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Systemic Dissemination and Persistence of Th2 and Type 2 Cells in Response to Infection with a Strictly Enteric Nematode Parasite

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Oral infection with the nematode parasite *Heligmosomoides polygyrus* *H. polygyrus* is entirely restricted to the small intestine. Although the evoked Th2 response has been extensively studied in secondary lymphoid organs, little is known about the systemic dissemination of Th2 cells or type 2 associated eosinophils and basophils. In this study we use bicistronic 4get IL-4 reporter mice to directly visualize the evoked Th2 response has been extensively studied in secondary lymphoid organs, little is known about the systemic dissemination as depot sites for Th2 cells, particularly cells generated in response to infection. We observed that CD4+/GFP+ Th2 cells spread systemically and found that these cells accumulated in nonlymphoid “hot spots” in the liver, the lung airways, and the peritoneal cavity. Interestingly, the total number of Th2 cells in the peritoneal cavity was comparable to those found in the draining mesenteric lymph node or the spleen. Peritoneal Th2 cells were distinguished by an exceptionally low apoptotic potential and high expression of the intestinal homing receptor α4β7 integrin. CD4+/GFP+ Th2 cells from these peripheral sites were fully functional as indicated by rapid IL-4 production upon polyclonal or Ag-specific restimulation. Th2 cells persisted in the intestinal tissue and the peritoneal cavity of drug-cured mice for weeks. The presence of peripheral memory Th2 cells in the intestine might be crucial for immunity to recall infections. These findings have important implications for the design of vaccination strategies because it may be necessary to establish and maintain memory CD4+ T cells at the potential future site of infection. The Journal of Immunology, 2005, 175: 5306–5313.

Over the past years it has become apparent that nonlymphoid tissues play important roles in type 1 immune responses mounted by infected hosts (1–3). Using MHC class I tetramer reagents, a number of groups have followed the dissemination of Ag-specific CD8+ T cells to peripheral sites after infection with systemic (4) or tissue-restricted (5, 6) pathogens. Interestingly, these groups have shown that the CD8+ effector and memory cells accumulate and are maintained in peripheral tissues, even those that were never infected. Experiments to address whether nonlymphoid tissues also serve as reservoirs for effector and memory CD4+ T cells have been more difficult to perform because multimers used to detect Ag-specific CD4+ T cells are not widely available. However, experiments in which TCR transgenic CD4+ T cells were transferred into hosts that were then infected with influenza revealed the presence of Th1 effector and memory cells in peripheral tissues (7), suggesting that at least for type 1 immune responses to viral and intracellular bacterial pathogens, nonlymphoid tissues can serve as a reservoir for Ag-specific T cells.

In contrast to CD4+ and CD8+ T cells in type 1 responses, very little is known about whether or not peripheral tissues can function as depot sites for Th2 cells, particularly cells generated in response to localized infections. Gastrointestinal nematode parasites are widely used to study type 2 immunity (8–10). However, analysis of T cells responding to pathogens of this type has been generally limited to the draining secondary lymphoid organs and the blood. This restriction is partially due to the fact that parasite Ags have not been identified, making it impossible to follow Ag-specific responses using transgenic T cells or tetramer and multimer reagents. In an attempt to overcome this limitation and track Ag-specific Th2 cells, we previously generated bicistronic 4get IL-4 reporter mice (11). IL-4 remains the signature cytokine of type 2 responses and plays a pivotal role in the initiation and maintenance of Th2 immunity by inducing its own expression in Ag-stimulated naive CD4+ T cells while concomitantly suppressing Th1 development (10). Importantly, we have shown that expression of the bicistronic GFP-IL-4 reporter is very rare in naive mice (11) and is only induced in CD4+ T cells that are activated in the presence of both Th2 polarizing conditions and TCR-mediated signals (11, 12). Therefore, CD4+/GFP+ T cells generated in vivo in response to parasite infection are likely to be specific for a cognate non-self epitope expressed by the parasite. This assumption is supported by data demonstrating that adoptively transferred CD4+/GFP+ T cells from *Nippostrongylus brasiliensis*-infected mice confer protection to T cell-deficient hosts that are otherwise susceptible to infection with this helmint (11). Therefore, we can use helmint-infected 4get IL-4 reporter mice to track polyclonal Ag-specific Th2 responses.

