  $\mu$ M 5-(and-6-) carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA SE; Molecular Probes, Leiden, The Netherlands). Again, cell suspensions containing 1–6  $\times$  10<sup>6</sup> CFSE-labeled tg T cells or B cells and 1–6  $\times$  10<sup>6</sup> unlabelled tg T or B cells were injected i.v. into unirradiated, age-matched IgH<sup>b</sup> BALB/c recipients as earlier described.

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<sup>3</sup> Abbreviations used in this paper: Alum, aluminum hydroxide; tg, transgenic; HEL, hen egg lysozyme; PLN, peripheral lymph node.

### Ags and Ag administration

Chicken OVA (OVA, Fraction V) was obtained from Sigma-Aldrich and HEL from Biozyme (Gwent, U.K.). Conjugated OVA-HEL 0.5 mM was prepared as previously described (15). Animals were injected s.c. with 130  $\mu\text{g}$  of chicken OVA-HEL in 1% Alum (Brenntag Biosector, Frederikssund, Denmark) with or without 1  $\mu\text{g}$  of IL-12 (PeptoTech, London, U.K.). To enhance intracellular cytokine staining, animals received 100  $\mu\text{g}$  of OVA<sub>323-339</sub> (Sigma-Genosys, Cambridge, U.K.) i.v. 1–2 h before sacrifice.

### Flow cytometry

PLN were harvested between days 1 and 7 after Ag exposure. Cell suspensions were prepared as previously described. Aliquots of cells were incubated with FcR blocking buffer (anti-CD16/32 hybridoma (2.4G2) supernatant; 10% mouse serum (Diagnostics Scotland, Edinburgh, U.K.); and 0.1% azide (Sigma-Aldrich)) for 5 min at 4°C. For detection of CD4<sup>+</sup> DO11.10 tg T cells, the cell suspensions were incubated with PE-conjugated anti-CD4 (BD Pharmingen, Oxford, U.K.) and biotinylated clonotypic anti-TCR Ab, KJ1.26 (24) for 20 min at 4°C. The cells were washed in FACS buffer (PBS, 2% FCS, and 0.1% azide) then incubated with FITC-conjugated Streptavidin (Vector Laboratories, Peterborough, U.K.) for 20 min at 4°C. Anti-B220-PE (BD Pharmingen) and biotinylated-HEL or anti-IgM<sup>a</sup> (BD Pharmingen) were used to identify MD4 B cells as previously discussed. Two-color analysis was performed on 20,000 events. For intracellular cytokine staining, cells were isolated from PLN as described, then incubated with 1  $\mu\text{g}/\text{ml}$  brefeldin A (Sigma-Aldrich) and 5  $\mu\text{M}$  OVA<sub>323-339</sub> for 4–5 h at 37°C. Tg T cells were stained for surface markers as described but using PerCP-CD4 (BD Pharmingen). Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instructions. After washing, cells were further stained with anti-IFN- $\gamma$  PE, anti-IL-4 PE, or an isotype control of irrelevant specificity (all BD Pharmingen). At least 2000 KJ1.26<sup>+</sup>CD4<sup>+</sup> cells were collected per sample for analysis.

### Abs ELISAs

To detect tg B cell-derived anti-HEL IgM<sup>a</sup> in serum, Immulon 2 plates (Costar; Corning, Corning, NY) were coated with HEL (20  $\mu\text{g}/\text{ml}$ ) in PBS at 4°C overnight. Plates were then washed at least three times with PBS Tween 0.05% (Sigma-Aldrich) before being blocked with PBS-FCS 10% (v/v) for 1 h at 37°C. Plates were washed and incubated with diluted serum samples (1/400) for 3 h at 37°C before further washing. IgM<sup>a</sup> levels in serum were determined by incubation with biotinylated anti-IgM<sup>a</sup> (2  $\mu\text{g}/\text{ml}$ ; BD Pharmingen) for 1 h at 37°C. Plates were then washed and incubated with Extravidin-HRP (1/1000; Sigma-Aldrich) for 1 h at 37°C. Plates were washed again and TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. To detect anti-OVA Abs produced by endogenous B cells the above protocol was followed but the plates were coated with OVA (20  $\mu\text{g}/\text{ml}$ ) and biotinylated anti-IgG2a (1/500; BD Pharmingen) or biotinylated anti-IgG1 (1/1600; Serotec, Oxford, U.K.) was used for detection. Absorbances were read on a plate reader at 630 nm.

