The Role of OX40 Ligand Interactions in the Development of the Th2 Response to the Gastrointestinal Nematode Parasite Heligmosomoides polygyrus

Melinda J. Ekkens, Zhugong Liu, Qian Liu, Jeannette Whitmire, Shiyun Xiao, Anthony Foster, John Pesce, Jansie VanNoy, Arlene H. Sharpe, Joseph F. Urban and William C. Gause

*J Immunol* 2003; 170:384-393; doi: 10.4049/jimmunol.170.1.384
http://www.jimmunol.org/content/170/1/384

**References**

This article cites 48 articles, 35 of which you can access for free at:
http://www.jimmunol.org/content/170/1/384.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The role of OX40 ligand (OX40L) deficiency on the development of Th2 cells during the Th2 immune response to the intestinal nematode parasite Heligmosomoides polygyrus.

Melinda J. Ekkens,* Zhugong Liu,* Qian Liu,* Jeannette Whitmire,* Shiyun Xiao,* Anthony Foster,* John Pesce,* Jansie VanNoy,* Arlene H. Sharpe,† Joseph F. Urban,‡ and William C. Gause3,*

In these studies, we examined the effects of OX40 ligand (OX40L) deficiency on the development of Th2 cells during the Th2 immune response to the intestinal nematode parasite Heligmosomoides polygyrus. Elevations in IL-4 production and total and Ag-specific serum IgE levels were partially inhibited during both the primary and memory immune responses to H. polygyrus in OX40L−/− mice. The host-protective memory response was compromised in OX40L−/− mice, as decreased worm expulsion and increased egg production were observed when H. polygyrus-inoculated OX40L+/− mice were compared to H. polygyrus-inoculated OX40L−/− mice. To further examine the nature of the IL-4 defect during priming, adoptively transferred DO11.10 T cells were analyzed in the context of the H. polygyrus response. Although Ag-specific T cell IL-4 production was reduced in the OX40L−/− mice following immunization with OVA peptide plus H. polygyrus, Ag-specific T cell expansion, cell cycle progression, CXCR5 expression, and migration were comparable between OX40L+/+ and OX40L−/− mice inoculated with OVA and H. polygyrus. These studies suggest an important role for OX40/OX40L interactions in specifically promoting IL-4 production, as well as associated IgE elevations, in Th2 responses to H. polygyrus. However, OX40L interactions were not required for serum IgG1 elevations, increases in germinal center formation, and Ag-specific Th2 cell expansion and migration to the B cell zone.


The development of IL-4 producing T cells and the associated Th2 response requires signaling through the TCR and costimulatory molecules. Although the CD28/B7 costimulatory molecule interactions comprise the most well-defined costimulatory molecules, recent studies have suggested that a number of other costimulatory molecules may be important during the initiation and progression of the Th2 response. In particular, OX40 (CD134), a member of the TNF superfamily, has been shown to play an important role in Ag-specific T cell activation and costimulation. OX40 is expressed on activated T cells (1, 2), while its ligand (OX40 ligand (OX40L); CD134 ligand) is expressed on activated B cells (1, 2), dendritic cells (3, 4), and endothelial cells (5, 6).

Several studies have suggested that OX40/OX40L interactions may be particularly important in promoting the development of OX40-producing T cells (7–9). In vivo administration of an anti-OX40L Ab decreased Th2 cytokine and Ab production during the primary immune response to Leishmania major with an increase in Th1 cytokine production associated with enhanced protection (4). However, more recent studies have demonstrated that protective immunity and the associated primary Th2 response to Nippostrongylus brasiliensis is not inhibited in OX40L−/− mice (10). As yet, the role of OX40L interactions in the development of the memory Th2 response during infectious disease has not been examined in vivo. Recent studies have suggested that OX40/OX40L interactions may be particularly important in sustaining T cell function at later stages of the primary immune response and during the memory response (8, 10–15). Gramaglia et al. (15) demonstrated that T cells from OX40-deficient mice produce IL-2 and proliferate normally, but, as the response proceeds, T cell expansion and associated increased cytokine production is not sustained in vitro. Furthermore, Rogers et al. (16) demonstrated that reduced T cell survival in OX40−/− mice was due to decreased levels of the antiapoptotic proteins Bcl-xL and Bcl-2. In addition, the frequency of Ag-specific T cells was reduced in the absence of OX40 during both the primary and secondary immune responses to keyhole limpet hemocyanin (15).

