Immunity, Vol. 23, 419–429, October, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.immuni.2005.09.006

A Two-Step Process for Cytokine Production Revealed by IL-4 Dual-Reporter Mice

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Summary

To monitor IL-4 expression at the single-cell level, we generated mice with insertions of different reporter genes into both copies of the *Il4* gene that permitted the simultaneous analysis of IL-4 transcripts via GFP and IL-4 protein secretion by use of huCD2. Innate and adaptive cells competent for IL-4 production were marked by GFP, while cells that presently or recently secreted IL-4 additionally displayed huCD2. After challenge with the strictly enteric helminth, *Heligmosomoides polygyrus*, GFP-positive innate and adaptive cells disseminated widely, but IL-4 secretion was predominantly mediated by CD4$^+$ T cells in the intestines and draining lymphoid organs. IL-4-competent cells persisted in cured animals, and memory responses reflected rapid cytokine production at the site of rechallenge. These data reveal a two-step process for cytokine production: the first generating poised cells that disseminate systemically and the second inducing the rapid production of the cytokine in response to local stimulation.

Introduction

Interleukin (IL)-4 is the signature cytokine of type 2 immunity and remains the canonical marker for Th2 cells (Abbas et al., 1996). Other sources of IL-4 include NK T cells, basophils, mast cells, and eosinophils (Brown and Hural, 1997). IL-4 is often produced in concert with IL-5 and IL-13 located in a contiguous cytokine gene cluster (Abbas et al., 1996; Ansel et al., 2003; Mohrs et al., 2001a). These cytokines are associated with the immunopathologies of allergy and asthma but are also required for immunity to parasitic infections (Finkelman et al., 1997; Kinet, 2002; Wills-Karp, 1999).

One paradigm example of IL-4-mediated type 2 immunity is the experimental infection with the strictly enteric nematode, *Heligmosomoides polygyrus* (*Hp*). Mice on a variety of inbred backgrounds are susceptible and develop persistent infection (Finkelman et al., 1997; Gause et al., 2003). After drug cure, however, rechallenged animals mount an accelerated and enhanced memory response, which results in immunity dependent on both CD4$^+$ T cells and IL-4 (Urban et al., 1991a, 1991b). Indeed, CD4$^+$ T cells with a type 2 cytokine mRNA profile accumulate early at the host:parasite interface in a protective memory response (Morimoto et al., 2004). Collectively, these observations suggest that the rapid production of IL-4 by CD4$^+$ Th2 memory cells at the peripheral site of infection is critical for immunity.

Despite the importance of IL-4 for type 2 immunity and immunopathology, it has been extremely difficult to identify IL-4-expressing cells in vivo, because cytokines are rapidly secreted. We previously generated bicistronic IL-4 reporter mice (4get) by the targeted addition of an IRES-GFP element to the 3′ untranslated region of the endogenous *Il4* gene (Mohrs et al., 2001b). The 4get reporter is faithfully expressed under IL-4-inducing conditions, such as the activation of naive CD4$^+$ T cells under Th2 polarizing conditions or the accumulation of IL-4-expressing innate and Th2 cells after infection with nematode parasites (Gessner et al., 2005; Mohrs et al., 2001b; Shinkai et al., 2002; Voehringer et al., 2004). However, it was puzzling that GFP fluorescence was maintained independently of stimulation, whereas the production of IL-4 protein, although restricted to GFP$^+$ cells, was observed only upon stimulation (Gessner et al., 2005; Mohrs et al., 2001b; Voehringer et al., 2004). Other IL-4 "knockin" reporter that were generated by IL-4 replacement rather than bicistronic addition (Ho et al., 1999; Hu-Li et al., 2001; Rivière et al., 1998) were expressed only upon stimulation, like IL-4 protein production. Intriguingly, the persistent GFP expression in 4get cells correlated with the presence of high IL-4 mRNA levels in Th2 cells days after the initial stimulation, even when IL-4 protein production was not detected (Gessner et al., 2005; Grogan et al., 2001). This striking correlation suggested that the particular configuration of the bicistronic 4get reporter links GFP fluorescence to *Il4* gene expression and the presence of IL-4 transcripts, whereas IL-4-substituting reporters mirror IL-4 protein production. This assumption is further supported by data showing that resting innate IL-4 producers such as NK T cells, basophils, eosinophils, and mast cells, which are constitutively GFP$^+$ in 4get mice, also contain IL-4 mRNA, but do not produce IL-4 protein unless stimulated (Gessner et al., 2005; Stetson et al., 2003; Voehringer et al., 2004).

To investigate this further, we generated IL-4 reporter mice, designated KN2 (knockin huCD2), by replacing the first two exons of IL-4 with a huCD2-encoding sequence. Heterozygous 4get/KN2 mice allowed us to visualize the expression of both reporters simultaneously in single cells while IL-4 protein production is preserved on the bicistronic 4get allele (Mohrs et al., 2001b). Using these IL-4 dual-reporter mice, we show that CD4$^+$/GFP$^+$ Th2 cells disseminate and persist widely in response to infection with the strictly enteric parasite, *H. polygyrus*. In contrast, IL-4 protein-producing Th2 cells are restricted to sites of antigen accumulation, such as the draining lymphoid organs and the intestinal tissue. Our data reveal that the production of IL-4 oc-

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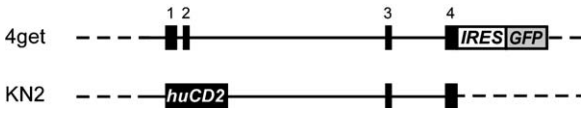

Figure 1. Schematic of 4get/KN2 IL-4 Dual-Reporter Mice

In 4get/KN2 IL-4 dual-reporter mice, one *Il4* allele is marked by the addition of the bicistronic IRES-GFP reporter (4get). The first two exons of the other allele are replaced with the huCD2 reporter (KN2) cassette. 4get/KN2 mice have a functional IL-4 copy as part of the bicistronic 4get allele. Filled boxes with numbers indicate exons. See Figure S1 for details.

curs in all analyzed IL-4 producers in two distinct, highly regulated steps.