We (11, 13) and others (14) have previously used 4get mice to study the Th2 immune response in key lymphoid and peripheral tissues after infection with the nematode parasite *N. brasilienensis*. However, this model is not suitable to study the systemic response of Th2 cells to a localized infection because this parasite migrates systemically via the blood through multiple tissues before eventually reaching the gut (8, 9). In contrast to *N. brasilienensis*, all parasitic stages of the gastrointestinal helmint *Heligmosomoides polygyrus* (*Hp*4) from the ingested third stage larvae to the adult...
worms are entirely restricted to the small intestine (8, 9, 15). Moreover, H. pylori is a natural mouse pathogen that establishes chronic infection whereas N. brasiliensis does not naturally infect mice and even primary infections are spontaneously resolved (8, 9). Therefore, the elicited immune response to H. pylori infection reflects the co-evolution established host-parasite relationship and constitutes a natural experimental infection system.

In the present study we used the bicistronic 4get IL-4 reporter mice to analyze the systemic dissemination of Th2 cells after acute H. pylori infection. Furthermore, we followed H. pylori-specific Th2 cells in vivo during the chronic stage of infection and the formation of memory after eliminating the parasite with an anthelmintic drug. We also analyzed the appearance and dissemination of eosinophils and basophils, which are tightly associated with the immune response to H. pylori infection (8, 9). These granulocytes share the potential for IL-4 production and can in 4get mice be unambiguously identified by their GFP fluorescence in combination with characteristic surface markers (14, 16). Our data clearly show that the Th2 cells disseminate systemically upon primary infection with a tissue-restricted parasite and can persist for long periods of time in both lymphoid and nonlymphoid tissues in drug-cured mice, indicating that nonlymphoid tissues serve as reservoirs for memory Th2 cells as well as Th1 and CD8+ T cells.

Materials and Methods

Mice and parasites

4get mice (11) were backcrossed to BALB/c for 10 generations and kept under specific pathogen-free conditions in filter top cages in the animal facility of Trudeau Institute and were used at 8–12 wk of age. Infective third stage larvae of H. pylori were prepared as described (17, 18). Experimental animals were inoculated by oral gavage with 200 larvae. Crude parasite extracts were prepared from extensively washed adult worms by vigorous mechanical disruption using a tissue grinder (18, 19). H. pylori larvae were killed before homogenization by repeated freeze-thaw cycles. Both extracts were used at 20 μg/ml. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee at Trudeau Institute.

Tissue sampling and preparation

Single cell suspensions were prepared from the lymph nodes, Peyer’s patch (PP), spleen, and the bone marrow by mechanical disruption through a cell strainer. Erythrocytes were removed by ammonium chloride lysis. Peritoneal exudate cells (PEC) and pleural exudate cells (PLC) by incubation in complete RPMI 1640 (10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin) at 37°C, 5% CO2 for 2 h. Intraepithelial lymphocytes (IEL) and cells from the lamina propria (LP) were isolated from the small intestine after removal of the PP as described (21). Briefly, the small intestine was cut longitudinally, washed with CMF (Ca2+ and Mg2+ free, HBSS), subsequently stirred with complete RPMI 1640 for 30 min at 37°C, and finally briefly vortexed. This step was repeated and the IEL fractions were pooled. LP lymphocytes were released by repeated digestion with collagenase VIII (100 U/ml; Sigma-Aldrich) in complete RPMI 1640 for 60 min at 37°C. Intraepithelial lymphocytes (IEL), and LP lymphocytes were enriched in the interphase of a discontinuous 60%/40% Percoll (Amersham Biosciences) gradient spun at 1200 ×g for 20 min at room temperature. Cells that were prepared for annexin V staining were kept in complete RPMI 1640 whenever possible.