In addition to regulating T cell survival, several studies suggest that OX40 signaling functions in the regulation of T cell homing within secondary lymphoid organs. In particular, CD28-dependent OX40 signaling has been correlated with the up-regulation of CXC5R on CD4+ T cells, which results in homing of these T cells toward the B cell-rich follicles where they provide the necessary signals for germinal center (GC) formation (7, 17, 18). In a study by Brocker et al. (18), OX40L transgenic mice demonstrated an accumulation of activated CD4+ OX40+ T cells in the B cell follicles of secondary lymphoid organs following antigenic stimulation.
To directly examine the role of OX40/OX40L interactions during in vivo primary and memory Th2 immune responses, we infected OX40L−/− and OX40L+/+ BALB/c mice with the murine gastrointestinal nematode parasite *Heligmosomoides polygyrus*. The parasite *H. polygyrus* is a useful model for studying development of primary and memory Th2 responses during infectious disease, because the primary host response is associated with chronic infection, while the memory immune response is a host-protective response characterized by CD4-dependent increases in IL-4 production that effectively limit adult worm maturation and egg production (19). Although the mechanism of host protection is unclear, it appears to depend primarily on direct effects of IL-4 and/or IL-13 on intestinal tissue (19, 20). Infection with *H. polygyrus* is characterized by CD4-dependent increases in Th2 cytokines, serum IgE and IgG1, and GC formation in the mesenteric lymph node (MLN) (21-23). In these studies, we examined whether Th2 node (MLN) (21–23) characterized by CD4-dependent increases in Th2 cytokines, serum IgE on intestinal tissue (19, 20). Infection with *H. polygyrus* was then inoculated at 5 x 10⁶ cells/ml in PBS plus 0.1% BSA, and labeled with 10 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C; labeling was terminated by washing in cold RPMI plus 10% FCS (Life Technologies, Grand Island, NY). Single-cell suspensions (5 x 10⁶) of OVA-specific KJ1-26 CD4+ T cells were then injected i.v. into recipient mice.

### Quantitation of serum IgS
Total serum IgG1, IgG2a, and IgE levels were quantitated by ELISA (26). Ag-specific Ab levels were measured using a modified ELISA as described previously (27).

### ELISPOT
The frequency of IL-4-producing cells was determined by an ELISPOT assay as previously described (26, 28). Alternatively, the ELISPOT assay was modified to include the capability to quantitate IL-4-producing cells following in vitro restimulation with OVA peptide. Briefly, lymph node cells were first cultured for 3 days on anti-IL-4-coated plates with or without 10 μg/ml OVA peptide. The OVA peptide was then washed away with PBS and PBS plus Tween 20 before addition of the secondary anti-IL-4 Ab diluted (4 μg/ml) in PBS plus 0.05% Tween plus 5% FCS, and added at 100 μl/well. Following an overnight incubation at 4°C, plates were washed, and a 1/2000 dilution of streptavidin-alkaline phosphatase (Jackson Immunoresearch Laboratories, West Grove, PA) was added. Plates were then developed, and results were counted as previously described (26, 28).

### Cell labeling and FACS analysis
The draining cervical lymph nodes were removed from individual mice, and single-cell suspensions were pooled within a treatment group. Lymph node cells were washed and 1 x 10⁶ cells were blocked with Fc Block (BD PharmMingen, San Diego, CA) before incubation with the following fluorescent Abs: CyChrome anti-CXCR5 (BD Pharmingen), PE anti-CD45R0 (BD Pharmingen), KJ1-26-PE (Caltag Laboratories, Burlingame, CA), KJ1-26-Tri-Color (Caltag Laboratories), and rabbit-anti-CXCR5 (polyclonal; a kind gift from Dr. J. Cyster (Department of Microbiology and Immunology, University of California, San Francisco, CA)), which was followed by PE-anti-rabbit-Ig (Serotec, Raleigh, NC). After washes, cells were fixed with 1% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) and analyzed by flow cytometry using an EPICS XL-MCL (Beckman Coulter, Fullerton, CA). Twenty thousand events were collected and analysis of activation marker expression was performed by gating on the CD4+ population. The total number of KJ1-26+ cells per draining lymph node was determined as the percentage of KJ1-26+ CD4+ cells multiplied by the total number of cells per lymph node. For CFSE-labeled cells, anti-CD4-CyChrome and KJ1-26-PE were used to distinguish the DO11.10 T cells. Cell cycle progression was monitored by measuring sequential reductions in CFSE fluorescence (29) of KJ1-26+ CD4+ cells using ModFit (Verity Software House, Topsham, ME) software.

### Immunohistological analysis
Immunohistological staining and GC quantitation of the MLN were performed as described previously (22, 30). In other experiments, the draining cervical lymph nodes were harvested from individual mice and frozen in liquid nitrogen, and 8-μm tissue sections were obtained from near the center of the cervical lymph node using a HM505E cryostat (Richard-Allan Scientific, Kalamazoo, MI). Tissue sections were allowed to dry at room temperature for 30 min, fixed in cold acetone for 10 min, and stored at −80°C. Cervical lymph node tissue sections were then dual-stained with PE-KJ1-26 (Caltag Laboratories) and biotinylated-anti-B220 (RA3-6B2; BD Pharmingen), followed by streptavidin Alexa-647 (Molecular Probes, San Diego, CA).

### Materials and Methods
#### Mice
All experiments were performed using BALB/c mice genetically deficient for OX40L (OX40L−/−) or normal BALB/c controls; four to five mice were used per treatment group. Breeding pairs of BALB/c OX40L+/+ mice and BALB/c DO11.10 TCR transgenic mice were obtained from Dr. A. Sharpe (Brigham and Women’s Hospital and Harvard Medical School), while the BALB/c controls were obtained from The Jackson Laboratory (Bar Harbor, ME). T cells from DO11.10 TCR transgenic OX40L+/+ mice were transferred to OX40L−/− mice to directly examine the role of OX40/OX40L interactions during the development and trafficking of Ag-specific T cells following primary inoculation with *H. polygyrus*. These studies demonstrated a reduction in Ag-specific IL-4 production, although Ag-specific T cell expansion, cell cycle progression, CXCR5 expression, and migration were comparable between OX40L+/+ and OX40L−/− mice primed with both *H. polygyrus* and OVA peptide. In summary, these studies suggest that the absence of OX40L does not impair the ability of Ag-specific T cells to proliferate and migrate toward the B cell follicles of secondary lymphoid organs, but does impair the ability of T cells to produce IL-4 and provide B cell help leading to increased IgE production and host protection.