### Immunohistochemistry

PLN were frozen in liquid nitrogen in OCT embedding medium (Miles, Elkart, IN) in cryomoulds (Miles) and stored at  $-70^{\circ}\text{C}$ . The 6- to 10- $\mu\text{m}$  tissue sections were cut on a cryostat (ThermoShandon, Cheshire, U.K.) and stored at  $-20^{\circ}\text{C}$ . Sections were brought to room temperature in acetone for 10 min, air dried, and rehydrated with PBS before being incubated in 0.1% azide/3% H<sub>2</sub>O<sub>2</sub> for 45 min, changing the solution three times, to block endogenous peroxidase. Avidin solution (Vector Laboratories) was added for 15 min to block unmasked endogenous biotin, then biotin solution (Vector Laboratories) was added to block excess avidin. Finally tissues were incubated with Fc blocking buffer (as described for flow cytometry) for 30 min. Sections were washed in PBS after each treatment. Tg T cells were detected by incubation for 40 min with biotinylated KJ1.26 (1/250) before being washed in TNT wash buffer (0.1 M Tris-HCl, (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20; PerkinElmer, Boston, MA). Streptavidin-HRP (PerkinElmer) was then added for 30 min and sections washed again. Biotinylated tyramide (PerkinElmer) was then added for 10 min, before washing and adding Streptavidin-Alexa Fluor 647 (Molecular Probes). Hydrogen peroxide was added again for 30 min to block any residual HRP activity before sequential incubation with permeabilization buffer (2% FCS, 2 mM EDTA, pH 8.0, 0.5% saponin (Sigma-Aldrich)) for 30 min; 1% blocking reagent (PerkinElmer) for 15 min; rabbit anti-IFN- $\gamma$  (1/250; Serotec) or isotype control (Serotec) (diluted in 1% blocking reagent/0.1% saponin) overnight; anti-rabbit IgG-HRP (Cell Signaling Technology, Hertfordshire, U.K.) diluted in blocking reagent for 30 min. Sections were

thoroughly washed with TNT wash buffer between each step. Finally, Alexa Fluor 488-tyramide (Molecular Probes) was added before washing and mounting. To stain B cell areas, tissues were incubated with FITC-B220 (BD Pharmingen) for 30 min. For three-color intracellular cytokine staining, tissues were stained as previously described, but Tg T cells were detected with biotinylated KJ1.26 and Streptavidin-Alexa Fluor 647, IFN- $\gamma$  was detected with rabbit anti-IFN- $\gamma$  (1/250; Autogen Bioclear, Wiltshire, U.K.), anti-rabbit IgG-HRP and tyramide-Pacific blue and B cells were stained B220-FITC. However, to make colocalization more apparent B cell areas were depicted in blue and IFN- $\gamma$  in green. All photographs were taken at  $\times 20$  magnification.

### Laser scanning cytometry

Sections were stained as previously described. Tg T cells were detected with biotinylated KJ1.26 and Streptavidin-Alexa Fluor 647 and B cell follicles were detected with B220-FITC. Sections were then scanned on a Laser Scanning cytometer equipped with argon, helium, neon, and UV lasers (Compucyte, Cambridge, MA) and visualized with Openlab imaging system (Improvision, Coventry, U.K.). The localization of tg T cells and B cell follicles were plotted. Using these tissue maps the number of tg T cells in defined gates was calculated.

### Statistics

Results are expressed as mean  $\pm$  SEM. To test significance Student's unpaired *t* tests were performed. A value of  $p < 0.05$  was regarded as significant.