Flow cytometry

Flow cytometry reagents were purchased from Caltag Laboratories or BD Biosciences unless otherwise stated and clones are given in parentheses. All samples were first incubated with anti-CD16/32 (2.4G2) to block non-specific binding of Abs to FcγRIII/II. The following FACS reagents were used: anti-CD4 (clone RM4-5), anti-Ige (R35-72), anti-CCR3 (17B10; R&D Systems), anti-CD44 (IM7), anti-CD62 ligand (anti-CD62L, HEL-14), anti-CD9 (H1.2F3), anti-αβ integrin (DATK32), anti-CD95 (Jo2), and streptavidin-allophycocyanin. CCR7 expression was detected with a recombinant EBV-induced molecule 1-ligand chemokine, ELC-Ig fusion protein (22), and provided by S. Swain (7). Annexin V staining (BD Biosciences) and the IL-4 cytokine secretion assay (Milenyi Biotec) were performed according to the manufacturer’s instructions (23). Dead cells were identified by the addition of DAPI (4′,6′-diamidino-2-phenylindole, 0.1 μg/ml; Sigma-Aldrich) or propidium iodide (3 μg/ml; Sigma-Aldrich) and excluded from the analyses. Samples were acquired on a FACScalibur (BD Biosciences) flow cytometer, a FACSScan (BD Biosciences) flow cytometer equipped with a multicolor upgrade (Cytek Development) adding allophycocyanin and allophycocyanin-Cy7 to the fluorochrome panel or a CyAn (DakoCytomation) flow cytometer. Data were analyzed using FlowJo (Tree Star) software. Electronic compensation matrices for data acquired on the CyAn cytometer were determined using the FlowJo compensation platform based on proper single stain controls. Numbers in FACS plots indicate the percentage of cells for each specific quadrant and fractions of percentages were rounded to the nearest full digit. Mean fluorescence intensities (MFI) were only compared when samples were acquired in the same session with the same instrument settings.

Cell sorting and culture

Two weeks after H. pylori infection, adherent cells were depleted from the peritoneal cavity and mesenteric lymph node (mesLN) by plastic adherence. Nonadherent cells were subsequently stained with anti-CD3 and sorted by using a FACSvantage flow cytometer (BD Biosciences) equipped with DaVinci electronics. Where indicated, CD4+/GFP+ T cells were cultured on plates coated with anti-CD3ε (clone 145-2C11, 10 μg/ml).

RT-PCR

Total RNA was extracted by using the RNAqueous-4PCR kit (Ambion) and reverse transcribed with the Superscript II RNase H− kit (Invitrogen Life Technologies) using oligo(dt)16 priming. TaqMan reagents for GAPDH and β2-microglobulin have been described (16) and primers and probes Bc1-2 and Bax were Assays on Demand purchased from Applied Biosystems. Quantitative real-time RT-PCR was performed by using an ABI Prism 7700 Sequence BioDetector (PE Biosystems) according to the manufacturer’s instructions (TaqMan, PerkinElmer). All transcripts were analyzed in triplicates. Threshold cycle (Ct) values for GAPDH were routinely between 15 and 18 cycles, and normalization to β2-microglobulin gave similar results.

Results

Systemic dissemination of Th2 cells in response to infection with the strictly enteric parasite H. pylori