#### Parasites and OVA inoculation
Mice were inoculated per os with 200 infective third-stage *H. polygyrus* larvae using a rounded gavage tube, and adult worm numbers and egg production were quantitated as described previously (25). An antihelminthic, pyrantel pamoate (1–2 mg) was administered orally to expel *H. polygyrus* adults from the gut after primary infection and before secondary challenge infection (25). Alternatively, DO11.10 T cell-recipient mice were inoculated intracutaneously (in the ear) with 300 infective third-stage *H. polygyrus* larvae and/or 30 μg of OVA peptide. HPLC-purified OVA peptide 323–339 with the sequence I-S-Q-A-V-H-A-A-H-A-I-E-N-E-A-G-R-COOH was synthesized by the Biomedical Instrumentation Center at the Uniformed Services University of the Health Sciences.

#### DO11.10 adoptive cell transfer
Peripheral lymph node spleens and the spleen were harvested from DO11.10 TCR transgenic mice that were age- and sex-matched to adoptive transfer recipients, and single-cell suspensions were prepared by gently pressing the recipients, and single-cell suspensions were prepared by gently pressing the
Eugene, OR), and coarsely pulsed using FluoromountG (Southern Biotechnology Associates, Birmingham, AL). The surface of the entire cervical lymph node section was mapped using scanning software designed by Jozsef Czege in the Biomedical Instrumentation Center at the Uniformed Services University of the Health Sciences. Mapped regions of the lymph node were then individually photographed at ×20 magnification using a SPOT2 cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Leica DMRXA (Leica Microsystems, Bannockburn, IL) computerized fluorescence microscope, and using SPOTAdvance software (Diagnostic Instruments). Each fluorescent channel was photographed separately, and the three sets of ×20 images were merged using TIFFany3 software (Caffeine Software, Santa Clara, CA) to create the final picture of the whole cervical lymph node section.

### Cytokine gene expression by RT-PCR

Total RNA was prepared from tissues and reverse transcribed, as previously described (26). Real-time PCR commercial kits (Applied Biosystems, Foster City, CA) specific for different cytokines or rRNA were used and all data were normalized to constitutive rRNA values. The Applied Biosystems 7700 sequence detector (PE Applied Biosystems) was used for amplification of target mRNA and quantitation of differences between treatment groups was calculated according to the manufacturer’s instructions.

### Statistical analysis

Statistical differences (significance level of \( p < 0.05 \)) between groups were assessed using ANOVA and Tukey’s \( t \) test for pairwise comparisons. The software program SigmaStat (Jandel Scientific Software, San Rafael, CA) was used for all statistical analyses.

### Results

**Host-protective immunity is inhibited in \( H. \) polygyrus-challenged OX40L\\(^{-/-} \) mice, consistent with reduced elevations in IL-4 production during the primary and secondary immune responses to \( H. \) polygyrus**

Initial infection with \( H. \) polygyrus stimulates a chronic Th2 CD4-dependent immune response, which can be completely cleared by administration of an antihelminthic drug. Subsequent exposure to \( H. \) polygyrus results in a strong IL-4 dominant memory response, which impairs adult worm development resulting in reduced fecundity (19). Previous studies have suggested that, although B7 blockade inhibits the primary response, administration of the B7 antagonist, CTLA4Ig, at the time of challenge does not impair the memory response (22, 23, 31). More recent studies have demonstrated that the development of memory T cells in response to \( H. \) polygyrus (27) or lymphocytic choriomeningitis virus (32) does not require B7-1/B7-2 or CD28 interactions, suggesting that other costimulatory molecules may play a role in the development of memory T cells. In particular, several studies have suggested that OX40/OX40L interactions may function in the development of memory T cells (8, 15, 16, 33–35). To examine the role of OX40/OX40L interactions in the primary and memory Th2 immune responses to \( H. \) polygyrus, BALB/c OX40L\\(^{-/-} \) and BALB/c OX40L\\(^{-/-} \) mice were orally inoculated with 200 third-stage \( H. \) polygyrus larvae. All mice were treated with the antihelminthic pyrantel pamoate at 2–3 wk postinfection to expulse all parasites before a challenge oral dose of \( H. \) polygyrus given 5–6 wk after primary inoculation. Two additional control groups of OX40L\\(^{-/-} \) and OX40L\\(^{-/-} \) mice received an initial inoculum of \( H. \) polygyrus at the time of challenge, allowing direct comparison between the primary and secondary immune responses to \( H. \) polygyrus. At day 12 following \( H. \) polygyrus challenge inoculation, all treatment groups were sacrificed, and various immune parameters were assessed.