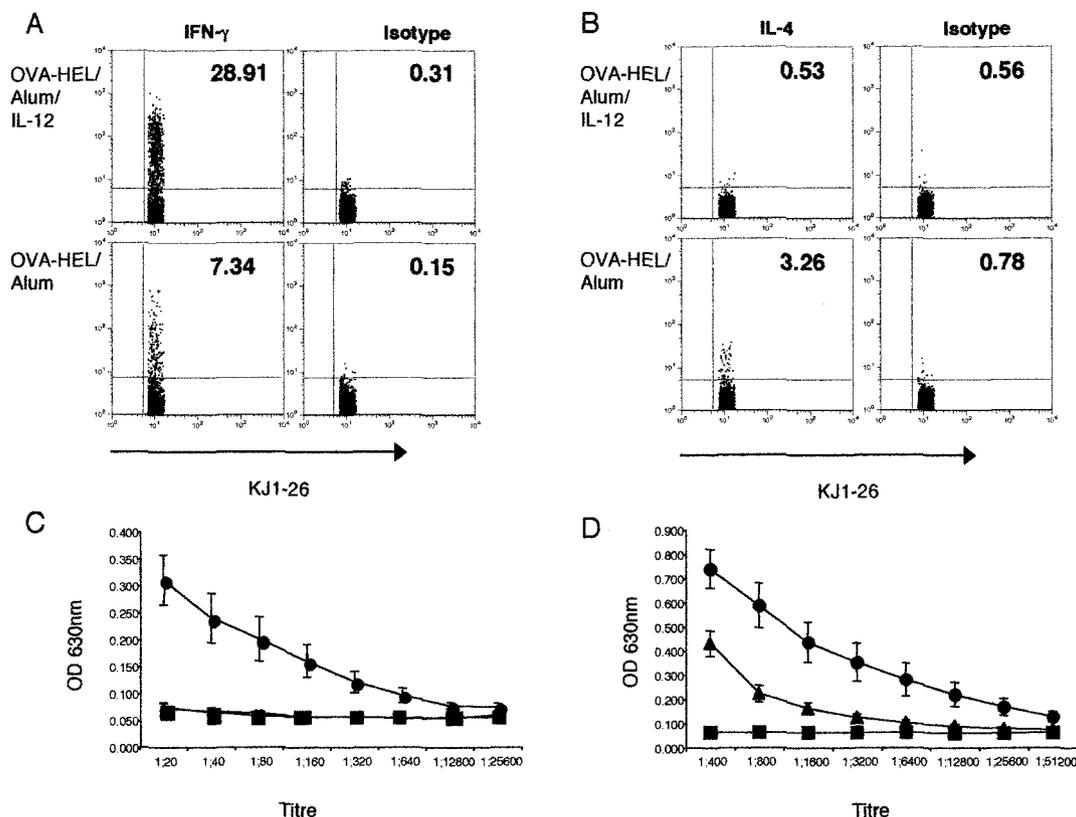
## Results

### *In vivo* generation of Th1 and Th2 T cell responses

To generate polarized responses *in vivo*, mice were transferred with OVA-specific CD4<sup>+</sup>KJ1.26<sup>+</sup> tg T cells 1 day before immunization with OVA-HEL/Alum/IL-12 to generate a Th1 type response or OVA-HEL/Alum to induce a Th2 type response. To confirm the phenotype of *in vivo* polarized tg cells, intracellular cytokine staining was performed on tg T cells isolated from PLNs 3 days postimmunization. Fig. 1A shows that tg T cells from OVA-HEL/Alum/IL-12 immunized mice produced significant levels of the prototypical Th1 cytokine IFN- $\gamma$  (when the results from three mice were averaged,  $23.53\% \pm 4.72$  CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells were IFN- $\gamma$ <sup>+</sup>) when compared with Th2 immunized mice (as the average from three mice was  $7.58\% \pm 0.12$  CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells being IFN- $\gamma$ <sup>+</sup>). In contrast, intracellular IL-4 was only detected in Th2 immunized animals (when the results from three mice were averaged,  $1.79\% \pm 0.74$  CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells were IL-4<sup>+</sup> in OVA-HEL/Alum immunized mice compared with  $0.40\% \pm 0.06$  in OVA-HEL/Alum plus IL-12 immunized mice) (Fig. 1B). To determine whether this polarized phenotype was apparent at a gross functional level *in vivo* we assessed the endogenous Ab response induced by each immunization regime. Fig. 1C shows that only Th1 immunized mice produce OVA-specific IgG2a 3 wk after immunization, whereas OVA-specific IgG1 can be detected in animals immunized with or without IL-12 (Fig. 1D). The presence of specific IgG1 in the absence of Th2 responses has been previously described (15, 25, 26) and rather than the presence of IgG1, the absence of IgG2a may be a better indicator of Th2 responses. These results confirm previous findings that these immunization regimes do indeed polarize stable Th1 and Th2 responses (19) *in vivo*.

### Th1 and Th2 cells clonally expand to similar levels

We next determined whether these immunization regimes induced similar T and B cell responses *in vivo*. Therefore, we examined the ability of *in vivo* polarized Th1 and Th2 cells to clonally expand. It can be seen in Fig. 2A that tg T cells undergo similar levels of clonal expansion and division (Fig. 2B) in both Th1 and Th2 immunized mice. This contrasts with our previous results in which *in vitro* polarized Th2 cells did not proliferate as well as Th1 cells, and may reflect inherent differences between *in vitro* and *in vivo*



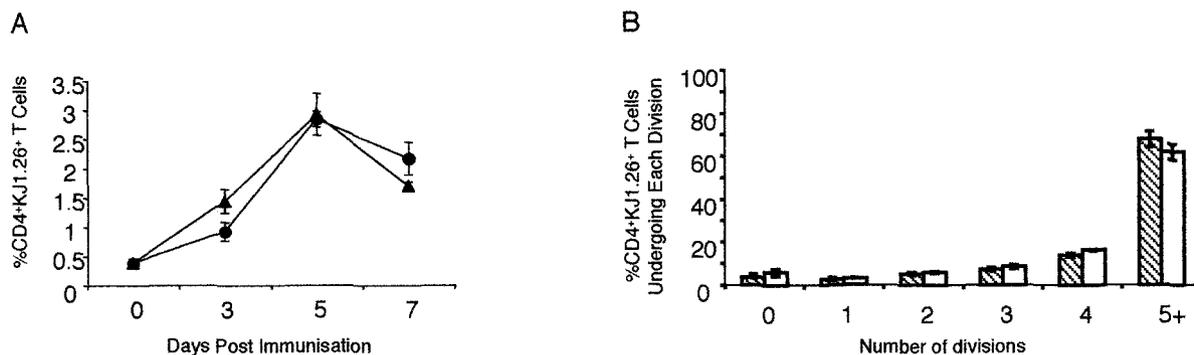
**FIGURE 1.** OVA/Alum/IL-12 immunization results in the generation of Th1 T cells. A total of  $1-6 \times 10^6$  tg T cells were cotransferred with  $1-6 \times 10^6$  B cells into naive recipients. One day later, animals were immunized s.c. with 130  $\mu$ g of OVA-HEL adsorbed to Alum with or without 1  $\mu$ g of IL-12 to induce a Th1 or Th2 response, respectively. Control animals received PBS. Five days after immunization, animals received 100  $\mu$ g of OVA<sub>323-339</sub> peptide i.v. 1-2 h before lymph nodes were harvested. Lymph node cells were then cultured *in vitro* with OVA<sub>323-339</sub> peptide and 1  $\mu$ g/ml brefeldin A for 4-5 h before CD4<sup>+</sup>KJ1.26<sup>+</sup> tg T cells were stained for the presence of IFN- $\gamma$  (A) or IL-4 (B). Data presented represent results from one animal, similar patterns were observed in at least two other mice per group (mean results are noted in study) and similar results were obtained in at least one further experiment. Serum was collected 14 days after immunization with OVA-HEL/Alum ( $\blacktriangle$ ) or OVA-HEL/Alum/IL-12 ( $\bullet$ ) and analyzed for the presence of OVA-specific IgG2a (C) IgG1 (D) Abs by ELISA. Control mice received PBS ( $\blacksquare$ ). Results are presented as mean  $\pm$  SEM for at least five mice per group. Similar results were obtained in two additional experiments.