To directly visualize the IL-4 response to primary infection with H. pylori, bicistronic 4get IL-4 reporter mice (11) were orally infected with 200 third-stage larvae, and various organs were analyzed 2 wk later by FACS. Although GFP+ cells were rare in most tissues of naïve mice (11) (Fig. 1A), the frequency of CD4+/GFP+ Th2 cells was substantially increased after infection in all lymphoid organs analyzed including the draining mesLN, the spleen, PBL, and PP (Fig. 1A). A high frequency of CD4+/GFP+ Th2 cells was also observed in tissues that are clearly associated with the gastrointestinal tract including the epithelium of the intestine (IEL), the LP, and the liver, which percolates blood entering from the intestine via the portal vein (24). Surprisingly, the frequency of Th2 cells was also extremely high in some tertiary sites that have no obvious connection with the infected intestine such as the lung airways (BAL cells) and the peritoneal cavity (PEC) (Fig. 1A). The accumulation of Th2 cells in the peritoneal cavity was selective because the Th2 cells did not accumulate to a similar extent in the pleural cavity, a serosal cavity like the peritoneal cavity.
In addition to CD4+ T cells, most organs from the Hp-infected mice contained a substantial fraction of CD4+ cells that contribute to the GFP+ population. We (13, 16) and others (14) have unambiguously identified basophils (GFP+/FceRI+/c-kit+/SSC<low>) and eosinophils (GFP+/CCR3+/SSC<high>) among these CD4+/GFP+ cells. Based on these markers we analyzed the contribution of CD4+/GFP+ Th2 cells, eosinophils and basophils to the total GFP+ population (Fig. 1B). Although CD4+/GFP+ Th2 lymphocytes dominated in the secondary lymphoid organs, the LP and the BAL, basophils and eosinophils prevailed in most other tissues. Although blood eosinophilia is a hallmark of the immune response to Hp (8, 9), eosinophils were even more prominent in the liver, peritoneal cavity and pleural cavity (Fig. 1B). A substantial fraction of the GFP+ cells that could not be identified by the aforementioned markers (referred to as "other") was found only in the bone marrow, epithelium of the intestine (IEL), and LP. Many of the other cells in the intestine and the LP were identified as mast cells (GFP+/FceRI+/c-kit+/SSC<high>) (data not shown and Ref. 16). These data show that, similar to what we observed with the Th2 cells, additional IL-4 expressing effector cells including basophils and eosinophils disseminate systemically and can be found in lymphoid as well as peripheral nonlymphoid tissues outside of the infected intestine.

Despite the presence of CD4+/GFP+ Th2 cells and GFP+ non-CD4+ cells in various tissues, there were significant differences in the MFI of the GFP IL-4 reporter (Fig. 1C). For example, PP and the mesLN consistently contained the brightest CD4+/GFP+ cells, whereas CD4+ T cells in the lung airways and pleural cavity were the least bright population. Because the expression of GFP correlates positively with the production of IL-4 upon stimulation (see below) (14, 16), CD4+/GFP+ cells in nonlymphoid sites are likely to produce lower amounts of IL-4. In contrast to CD4+ T lymphocytes, which showed substantial heterogeneity in GFP expression even within a given tissue, non-CD4+ cells were very homogeneous in GFP fluorescence (Fig. 1A and Ref. 16). Nonetheless, these cells also showed varying levels of GFP fluorescence in different tissues, most pronounced in the eosinophil population (Fig. 1C).

Taken together, these data show that IL-4-expressing Th2 cells are efficiently generated in response to Hp infection and accumulate at high frequencies in peripheral nonlymphoid tissues that are not sites of Hp infection.

The peritoneal cavity is a major site of type 2 immunity

Hp-infected 4get mice consistently revealed extremely high frequencies of GFP+ cells in the peritoneal cavity (23 ± 2%), the liver (28 ± 7%) and BAL (49 ± 5%) (Fig. 1A). Because the total number of recovered cells from the peritoneal cavity was consistently >10-fold the number isolated from the liver or pleural cavity and >1000-fold higher than the number found in BAL (data not shown), the largest number of GFP+ cells of all the peripheral tissues is found in the peritoneal cavity. In fact, the peritoneal cavity of infected mice contained ~30 times more GFP+ cells than the peritoneal cavity of naive mice (data not shown) and the total number of GFP+ cells present in the peritoneal cavity of infected mice was comparable to that found in secondary lymphoid organs such as the spleen and mesLN (data not shown), suggesting that the peritoneal cavity is a major site for the accumulation of IL-4-expressing effector cells. In agreement with this finding, we observed that the frequency of GFP+ Th2 cells within the CD4+ population was 62 ± 6% in the peritoneal cavity, 69 ± 7% in the liver, and 72 ± 8% in BAL. The number of CD4+/GFP+ Th2 cells in the peritoneal cavity increased ~60-fold after infection and was even greater than the increase in the number of cells observed in the mesLN or spleen (Fig. 2). Likewise, the total number of CD4+/GFP Th2 cells in infected mice was similar to that found in the mesLN or spleen. Thus, these data demonstrate that the peritoneal
cavity is a major peripheral site for the accumulation of Th2 cells responding to infection with Hp.