Previous studies have demonstrated that CD4\\(^{+} \)TCR-\\( \alpha\beta \) cells are the primary source of IL-4 production during the primary (21, 22, 25) and challenge (23, 25) immune responses to \( H. \) polygyrus. This cell population directly mediates host protection during the challenge response (25), resulting in an inhospitable enteric environment in which egg production by adult worms is inhibited and, in some cases, worms are expelled (23, 25). At day 12 following \( H. \) polygyrus challenge, the total number of eggs and adult worms per mouse were determined, as previously described (25). As seen in Fig. 1A, the number of adult worms was significantly (\( p < 0.01 \)) reduced in the \( H. \) polygyrus-challenged OX40L\\(^{-/-} \) mice, as compared with OX40L\\(^{-/-} \) mice that received only a primary \( H. \) polygyrus inoculation. However, comparable numbers of adult worms were observed in OX40L\\(^{-/-} \) mice during both the primary and memory immune responses to \( H. \) polygyrus, indicating that

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom)
adult worm expulsion was blocked in the *H. polygyrus*-challenged OX40L−/− mice. Although *H. polygyrus*-challenged OX40L+/+ and OX40L−/− mice demonstrated a significant (p < 0.05) reduction in adult worm egg production, as compared with OX40L+/+ and OX40L−/− mice given only a primary dose of *H. polygyrus*, egg production was significantly (p < 0.05) greater in *H. polygyrus*-challenged OX40L−/− mice, as compared with *H. polygyrus*-challenged OX40L+/+ mice (Fig. 1B). These findings suggested that the *H. polygyrus* memory response that developed in the OX40L-deficient mice was unable to mediate effective adult worm expulsion, while partially limiting adult worm egg production.

Previous studies have suggested that IL-4 plays an important role in the secondary host-protective response to *H. polygyrus* (20, 36, 37). To determine whether elevations in IL-4 protein levels were observed in the absence of OX40/OX40L interactions, MLNs were collected from individual mice at day 12 following challenge inoculation, and the number of IL-4-producing cells was determined by the ELISPOT assay, in which cells were cultured for 4 h without restimulation. As compared with OX40L+/+ mice inoculated with *H. polygyrus*, elevations in IL-4 protein levels were reduced (p < 0.01) in the OX40L−/− mice given either a primary or challenge dose of *H. polygyrus* (Fig. 1C). This finding was consistent with the impaired host-protective response observed in the OX40L−/− mice and suggested that OX40/OX40L interactions were required for optimal IL-4 production by both primary effector and memory effector T cells. No elevations in IFN-γ expression were detected in *H. polygyrus*-inoculated OX40L−/− mice as measured by RT-PCR (data not shown).

Elevations in total and Ag-specific serum IgE levels are reduced, while elevations in Ag-specific serum IgG1 levels and GC formation are comparable between OX40L+/+ and OX40L−/− mice primed or challenged with *H. polygyrus*

Previous studies with *H. polygyrus* have demonstrated that production of serum IgG1 and IgE is dependent on the presence of CD4+ T cells (38), while IgE, but not IgG1, is dependent on IL-4 (39, 40). Furthermore, CD4+ T cells are required for the production of IgE and IgG1 during both the primary (38) and secondary (25) immune responses to *H. polygyrus*. To determine whether the humoral immune response was impaired in the absence of OX40/OX40L interactions due to reduced levels of IL-4, total and Ag-specific serum Ig levels were measured by ELISA at day 12 following *H. polygyrus* challenge inoculation of OX40L+/+ and OX40L−/− mice. As seen in Fig. 2A, elevations in total serum IgE levels were significantly (p < 0.01) increased in *H. polygyrus*-challenged OX40L+/+ and OX40L−/− mice, as compared with OX40L+/+ and OX40L−/− mice given a primary inoculation of *H. polygyrus*. However, elevations in total serum IgE levels were significantly (p < 0.01) reduced during both the primary and secondary immune response to *H. polygyrus* in OX40L−/− mice, as compared with OX40L+/+ mice inoculated with *H. polygyrus* (Fig. 2B). Elevations in Ag-specific serum IgE levels were also significantly (p < 0.01) reduced in *H. polygyrus*-challenged OX40L+/+ mice, as compared with *H. polygyrus*-challenged OX40L−/− mice (Figs. 2B). Total serum IgG1 levels were significantly (p < 0.01) increased in *H. polygyrus*-challenged OX40L+/+ and OX40L−/− mice as compared with OX40L+/+ and OX40L−/− mice given only a primary dose of *H. polygyrus*.

**Fig. 2.** Elevations in total and Ag-specific serum IgE levels are reduced, while elevations in Ag-specific serum IgG1 levels and GC formation are comparable between OX40L+/+ and OX40L−/− mice infected with *H. polygyrus*. OX40L−/− mice infected with *H. polygyrus* as described in Fig. 1. At day 12 postchallenge, individual mice (five mice per treatment group) were bled and examined for increases in total and Ag-specific serum Ig levels. Total (A) and Ag-specific (B) serum IgE, and total (C) and Ag-specific (D) serum IgG1 levels were determined by ELISA, as described in Materials and Methods. A value of “0” represents nondetectable levels. Results are expressed as the mean and SE for each treatment group.
(Fig. 2C), while elevations in Ag-specific serum IgG1 levels were comparable between H. polygyrus-challenged OX40L+/+ and H. polygyrus-challenged OX40L−/− mice (Fig. 2D). Similar results were obtained in an independent experiment. These data suggested that, in the absence of OX40/OX40L interactions, the humoral response, particularly with respect to IL-4-dependent class-switching to IgE, was impaired during both the primary and secondary immune responses to H. polygyrus.