polarized T cells and/or the use of different adjuvants in the two studies (15).

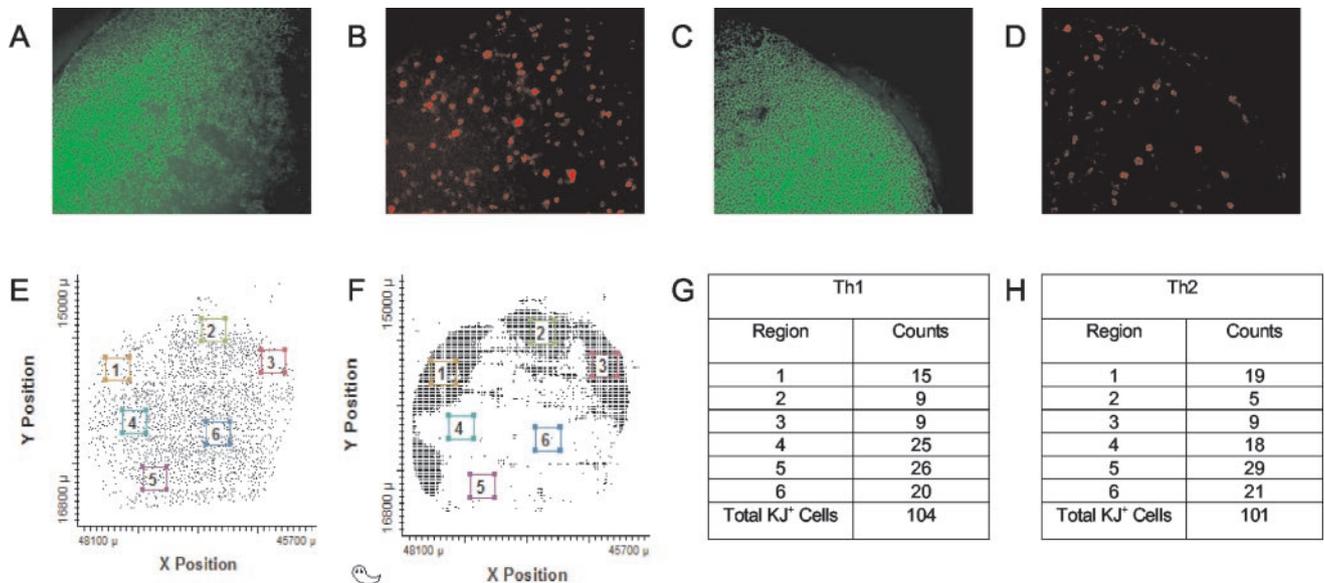
#### Localization of Tg T cells in Th1 and Th2 immunized animals

As the capacity of T cells to help B cells may relate to their ability to migrate into B cell follicles, we directly observed the location of

*in vivo* generated Th1 and Th2 cells. We immunized recipient animals with OVA-HEL/Alum with or without IL-12 and assessed the location of tg T cells *in situ*. Tg T cells were detected in B cell follicles of both Th1 (Fig. 3, A and B) and Th2 (Fig. 3, C and D) immunized animals. The localization of TCR tg T cells was quantified by laser scanning cytometry, an example of which is shown



**FIGURE 2.** *In vivo* generated Th1 and Th2 cells expand and divide to a similar extent. PLN were harvested from mice, as described in *Materials and Methods*, for 3, 5, and 7 days after immunization with OVA-HEL/Alum or OVA-HEL/Alum/IL-12. CD4<sup>+</sup>KJ1.26<sup>+</sup> tg T cell clonal expansion was assessed by FACS (A). Results from PBS-injected mice from each time point were averaged and presented as day 0. Results are presented as mean  $\pm$  SEM for at least three mice per group. Similar results were obtained in four additional experiments. Mice were transferred with  $1-6 \times 10^6$  CFSE-labeled CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells. B, Three days after immunization PLNs were harvested and the CFSE division of CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells in Th1 ( $\square$ ) and Th2 ( $\boxtimes$ ) immunized animals was assessed by FACS. Results are presented as the mean  $\pm$  SEM for at least three mice per group. Similar results were obtained in one further experiment.