Peritoneal Th2 cells have a distinct phenotype

Conventional methods used to identify cytokine-expressing cells at the single cell level require in vitro restimulation (23, 25, 26), which alters the expression patterns of surface Ags (27, 28) and renders effector T cells susceptible to activation-induced cell death (AICD) (29, 30), thereby precluding phenotypic characterization of these cells directly ex vivo. However, using the 4get reporter mice, we can detect and phenotype any IL-4-expressing cell directly ex vivo without further restimulation (11, 13). Therefore, we performed phenotypic analysis to determine whether CD4+CD45R+Th2 cells found in the peritoneal cavity of Hp-infected mice can be distinguished from the Th2 cells present in secondary lymphoid tissues. As expected (11), CD4+CD45R+Th2 cells isolated from all tissues 2 wk postinfection presented an activated surface phenotype with high CD44 expression (data not shown). In contrast, as shown in Fig. 3A, the frequency of CD4+CD45R+Th2 cells expressing the acute activation marker CD69, which is rapidly induced by TCR stimulation (28), was consistently lower in the peritoneal cavity compared with the draining mesLN and the liver, which accumulates activated and apoptotic T cells (20, 24).

The intestine is the only infected tissue after oral challenge with Hp (8, 9, 15) and is the major peripheral site where effector Th2 cells would be expected to accumulate. Because the combinatorial αβ7 integrin has a key role in the homing of lymphocytes to the intestine (31), we analyzed the surface expression of αβ7 on CD4+ T cells. As expected (31), CD4+CD45R+Th2 cells in the LP and in PBL expressed substantial levels of αβ7 (Fig. 3B) but were αβ7− in the lung airways. Unexpectedly, the frequency of CD4+CD45R+Th2 cells expressing αβ7 was the highest in the peritoneal cavity (Fig. 3B). The preferential accumulation of GFP+ Th2 cells in the peritoneal cavity could reflect the different tissue homing properties of effector memory T cells versus central memory T cells (32). Effector memory T cells express low levels of CD62L and CCR7 and home preferentially to nonlymphoid tissues, whereas central memory T cells express high levels of CD62L and CCR7 and migrate through secondary lymphoid tissues. Therefore we analyzed the expression of these molecules on CD4+ T cells in the mesLN and the peritoneal cavity. However, as shown in Fig. 3C, there was no apparent difference in the expression of these markers on CD4+GFP+ cells suggesting that the peritoneal cavity does not selectively accumulate effector memory T cells.

Together, these data show that CD4+GFP+ Th2 cells in the peritoneal cavity can be distinguished from Th2 cells in secondary lymphoid tissues and from other peripheral sites because they show a low level of acute activation and are highly enriched for the expression of the αβ7 integrin, a molecule required for intestinal homing.

Peritoneal Th2 cells have a low apoptotic potential

Because CD4+CD45R+ Th2 cells with an activated (CD44high, CD62Llow) phenotype were present in all tissues and activated effector T cells are more susceptible to apoptosis (29), we determined the apoptotic potential of CD4+ T cells in various organs by staining with Annexin V for the presence of phosphatidylserine on the cell surface (33) (Fig. 4A). CD4+CD45R+Th2 cells in the peritoneal cavity and the BAL consistently had the lowest frequency of Annexin V staining whereas the highest frequency of apoptotic Th2 cells was found in the liver. These data suggested that peritoneal CD4+CD45R+Th2 cells have a low apoptotic potential. In agreement with this assumption peritoneal CD4+CD45R+Th2 cells expressed low levels of the death receptor CD95 (Fig. 4B) and had an increased transcript abundance of the antiapoptotic factor Bcl-2 vs the proapoptotic factor Bax (Fig. 4C) as compared with mesenteric Th2 cells (34, 35). To directly challenge the sensitivity of peritoneal and mesenteric CD4+CD45R+Th2 cells to AICD the respective populations were sorted and stimulated with plate-bound anti-CD3. As shown in Fig. 4D, there was less cell death in the cultures of peritoneal cells after 24 and 48 h which was reflected in the substantially increased number of viable cells (Fig. 4E). Furthermore, all peritoneal CD4+CD45R+Th2 cells up-regulated the expression of IL-4-GFP within 24 h, whereas only a small fraction of mesenteric Th2 cells increased in brightness, potentially reflecting a responsive subset (Fig. 4F). This interpretation is supported by the fact that GFP fluorescence between the cultures was similar after 48 h, while the total cell number was substantially lower in mesLN cultures (Fig. 4E).