Previous studies have suggested that OX40/OX40L interactions may be required for the development of GCs in the peripheral lymphoid tissues during the Th2 immune response (17). To directly examine whether GC formation was impaired in OX40L−/− mice following H. polygyrus infection, MLNs were removed from individual OX40L+/+ and OX40L−/− mice at day 12 following H. polygyrus challenge inoculation, and changes in GC volume were quantitated as previously described (22, 30). As shown in Table I, GC formation was markedly increased during both the primary and secondary immune responses in H. polygyrus-inoculated OX40L+/+ mice, and was comparable to that observed in OX40L+/+ mice infected with primary or challenge doses of H. polygyrus. These findings suggested that GC formation does not require OX40L interactions during either the primary or memory in vivo Th2 immune response to H. polygyrus, although OX40/OX40L interactions are required for efficient production of total and Ag-specific serum IgE during both the primary and secondary immune responses to H. polygyrus. These data suggested that the impaired humoral immune response to H. polygyrus, in the absence of OX40L interactions, was not due to a defect in GC formation, but rather was mediated by the impaired ability of CD4+ T cells to produce IL-4, resulting in decreased total and Ag-specific IgE serum levels and impaired host protection in OX40L−/− mice. These experiments were repeated two times with similar results.

Elevations in Ag-specific DO11.10 T cell IL-4 levels are reduced in OX40L−/− mice inoculated with a combination of OVA peptide and H. polygyrus, although DO11.10 T cell expansion and cell cycle progression are comparable between OX40L+/+ and OX40L−/− mice

The previous studies demonstrated impaired production of IL-4 and IgE during both the primary and memory immune responses to H. polygyrus in OX40L-deficient mice, suggesting a preferential role for OX40L in development of IL-4-producing T cells. To directly examine the development and trafficking of Ag-specific T cells in the absence of OX40L, OVA-specific DO11.10 T cell-recipient mice were immunized intracutaneously in the ear with OVA plus H. polygyrus. This approach, where OVA is the peptide Ag and H. polygyrus acts as the adjuvant for the OVA-specific DO11.10 T cells, provides a useful model system to examine the requirements for OX40/OX40L costimulation during the development of Ag-specific T cells.

CD4+ T cells were purified from BALB/c DO11.10 TCR transgenic mice, and labeled with CFSE before transfer into BALB/c OX40L+/+ and BALB/c OX40L−/− recipient mice. Two days later, recipient OX40L+/+ and OX40L−/− mice were inoculated in the ear with OVA peptide alone, H. polygyrus alone, or a combination of OVA peptide and H. polygyrus. At day 7 after inoculation with OVA and H. polygyrus (day 9 following DO11.10 T cell transfer), all mice were sacrificed, and the draining cervical lymph node was removed for analysis. Previous studies have

### Table I. Elevations in GC formation are comparable during both the primary and secondary immune responses in H. polygyrus-inoculated OX40L+/+ mice and OX40L−/− mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OX40L+/+ (%)</th>
<th>OX40L−/− (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>&lt;5%</td>
<td>7.7 ± 1.50</td>
</tr>
<tr>
<td>’Rx, Hp1’</td>
<td>31.6 ± 4.46</td>
<td>27.2 ± 5.10</td>
</tr>
<tr>
<td>’Hp1’ plus Rx, Hp2’</td>
<td>18.4 ± 2.91</td>
<td>23.3 ± 2.71</td>
</tr>
</tbody>
</table>

* MLN GCs stained with peanut agglutinin (PNA) were quantitated volumetrically for each animal (five mice per treatment group) as described in Materials and Methods. A value of <5% represents minimal GC formation. Results are expressed as the mean and SE for each treatment group, and similar results were obtained in additional experiments.

* Control groups of OX40L+/+ and OX40L−/− mice received the initial inoculum of H. polygyrus at the time of challenge, and MLN were obtained at day 12 after primary inoculation, allowing direct comparison between the primary (Hp1’) and secondary (Hp2’) immune responses to H. polygyrus.

*’Ox40L+/+’ and OX40L−/− mice were orally infected with 200 third-stage H. polygyrus larvae which were subsequently expelled with the anthelmintic drug pyrantel pamoate, and 2-3 wk after expulsion, the mice were administered a challenge dose of H. polygyrus (Hp2’). At day 12 postchallenge, MLNs were obtained from individual mice (five mice per treatment group) and frozen in liquid nitrogen.