**FIGURE 3.** Localization of Ag-specific T cells in Th1 and Th2 immunized animals. Animals were transferred as described in *Materials and Methods* and immunized with OVA-HEL/Alum or OVA-HEL/Alum/IL-12. Five days after immunization PLN were harvested and frozen. Tg T cells were detected on sections from Th1 (A and B) and Th2 immunized animals (C–F) using KJ1.26 (red) to stain Tg T cells and anti-B220 (green) to stain B cell follicles. Using a Laser Scanning cytometer, the location of tg T cells (B, D, and E) and B cell follicles (A, C, and F) were determined. Gates were then drawn in B cell follicles (gates 1–3) and extrafollicular regions (gates 4–6). The number of tg T cells within follicular gates was then compared between Th1 (G) and Th2 (H) immunized animals.

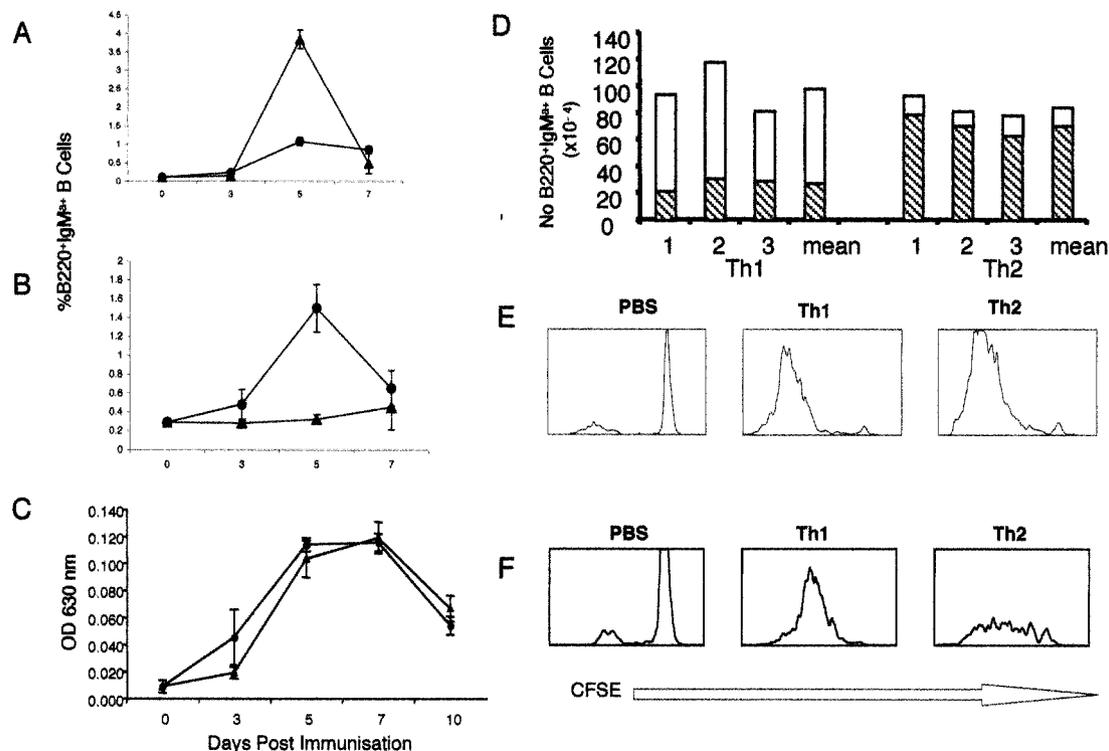
in Fig. 3, E–H. When several animals ( $n = 3$ ) from each group were analyzed in this way, it was evident that there was no significant difference in the number of T cells per unit area of B cell follicle when Th1 and Th2 immunized animals were compared (the average total T cell number per 12 arbitrary unit areas being  $66 \pm 7$  vs  $71 \pm 18$  in Th1 vs Th2 immunized animals), although as expected, both groups displayed increased T cell follicular migration in comparison with unimmunized controls ( $8 \pm 6$ ). Therefore, in vivo polarized Th1 and Th2 cells clonally expand and migrate to a similar degree after immunization.