Thus, peritoneal Th2 cells have low apoptotic potential, are less susceptible to AICD and rapidly increase IL-4 expression upon stimulation.
Rapid IL-4 production by GFP⁺ Th2 cells in lymphoid and nonlymphoid sites

Next we analyzed the potential of individual CD4⁺ T cells from various organs to rapidly secrete IL-4 upon stimulation. As shown in Fig. 5A, the secretion of IL-4 by CD4⁺ T cells from all tissues was minimal in the absence of stimulation. However, CD4⁺/GFP⁺ Th2 cells from all analyzed organs rapidly secreted IL-4 upon polyclonal stimulation with PMA plus ionomycin (Fig. 5A) or anti-CD3 (data not shown). The production of IL-4 was restricted to GFP⁺ cells and correlated positively with the brightness of GFP fluorescence (Fig. 5A). Thus, the GFP⁺/CD4⁺ T cells found in peripheral sites such as the peritoneal cavity are functional and capable of rapidly producing IL-4 upon restimulation.

In contrast to many type 1 pathogens, the antigenic epitopes of most type 2 pathogens such as gastrointestinal worms have not yet been identified. To demonstrate the presence of Ag-specific T cells in the peritoneal CD4⁺/GFP⁺ population, we stimulated peritoneal cells, which contain large numbers of MHC class II-bearing macrophages and B cells (data not shown), with crude parasite extracts (18, 19) and analyzed whether the GFP⁺/CD4⁺ Th2 cells secreted IL-4. Crude extracts prepared from both adult worms or larvae induced the secretion of IL-4 by CD4⁺/GFP⁺ Th2 cells from the PEC, whereas cytokine production was minimal without the addition of extracts (Fig. 5B). Similar to what we observed after polyclonal restimulation with PMA plus ionomycin or anti-CD3, the production of IL-4 by CD4⁺ T cells restimulated with Hp Ags was restricted to GFP⁺ cells and correlated positively with the GFP brightness. Taken together, these data show that CD4⁺/GFP⁺ Th2 cells isolated from various lymphoid or nonlymphoid tissues rapidly produce IL-4 after either polyclonal restimulation or Hp-specific restimulation, indicating that Ag-specific CD4⁺ Th2 cells accumulate in lymphoid and nonlymphoid tissues.

Th2 cells persist in drug-cured mice

Although primary infections with Hp are chronic, drug-cured mice are immune to challenge inocula (8, 9). Because immunity is dependent on both CD4⁺ T cells and IL-4 (9, 17, 36), we analyzed the persistence of CD4⁺/GFP⁺ Th2 cells in various organs of animals that were cured 4 wk earlier and compared them with chronically infected mice. As shown in Fig. 6A, CD4⁺/GFP⁺ Th2 cells were found at high frequencies in lymphoid and nonlymphoid tissues of chronically infected as well as drug-cured mice. The frequency of GFP⁺ Th2 cells within the CD4⁺ population was reduced in some tissues of drug-cured mice including the mesLN and PBL (Fig. 6A). However, the frequency and total number of CD4⁺/GFP⁺ Th2 cells in nonlymphoid tissues including the LP, BAL, and the peritoneal cavity was comparable between the infected and “memory” mice (Fig. 6, A and B and data not shown). A similar pattern was apparent with respect to CD4⁺/GFP⁺ cells that were less abundant in PBL, bone marrow, and the liver of the drug-cured mice but persisted at equal frequencies in the pleural and peritoneal cavities (Fig. 6A). Despite the reduced frequency and number of CD4⁺/GFP⁺ Th2 cells and CD4⁺/GFP⁺ cells in...
some tissues of drug-cured mice, both populations were still far more abundant than in naive animals (Figs. 1A, 2, and 6, A and B). The vast majority of CD4+/GFP+ cells were even several weeks after the drug-mediated elimination of the worm in all examined tissues CD62Llow (Fig. 6C and data not shown). In contrast, mesenteric and peritoneal CD4+/GFP+ cells were heterogeneous in their expression of CCR7 and can therefore not be categorized into effector memory or central memory T cells (32). Unexpectedly, peritoneal CD4+/GFP+ cells stained brighter for CCR7 despite their presence in a tertiary site. The CD4+/GFP+ Th2 cells that were present in the mesLN or the peritoneal cavity of drug-cured mice were fully functional as demonstrated by the rapid secretion of IL-4 upon stimulation (Fig. 6D). Interestingly, peritoneal Th2 cells secreted IL-4 more efficiently than lymphocytes isolated from the mesLN of the same animals.