**FIGURE 3.** Elevations in Ag-specific DO11.10 T cell IL-4 levels, but not DO11.10 T cell expansion, are reduced in OX40L−/− mice, as compared with OX40L+/+ mice inoculated with OVA plus H. polygyrus. CD4+ T cells were purified from BALB/c DO11.10 TCR transgenic mice and labeled with CFSE before transfer to BALB/c OX40L−/− or BALB/c OX40L+/+ recipient mice. Two days later, mice were inoculated intracutaneously in the ear with OVA peptide alone, H. polygyrus alone, or a combination of OVA and H. polygyrus. At day 7 after inoculation, the draining cervical lymph node was removed from individual mice (four to five mice per treatment group) for analysis. A, Single-cell suspensions were cultured for 3 days with, or without, restimulation with OVA peptide before Ag-induced IL-4 protein levels were measured by the ELISPOT assay, as described in Materials and Methods. A value of "0" represents undetectable levels. Results are expressed as the mean and SE for each treatment group. B, Total number of DO11.10 T cells (KJ1-26+CD4+) in the draining cervical lymph node was determined by FACS analysis of single-cell suspensions of pooled treatment groups.
shown marked increases in IL-4, but not IFN-γ, mRNA in the cervical draining lymph node following intracutaneous immunization with *H. polygyrus* in the ear (data not shown). To assess IL-4 production by Ag-specific T cells, the draining cervical lymph nodes were removed from individual mice and single-cell suspensions were cultured for 3 days with or without OVA peptide restimulation; an ELISPOT assay was then performed using the cultured cells. The OVA peptide restimulation allowed measurement of IL-4 produced in response to Ag-specific stimulation. As shown in Fig. 3A, OX40L-/- and OX40L-/- mice given either OVA peptide alone or *H. polygyrus* alone demonstrated minimal IL-4 production when cultured in either medium alone or with OVA peptide. In contrast, OX40L-/+ and OX40L-/- mice inoculated with both OVA and *H. polygyrus* demonstrated a marked increase in IL-4 protein production when cultured with OVA peptide, although levels were significantly (p < 0.01) reduced in the OX40L-/- mice, as compared with the OX40L-/+ mice. These findings suggested that *H. polygyrus* was able to act as a potent adjuvant that, in combination with OVA peptide, induced a strong Ag-specific primary DO11.10 T cell IL-4 response in both OX40L-/+ and OX40L-/- mice. However, OX40/OX40L interactions were required for optimal production of Ag-specific-induced IL-4 following inoculation with OVA and *H. polygyrus*.

To determine whether the reduction in IL-4 protein production was due to a defect in Ag-specific T cell expansion in the absence of OX40/OX40L interactions, the total number of KJ1-26+CD4+ DO11.10 T cells per cervical lymph node was determined as described in Materials and Methods. DO11.10 T cell expansion was minimal in OX40L-/- and OX40L-/- mice inoculated with either OVA peptide alone or *H. polygyrus* alone (Fig. 3B). However, DO11.10 T cell expansion was markedly increased, and comparable, in OX40L-/+ and OX40L-/- mice inoculated with both OVA and *H. polygyrus* (Fig. 3B). This suggested that OX40/OX40L interactions were not required for in vivo expansion of Ag-specific T cells within the draining lymph node during the primary response. It should be further noted that the total number of CD4+ lymphocytes in OX40L-/+ and OX40L-/- mice was not significantly different in parallel treatment groups and that the percentage of DO11.10 T cells remained small (<2%; data not shown).

To examine the ability of OX40L-deficient T cells to undergo cell cycle progression, single-cell suspensions from the draining cervical lymph nodes were pooled and analyzed for DO11.10 cell CFSE fluorescence as described in Materials and Methods. As seen in Fig. 4A, OX40L-/+ and OX40L-/- mice that received only DO11.10 CFSE-labeled T cells demonstrated minimal cell cycling, with >90% of the transferred cells remaining in the parent
CXCR5 expression was measured by FACS analysis. Single-cell suspensions were pooled within treatment groups, and CFSE-labeled cells were stained with KJ1-26-TriColor and rabbit-anti-CXCR5, followed by anti-rabbit-IgG PE. CXCR5 expression was then analyzed on KJ1-26-gated CFSE+ cells; CFSE was used to follow cell cycle progression.

**FIGURE 5.** Elevations in DO11.10 T cell CXCR5 expression are comparable and cell cycle dependent in OX40L−/+ and OX40L−/− mice inoculated with a combination of OVA peptide and *H. polygyrus*. CD4+ DO11.10 T cells were labeled with CFSE and transferred to OX40L−/+ and OX40L−/− recipient mice, before inoculation with OVA and/or *H. polygyrus*, as described in Fig. 3. At day 7 after inoculation with OVA and *H. polygyrus*, draining cervical lymph nodes were harvested and CXCR5 expression was measured by FACS analysis. Single-cell suspensions were pooled within treatment groups, and CFSE-labeled cells were stained with KJ1-26-TriColor and rabbit-anti-CXCR5, followed by anti-rabbit-IgG PE. CXCR5 expression was then analyzed on KJ1-26-gated CFSE+ cells; CFSE was used to follow cell cycle progression.

**FIGURE 6.** To examine the role of OX40/OX40L interactions in the up-regulation of CXCR5 expression on Ag-specific Th2 cells, the draining cervical lymph nodes were removed from OX40L−/+ and OX40L−/− mice at day 7 postinoculation, and CXCR5 expression on KJ1-26+ CFSE+ cells was analyzed by FACS. As shown in Fig. 5, CXCR5 expression was comparable in OX40L−/+ and OX40L−/− mice inoculated with a combination of OVA and *H. polygyrus*, and furthermore, CXCR5 was expressed in a cell cycle-dependent manner. CXCR5 expression in OX40L−/+ and OX40L−/− mice that received only DO11.10 CFSE-labeled T cells was greatly reduced and similar to that observed in OX40L−/+ and OX40L−/− mice inoculated with either OVA peptide alone or *H. polygyrus* alone (Fig. 5 and data not shown). These findings suggested that Ag-specific T cells were able to up-regulate CXCR5 expression during an in vivo primary Th2 response in the absence of OX40L interactions.