#### Both Th1 and Th2 cells support B cell clonal expansion and Ab production

As the ability of Th1 cells to support B cell responses in vivo has been controversial, we cotransferred OVA-specific tg T cells and HEL-specific tg B cells into naive recipients to directly assess the ability of Th1 and Th2 cells to provide help for Ag-specific B cells. These animals were then immunized with conjugated OVA-HEL/Alum with or without IL-12 to produce a Th1 or Th2 response, respectively. Previous studies have shown that the ensuing B cell response is dependent upon help from tg T cells (22). Fig. 4A shows that both Th1 and Th2 T cells support B cell responses in the PLN of immunized mice. Th2 cells support significantly more B cell clonal expansion than Th1 cells. However, there was no apparent difference in cell division supported by Th1 or Th2 cells (Fig. 4E). Furthermore, Ab production from tg B cells is similar in both cases (Fig. 4C). Interestingly, when the spleen is assessed the opposite is observed with more tg B cells (Fig. 4B) and more tg B cell division (Fig. 4F) being observed in Th1 immunized mice. Thus, when the tg B cell numbers from both sites are assessed together (Fig. 4D) it can be seen that Th1 and Th2 cells support similar levels of B cell clonal expansion. Therefore, Th1 and Th2 cells are able to support B cell expansion to a similar degree but this may comprise different contributions from distinct lymphoid organs.

#### Location of Th1 and Th2 T cells in vivo

Although we have shown that at a gross level tg T cells in OVA-HEL/Alum with IL-12 immunized animals behave as Th1 cells and T cells in OVA-HEL/Alum immunized animals behave as Th2 cells, we have not definitively shown that in OVA-HEL/Alum plus IL-12 immunized animals the follicular T cells have a Th1 phenotype. Therefore, we performed intracellular cytokine analysis on tg T cells in situ to determine whether IFN- $\gamma$  secreting cells migrate to follicles to interact with Ag-specific B cells. Animals were transferred with tg T cells and B cells and immunized with OVA-HEL/Alum with or without IL-12. Five days later animals received  $100 \mu\text{g}$  of OVA<sub>323–339</sub> i.v. 1–2 h before lymph nodes were harvested. This peptide pulse was performed as in the absence of in vitro restimulation it improved detection of intracellular cytokines by FACS (data not shown). Sections were stained with B220-FITC to detect B cell follicles as shown in Fig. 5A, and all subsequent pictures cover follicular areas (Fig. 5, B–F). Serial sections were then stained with KJ1.26 (red) (Fig. 5, B–E) and IFN- $\gamma$  (green) (Fig. 5, B, D–F) or an isotype control (Fig. 5C). Tg T cells can be seen in all transferred animals (Fig. 5, B–F), however, tg T cells only enter follicles after immunization with Ag and adjuvant (Fig. 5, B–D and F). No KJ1.26<sup>+</sup>IFN- $\gamma$ <sup>+</sup> tg T cells were observed in OVA-HEL/Alum (Fig. 5F) or PBS (Fig. 5E) treated animals. However, Fig. 5B clearly shows IFN- $\gamma$ <sup>+</sup> tg T cells in B cell follicles 5 days following OVA-HEL/Alum plus IL-12 immunization, in addition IFN- $\gamma$  production is also evident in non-tg cells. As there is no way to limit the effect of the immunization to only transferred cells, these may be the recipient's own Ag-specific lymphocytes, however, it is more probable that these are innate immune cells such as NK cells. Furthermore, three-color staining definitively identified these cells within follicles (Fig. 5D). Thus we have provided direct evidence that Th1 cells are capable of migrating to follicles in which they are ideally placed to support B cell clonal expansion and Ab production.



**FIGURE 4.** In vivo generated Th1 and Th2 cells provide help for B cell clonal expansion and Ab production. Lymph nodes and spleen were harvested from transferred mice 3, 5, and 7 days after immunization with OVA-HEL/Alum ( $\blacktriangle$ ) or OVA-HEL/Alum/IL-12 ( $\bullet$ ). B220<sup>+</sup>IgM<sup>+</sup> tg B cell clonal expansion was assessed by FACS in PLN (A) and spleen (B). C, HEL-specific IgM<sup>+</sup> levels were assessed from serum as described in *Materials and Methods*. Results from PBS injected mice from each time point were averaged and presented as day 0. Results are presented as mean  $\pm$  SEM for at least three mice per group. Similar results were obtained in four additional experiments. D, Total tg B cell numbers in spleen ( $\square$ ) and PLNs ( $\text{hatched}$ ) was assessed by FACS. Results from three individual mice are presented followed by the average from the three. Mice were transferred with  $1-6 \times 10^6$  CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells and  $1-6 \times 10^6$  CFSE-labeled B220<sup>+</sup>IgM<sup>+</sup> B cells. Five days after immunization, organs were harvested and the CFSE intensity and hence division of B220<sup>+</sup>IgM<sup>+</sup> B cells in PLN (E) and spleen (F) was assessed by FACS. Similar patterns were observed in at least two other mice per group. Similar results were obtained in one further experiment.