Thus, CD4+/GFP+ Th2 cells persist in lymphoid and nonlymphoid tissues of drug-cured mice for weeks after the adult worm is eliminated. These cells appear to be functional memory cells as they are competent to produce IL-4 within hours of stimulation. Therefore, these data suggest that Th2 cells are able to migrate and accumulate in peripheral sites that were never infected and can persist in those sites as for prolonged periods of time.

**Discussion**

In the present study we show that Ag-specific Th2 cells disseminate systemically in response to the strictly enteric infection with the helminth parasite *Hp*. As expected (8, 9, 11), primary infection with *Hp* resulted in high frequencies of CD4+/GFP+ Th2 cells in the secondary lymphoid organs draining the site of infection. However, we also found that Th2 cells preferentially accumulate in some peripheral “hot spots” such as the liver, the lung airways, and the peritoneal cavity, which are sites that do not have an obvious association with the infected organ. In fact, the total number of Th2 cells found in the peritoneal cavity of *Hp*-infected mice was comparable to that observed in the draining mesLN or the spleen. Although CD4+/GFP+ T cells were abundant in the peritoneal cavity, these T cells, as well as those found in the liver, PLC, and BAL expressed lower levels of GFP compared with the T cells found in secondary lymphoid tissues and effector sites like the IEL and LP. Because the level of GFP fluorescence correlates positively with the secretion of IL-4 (14, 16) (Figs. 5, A and B, and 6D), the Th2 cells found in the peripheral tissues most likely have a lower potential to secrete IL-4 compared with the Th2 cells present in the draining lymph nodes and effector site. Thus, although Th2 cells can accumulate in peripheral sites, these T cells appear to be phenotypically and perhaps functionally distinct from those found in lymphoid tissues and in the infected effector site.

Although the Th2 cells accumulate with the highest efficiency in the BAL (Fig. 1A), the total number of T cells found in this site is quite low, particularly when compared with other peripheral sites such as the liver or peritoneal cavity. At present we do not know why Th2 cells accumulate with such a high frequency in the BAL, however, we speculate that the recruitment is not Ag-specific (37) and likely represents irreversible recruitment rather than local expansion, because CD4+ T cells do not divide in the lung airways or return to secondary lymph organs (38).

In contrast, the liver may represent a nonlymphoid site for generating and/or accumulating Th2 effectors. In fact, it has previously...
been reported that Th2 cells can be found in the livers of N. brasiliensis-infected mice (39). However, in contrast to Hp, N. brasiliensis migrates systemically via the blood from the s.c. site of infection before eventually entering the intestine (8, 9, 11). Therefore, one might expect to find a type 2 response in the liver of N. brasiliensis-infected mice. Even though the liver is never infected by Hp, the accumulation of Th2 cells in the liver is selective because the frequency of CD4\(^+\)/GFP\(^+\) T cells was higher than that observed in the circulating blood. It is conceivable that the liver, which percolates blood entering from the intestine via the portal vein, could play an important role in Ag presentation and T cell activation (40) as indicated by the high frequency of GFP\(^+\)/CD4\(^+\) cells expressing CD69 (Fig. 3A). Alternatively the liver might be important for the elimination of apoptotic effector cells as indicated by the high frequency of Annexin V\(^+\) cells (Fig. 4A) (24). In either case, the liver appears to be a site where activated effector Th2 cells accumulate.