To examine CD4+ T cell migration in the absence of OX40L, draining cervical lymph nodes were removed from individual mice at day 7 following inoculation with OVA peptide or *H. polygyrus*. The draining cervical lymph nodes were sectioned and stained with PE-KJ1-26 and biotinylated-anti-B220, followed by streptavidin-Alexa647. The entire surface of the lymph node was then digitally photographed as described in Materials and Methods. Consistent with the FACS analysis of CFSE staining (Fig. 4), control OX40L−/+ and OX40L−/− mice that received only DO11.10 CFSE-labeled T cells maintained a preponderance of green (CFSE) fluorescing cells, indicating minimal cell cycling (Fig. 6A and data not shown). As shown in Fig. 6B, inoculation of DO11.10 T cell-recipient OX40L−/− mice with a combination of OVA and *H. polygyrus* resulted in considerable DO11.10 T cell proliferation (loss of green CFSE staining and associated increased detection of red KJ1-26+ staining) and migration of DO11.10 T cells into the T:B cell zone of the draining cervical lymph node; it is noteworthy that only the proliferating DO11.10 T cells, which had lost CFSE fluorescence, migrated into the B cell zone of the draining lymph
FIGURE 6. Migration of activated Ag-specific T cells to the B cell zone of the draining cervical lymph node is pronounced in OX40L<sup>+/−</sup> mice inoculated with a combination of OVA peptide and <i>H. polygyrus</i>. Draining cervical lymph nodes were removed from individual DO11.10 T cell-recipient mice (four to five mice per treatment group) at day 7 after inoculation of OX40L<sup>−/−</sup> mice with OVA peptide and/or <i>H. polygyrus</i>, as described in Fig. 3. The draining cervical lymph nodes were then sectioned at 8 μm and stained with PE-anti-KJ126 (red) and biotinylated-anti-B220, followed by streptavidin-Alexa647 (blue). The surface of the entire tissue section was digitally mapped and photographed at ×20 magnification with a Leica DMRXA fluorescence microscope and individual fluorescent channels and images were merged using TIFFany3 software to create the final picture, as described in Materials and Methods. Lymph node sections were taken in the largest central region of each lymph node and are depicted proportional to their actual size. A, OX40L<sup>−/−</sup> nonimmunized mice receiving DO11.10 T cells exhibited minimal cell cycling or migration to B cell regions as evidenced by the large number of CFSE<sup>−</sup> (green) cells outside the B cell zone (blue). B, DO11.10 T cell-recipient OX40L<sup>−/−</sup> mice inoculated with both OVA peptide and <i>H. polygyrus</i> demonstrated markedly increased Ag-specific DO11.10 T cell expansion and cell cycling associated with migration of KJ1-26<sup>+</sup> (red) DO11.10 T cells into the B cell follicles (blue) of the draining cervical lymph node. Note that, as the individual cells proliferate in response to the combination of OVA and <i>H. polygyrus</i> in the OX40L<sup>−/−</sup> mice (B), the CFSE (green) is progressively diluted, unmasking the KJ1-26 Ab (red) staining. These experiments were repeated two times with similar results.

Discussion

These studies demonstrate that, during primary and secondary immune responses to <i>H. polygyrus</i> in OX40L<sup>−/−</sup> mice, elevations in both IL-4 production and total and Ag-specific serum IgE are significantly reduced, while elevations in serum IgG1 levels and GC formation are primarily intact. Memory effector T cell function, which mediates adult worm expulsion and reduced egg production, was also decreased. In additional studies, adoptively transferred DO11.10 transgenic T cells demonstrated a reduction in Ag-specific T cell IL-4 production following primary immunization of OX40L<sup>−/−</sup> mice with OVA plus <i>H. polygyrus</i>; however, surprisingly, Ag-specific T cell expansion, cell cycle progression, and migration were comparable between OX40L<sup>+/+</sup> and OX40L<sup>−/−</sup> mice primed with OVA and <i>H. polygyrus</i>. A number of studies have suggested that OX40/OX40L interactions influence T cell differentiation downstream of B7-1/B7-2 costimulation. In particular, several studies have suggested that OX40L interactions may be required for CD4<sup>+</sup> T cell up-regulation of CXCR5 and consequent migration from the T cell-rich region of the lymph node to the T:B cell zone, where T cell help leading to Ig class switching and plasma cell differentiation is initiated (7, 11, 17, 18, 41). Our studies did find pronounced decreases in serum IgE elevations and CD4<sup>+</sup> T cell IL-4 production in <i>H. polygyrus</i>-primed OX40L<sup>−/−</sup> mice, although elevations in serum IgG1 and GC formation were sustained. This is in contrast to findings in OX40L<sup>−/−</sup> mice immunized with the intestinal nematode parasite, <i>N. brasiliensis</i>, where both IL-4 production and serum IgE levels were comparable to those observed in <i>N. brasiliensis</i>-inoculated OX40L<sup>−/−</sup> mice (10). We also observed little difference in these parameters in <i>N. brasiliensis</i>-inoculated OX40L<sup>−/−</sup> mice (data not shown), indicating a difference in OX40L costimulatory molecule requirements for Th2 cell differentiation in the immune response to these two pathogens. Interestingly, the primary immune responses to these two gastrointestinal nematode parasites also differ in B7-1/B7-2 costimulatory molecule requirements: the primary immune response to <i>N. brasiliensis</i> is B7 independent (42), while the primary immune response to <i>H. polygyrus</i> is B7 dependent (22, 31). Although these parasites are both intestinal nematodes, the life cycles and Th2 immune responses to <i>N. brasiliensis</i> and <i>H. polygyrus</i> are actually quite node. OX40L<sup>+/+</sup> mice inoculated with OVA peptide and <i>H. polygyrus</i> demonstrated a similar pattern to that observed in OX40L<sup>−/−</sup> mice inoculated with OVA and <i>H. polygyrus</i>, with considerable expansion and migration of DO11.10 T cells. OX40L<sup>+/+</sup> and OX40L<sup>−/−</sup> mice inoculated with either OVA peptide alone or <i>H. polygyrus</i> alone demonstrated minimal DO11.10 T cell expansion or migration (data not shown). Thus, Ag-specific CD4<sup>+</sup> T cell migration to the B cell zone is not OX40L-dependent during the primary Th2 immune response. These experiments were performed two times, at days 7 and 12 after immunization, with similar results.
different. *N. brasiliensis* is inoculated s.c. and migrates to the lung and finally to the gut, while *H. polygyrus* is orally inoculated and is a strictly enteric parasite (19, 43, 44). Thus, additional APC populations may mediate T cell activation during the *N. brasiliensis* immune response. Furthermore, *N. brasiliensis* triggers an acute host-protective primary Th2 response while *H. polygyrus* infection results in a chronic primary Th2 response and host-protective secondary response. Our findings with *H. polygyrus* are more consistent with those of Akiba et al. (4) who showed that administration of anti-OX40L Ab partially inhibited the development of the B7-dependent Th2 immune response to *Leishmania major* in BALB/c mice, resulting in suppression of IL-4 and total serum IgE.