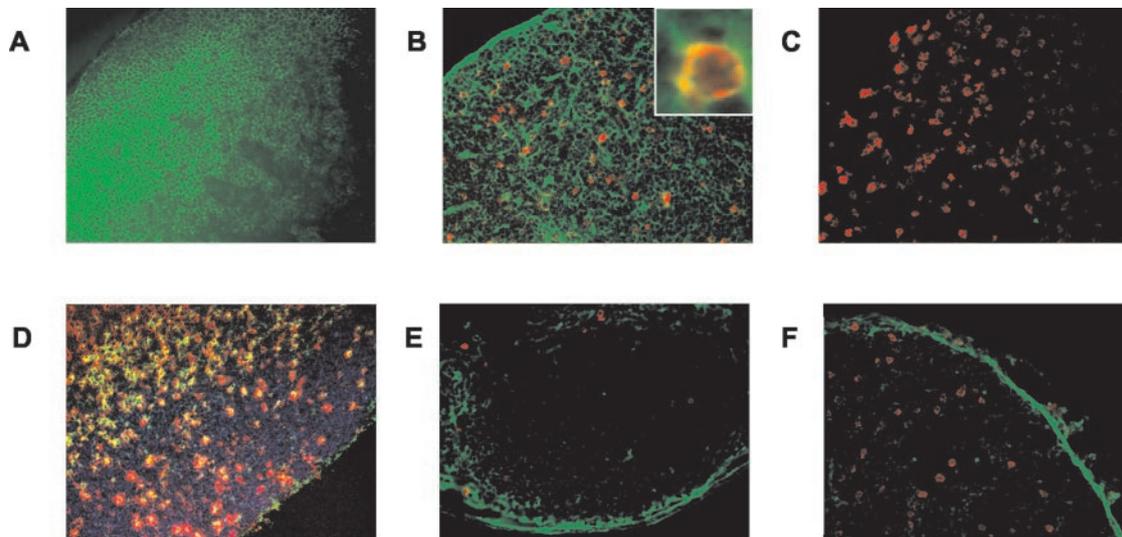
## Discussion

We have successfully polarized naive TCR tg T cells toward Th2 and Th1 phenotypes in vivo using Ag prepared in Alum or Alum and IL-12, respectively. Using an adoptive transfer system, we have then tracked these cells throughout a developing immune response. We have shown that during the early immune response, TCR tg T cells adopt the expected cytokine profiles with T cells in Th1 immunized animals producing IFN- $\gamma$  and Th2 immunization producing T cells secreting IL-4. Furthermore, at a gross level, Th1 cells supported IgG2a and IgG1 production, whereas Th2 cells supported only IgG1 production. We have also assessed the ability of these cells to interact with B cells and provide evidence that both in vivo generated Th1 and Th2 cells migrate into B cell follicles to support B cell clonal expansion and Ab production to a similar extent. Critically, we have shown that IFN- $\gamma$ <sup>+</sup> TCR tg cells are present within B cell follicles where they are ideally placed to participate in cognate interactions with Ag-specific B cells.

The original descriptions of Th1 and Th2 cells clearly stated that although Th1 cells were primarily involved in cell-mediated immune responses, they were obligatory for IFN- $\gamma$  production to support the production of complement fixing Abs such as murine IgG2a (3–10, 12, 13). More recent evidence has suggested that Th1 cells are unable to interact with B cells as they preferentially express CCR7 and are thus held in the T cell areas of the spleen; in contrast, Th2 cells do not express this chemokine receptor and are subsequently able to localize with B cells by expressing CXCR5 (14). However, T cell localization appeared to be different in the spleen vs lymph nodes, with both Th1 and Th2 cells in close prox-

imity to B cells and therefore capable of providing help for B cells in the lymph nodes (14). Furthermore, as B cell clonal expansion in extrafollicular foci is not associated with somatic hypermutation (27), if IFN- $\gamma$ -producing Th1 cells do not enter follicles it is difficult to envisage how high affinity IgG2a Abs are produced. Nevertheless, current dogma appears to suggest that Th1 cells are incapable of mediating B cell help and that this function is entirely the domain of Th2 cells. Contrasting with this, studies of murine and human CD4<sup>+</sup> T cells found no evidence that Th1 vs Th2 cells preferentially migrate toward the CCR7 ligand CC chemokine ligand 21 (28). Furthermore, recent studies have indicated that differentiation into both Th1 and Th2 cells is accompanied by a progressive loss of CCR7 expression and increased expression of CXCR5 (although the frequency and kinetics of expression may be different on each cell type) (29), supporting the finding that Th1 and Th2 cells can migrate into B cell follicles and support B cell responses (15–17, 30). Alternatively, some studies have even suggested that B cell help is a function of an entirely separate Th cell subset, T cell follicular helper, which may not be Th1 or Th2 or may only become polarized after passage through a follicle (31–34). Clearly, this remains a controversial area but is of crucial importance when considering the rational design of vaccines and immunological therapies.