Although the liver has an obvious functional association with the infected intestine (24), we were surprised by the striking accumulation of CD4\(^+\)/GFP\(^+\) Th2 cells and eosinophils in the peritoneal cavity (Figs. 1, A and B, 2, and 6, A and B). The general cellular influx into the peritoneal cavity of Hp-infected mice has previously been described (41); however, the selective accumulation of Th2 cells and eosinophils has not been reported. The peritoneal CD4\(^+\)/GFP\(^+\) Th2 cells express high levels of the combinatorial \(\alpha_\beta_7\) integrin (Fig. 3B) required for intestinal homing (31) and therefore might be competent to transmigrate toward the site of infection. Interestingly, peritoneal eosinophils, but not blood-borne eosinophils, also express high levels of \(\alpha_\beta_7\) integrin (data not shown), suggesting that these cells may also be able to home to the infected tissue. Peritoneal Th2 cells could also be distinguished from the Th2 cells found in lymphoid tissues and the effector site because they did not appear to be recently activated by Ag (reduced frequency of CD69\(^+\) cells (Fig. 3A) and displayed an extremely low apoptotic potential (Fig. 4). Thus, the peritoneal cavity Th2 cells resemble memory or “effector-memory” type cells that persist for extended periods of time.

Indeed, one of the most striking findings of this study was that Th2 cells persisted at high frequencies for weeks in lymphoid and nonlymphoid tissues even when the intestinal parasite was completely eliminated by antihelminthic treatment (Fig. 6, A and B). The elimination of the gastrointestinal parasite did result in reduced frequencies of GFP\(^+\) cells in the blood, the liver, and the bone marrow. However, drug-cured mice had substantially higher frequencies and numbers of GFP\(^+\) cells in all tissues when compared with naive animals (Figs. 2 and 6B). Indeed, the frequencies of CD4\(^+\)/GFP\(^+\) Th2 cells in drug-cured mice were similar in most tissues to those found in chronically infected animals (Fig. 6A). Persistent CD4\(^+\)/GFP\(^+\) cells, especially those located at the former site of infection, might be important to confer immunity to rechallenge infections (8, 9). Morimoto et al. (42) have recently demonstrated that CD4\(^+\) T cells with a type 2 cytokine profile rapidly accumulate in a protective memory responses at the host: parasite interface. As we show, CD4\(^+\)/GFP\(^+\) Th2 cells persist in the intestine of drug-cured mice (Fig. 6A) and therefore are exquisitely positioned to accumulate rapidly at the sites of larval penetration upon rechallenge. The large numbers of CD4\(^+\)/GFP\(^+\) Th2 cells and eosinophils in the peritoneal cavity that express high levels of the \(\alpha_\beta_7\) integrin (Figs. 3B, and 6, A and B) required for intestinal homing might provide an additional reservoir of memory cells that can be quickly mobilized and recruited into the intestine. Alternatively, some of the persistent CD4\(^+\)/GFP\(^+\) Th2 cells might preferentially home to draining lymphoid tissues due to their high expression of CCR7 (Fig. 6C) where they promote a recall Th2 response by the local production of IL-4. In fact, the transfer of peritoneal exudates cells from Hp immune mice to naive recipients has been shown to confer partial protection (43).

Our findings have important implications for the design of immunization strategies because the localization of Th memory cells at the potential future site of challenge might be crucial to achieve optimal immunity. Indeed, although oral infection with Hp results in a high level of protection against challenge inocula (17, 36), the i.v. transfer of lymphocytes from immune animals into naive recipients results in low or no protection (43–45). Even the ectopic i.v., s.c., or i.p. immunization of mice with Hp does not result in efficient immunity within the same animal (46) (K. Mohrs and M. Mohrs, unpublished observation). These observations suggest that the mere presence of Ag-specific memory cells in an immunized animal is not sufficient for optimal immunity but that these cells must be recruited and maintained at peripheral sites, most likely the site of infection. Therefore it might be critical for immunization protocols to establish or recruit memory cells into the potential site of infection to establish maximal protection. Our data support this concept for mucosal type 2 immunity in the intestine but it is likely to be also relevant for type 1 immunity and other mucosal tissues including the lung (47).

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Disclosures

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References


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