To address the possibility that reduced serum IgE, and perhaps also T cell IL-4 production was a consequence of impaired migration to the T:B cell zone, DO11.10 adoptively transferred T cells were examined in the context of the primary Th2 response to *H. polygyrus*. Our findings showed that, in the absence of OX40L interactions, Ag-specific Th2 cells up-regulated CXCRI and migrated to the B cell zone, indicating that this chemokine-mediated migration pathway is intact in vivo, and comparable, in *H. polygyrus*-inoculated OX40L−/− and OX40L+/+ mice during the primary response.

Other studies involving in vitro restimulation have indicated that OX40L interactions are required to sustain T effector cell survival and cell cycle progression (8, 15, 16, 45). We directly examined this possibility in vivo by comparing cell cycle progression of Ag-specific T cells inoculated with *H. polygyrus* and OVA and found no difference between OX40L−/− and OX40L+/+ mice as late as 12 days after primary immunization. There were also no differences in the total number of DO11.10 T cells in the draining cervical lymph nodes. These studies suggest that, in the context of the immune response to an infectious pathogen that triggers a Th2 response, sustained T cell survival and expansion is not impaired by an absence of OX40L interactions during the primary response. The regulation of IL-4 expression by OX40L interactions in the *H. polygyrus* system is thus not directly related to T cell cycling or expansion. Other studies have also suggested that Th2 cell development is not directly linked to cell division (46, 47). Our studies suggest that other factors are important in regulating the acquisition of competence to produce optimal IL-4, independent of cell cycle progression. One possibility is that optimal IL-4 receptor expression or signaling, as well as optimal IL-4 expression, may require OX40/OX40L interactions.

The role of OX40L interactions in the memory Th2 response during infectious disease has not previously been examined. However, Ab production was impaired during the secondary immune response to the T-dependent Ag trinitrophenyl-keyhole limpet hemocyanin when OX40/OX40L interactions were inhibited with blocking Abs administered during both the primary and secondary immune responses (48). The *H. polygyrus* memory Th2 immune response also showed impaired Ab production in OX40L−/− mice. However, only IgE was markedly reduced, while serum IgG1 levels and GC formation remained pronounced. The reduced levels of serum IgE were probably a result of the reduced IL-4 levels also detected in the memory response. Previous studies have shown that CD4+ T cell-dependent IgE, but not IgG1 elevations, are IL-4-dependent during the *H. polygyrus* immune response (39, 40). Worm expulsion and decreases in adult worm fecundity, which are dependent on Th2 cytokines and occur primarily during the memory response (19), were also impaired. This is in contrast to studies of the CD4+ T cell-dependent memory response to *H. polygyrus* in B7-1/B7-2−/− mice, where decreases in adult worm fecundity and increases in T cell IL-4 production were not reduced (27). These findings suggest a preferential role for OX40/OX40L costimulatory molecules during the Th2 memory response. However, further studies are required to specifically determine whether OX40 interactions are required for the initial development of memory cells, the maintenance of memory cells, or the activation of memory cells during challenge.

Taken together, these studies suggest an important role for OX40/OX40L interactions in specifically promoting IL-4 production, as well as associated IgE elevations and host protection, during the Th2 response to *H. polygyrus*. However, other aspects of the Th2 immune response remain intact in the absence of OX40/OX40L costimulation.

**Acknowledgments**

We thank Dr. Jozsef Czege and Ms. Karen Wolcott in the Biomedical Instrumentation Center at the Uniformed Services University of the Health Sciences for their assistance. Dr. Jozsef Czege developed the mapping and scanning software used to photograph the whole cervical lymph node sections, and Ms. Karen Wolcott provided excellent assistance with FACS analysis.

**References**