Many of the studies described in this study used in vitro polarized Th1 and Th2 cells (14, 15), used different Ags for generating the Th1 vs Th2 responses (17), or did not definitively identify Ag-specific Th1 cells in B cell follicles (16). To address these issues, we combined a cotransfer system in which both Ag-specific



**FIGURE 5.** IFN- $\gamma^+$  tg Th1 cells migrate into T cell follicles and interact with HEL-specific B cells. PLNs from transferred mice were frozen 5 days after immunization with OVA-HEL/Alum/IL-12 (A–D), PBS (E), or OVA-HEL/Alum (F). Five days after immunization animals received 100  $\mu$ g of OVA<sub>323–339</sub> peptide i.v. 1–2 h before lymph nodes were harvested. Sections were prepared and labeled with KJ1.26 (red) and IFN- $\gamma$  (green) (B, E, and F) or an isotype control (C). A, Serial sections were stained with B220 (green) to locate follicles. D, For three-color staining, sections were stained with KJ1.26 (red), and IFN- $\gamma$  (green), and B220 (blue). Similar patterns were observed in at least two other mice per group. Similar results were obtained in two additional experiments.

T cells and B cells could be assessed directly with immunization regimes that allowed us to polarize cells *in vivo* using exactly the same conditions with the exception of IL-12 inclusion in Th1 group. Thus, we were able to observe Th1 and Th2 induction over a detailed time course *in vivo*. Intracellular cytokine staining showed that animals immunized with OVA-HEL/Alum plus IL-12 produced significantly more IFN- $\gamma^+$  tg T cells than OVA-HEL/Alum immunized animals. Furthermore, IL-4 $^+$  tg T cells were only detected in Th2 immunized animals. It is possible that some of the IFN- $\gamma^+$  T cells were of the Th0 phenotype which have been demonstrated *in vitro*. However, this phenotype does not appear to be stable *in vivo* (35), therefore, we propose that the IFN- $\gamma^+$  cells generated in our system are Th1 cells and the IL-4 $^+$  cells generated are Th2 cells. To determine whether these polarized phenotypes were stable after a single immunization, the endogenous Ab response was assessed. Three weeks after immunization, OVA-specific IgG2a was detected only in Th1 immunized animals, confirming that these immunizing regimes generated Th1 or Th2 cells and that the polarized phenotypes were stable. These data confirm previous findings in which following challenge immunizations, Ag adsorbed to Alum resulted in IgG1 production but no IgG2a production, whereas incorporation of IL-12 generated both IgG1 and IgG2a (18).

These results allowed us to characterize the respective immune responses and assess how *in vivo* generated Th1 and Th2 cells behaved. In contrast to our previous studies in which *in vitro* generated Th1 expanded significantly more than Th2 cells (15), *in vivo* generated Th1 and Th2 cells expanded to similar levels and divided to similar degrees following immunization. Thus, these results highlight the difference between cells that have been polarized *in vitro* and cells that have been polarized using differing immunization conditions *in vivo*. However, in confirmation of our previous studies, Th1 and Th2 tg T cells both entered B cell follicles. Furthermore, when follicular tg T cell numbers were quantified using Laser Scanning cytometry there was no difference between the Th1 and Th2 immunized animals.

The use of the cotransfer of TCR and B cell receptor tg T and B lymphocytes allowed us to analyze in detail whether *in vivo* polarized Th1 and Th2 cells were capable of supporting B cell responses. Characterization of the B cell response after Th1 or Th2 immunization has provided us with further evidence that both Th1 and Th2 cells support Ag-specific B cell clonal expansion, division and Ab production. Interestingly, when B cell expansion was analyzed in the PLN it appeared that Th2 cells supported more B cell clonal expansion and division than Th1 cells. However, in confirmation of previous studies (15), Ab production was similar in both cases. When we assessed B cell responses in the spleen, it became apparent that Th1 cells supported far more B cell clonal expansion in this organ. This would appear to contrast with other findings that Th1 cells do not enter B cell follicles to support B cell responses in the spleen (14), the contrast in these findings may result from the use of different adjuvants, different immunization routes, or because the latter study used *in vitro* polarized cells. When the overall numbers of Ag-specific B cells in the PLN and spleen was calculated, it was clear that Th1 and Th2 cells were able to support B cell clonal expansion to a similar degree, thus explaining the similar levels of serum Ab after each immunization.

Although we were able to show that after induction, Th1 and Th2 cells adopted the expected phenotype and that these phenotypes were stable and able to support B cell responses to a similar extent, we had not definitively shown that the follicular T cells in Th1 immunized animals were of the Th1 phenotype. Therefore, we performed intracellular cytokine staining *in situ* to confirm that the follicular T cells in Th1 immunized animals were indeed IFN- $\gamma^+$ . Our data, together with the recent finding that IgG2a $^+$  B cells are initially detected after immunization in the B cell follicle (16), support the hypothesis that IFN- $\gamma^+$  Th1 cells migrate into B cell follicles to provide cognate help for B cells.

Therefore, using a cotransfer system, we have shown that *in vivo* polarized Th1 and Th2 cells are able to support Ag-specific B cell clonal expansion, division and Ab production to a similar degree. Crucially, we have directly demonstrated that Ag-specific,

IFN- $\gamma$ <sup>+</sup> T cells migrate into B cell follicles in Th1 immunized animals.

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