Results

Generation of IL-4 Dual-Reporter Mice

The KN2 mice were generated by introducing a cDNA encoding human CD2 reporter protein into the endogenous *Il4* locus, thus disrupting IL-4 but leaving all known regulatory elements intact (see Figure S1 in the Supplemental Data available with this article online; Ansel et al., 2003, 2004; Lee et al., 2003; Mohrs et al., 2001a). As expected, cells from homozygous KN2 mice failed to produce IL-4 under conditions where IL-4 could be readily measured from heterozygous mice (Figure S1). The KN2 mice are similar to previously generated huCD2/IL-4 reporter mice (Rivière et al., 1998), except that the neomycin selection cassette was deleted in the KN2 line (Figure S1). Homozygous KN2 mice were interbred with 4get mice to generate heterozygous 4get/KN2 dual-reporter mice (Figure 1). The resulting mice have a functional copy of IL-4, as part of the bicistronic IL-4-GFP transcript (Mohrs et al., 2001b), and a replacement of the other *Il4* allele with the huCD2 reporter.

huCD2 Expression Is a Faithful Reporter for IL-4 Protein Secretion

CD4$^+$ T cells from naive 4get/KN2 mice were stimulated with anti-CD3 in vitro under Th1 (IL-12 plus anti-IL-4) or Th2 (IL-4 plus anti-IFN-γ) polarizing conditions in the presence of irradiated APCs. As expected (Mohrs et al., 2001b, 2003), after 6 days, the vast majority of Th2-primed cells expressed GFP, even in the absence of stimulation beyond priming on day 0 (Figure 2A). However, these cells neither secreted IL-4 nor did they express the huCD2 reporter. This is consistent with numerous publications showing that resting Th2 cells do not produce IL-4 protein or express an IL-4-replacing reporter (Ho et al., 1999; Min et al., 2004; Rivière et al., 1998). In contrast, when resting Th2 cells were stimulated with plate bound anti-CD3/CD28, they secreted IL-4 protein, expressed huCD2, and increased in GFP fluorescence (Figure 2A). Both IL-4 production and huCD2 expression were restricted to GFP$^+$ cells and correlated positively with the GFP brightness. Thus, the level of huCD2 expression reflects the amount of IL-4 protein secretion. Th1-polarized CD4$^+$ T cells did not express GFP (Mohrs et al., 2001b), huCD2, or IL-4, even

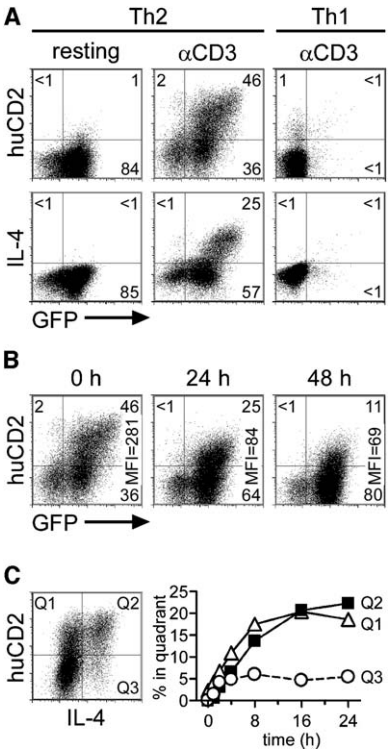

Figure 2. huCD2 Expression Faithfully Reflects IL-4 Protein Secretion

CD4$^+$ T cells were purified from naive 4get/KN2 mice and stimulated under Th2 (IL-4 + anti-IFN-γ) or Th1 (IL-12 + anti-IL-4) polarizing conditions.

(A) After 6 days, these cultures were transferred to uncoated (resting) or anti-CD3ε-coated (αCD3) wells and analyzed the next day for huCD2 expression (top) or IL-4 secretion (bottom) via a cytokine secretion assay.

(B) The plate bound anti-CD3ε stimulation was abruptly terminated by transfer to fresh, uncoated wells (0 hr), and the cells were analyzed 24 and 48 hr later. Vertical numbers indicate the MFI of GFP$^+$/huCD2$^+$ population.

(C) Th2 cultures were generated and restimulated as in (A) and (B) for the indicated periods of time (right). huCD2 expression and IL-4 secretion were determined as in (A). Representative FACS plot depicts the 8 hr time point.

after restimulation. To determine the half-life of the huCD2 reporter on the cell surface, the TCR-mediated stimulation was abruptly terminated by transferring the cells (Figure 2B; 0 hr) to uncoated wells. Although the cells remained GFP$^+$ (Figure 2B), both the frequency and the MFI_{huCD2} of huCD2$^+$ cells decreased rapidly. The decline of both parameters was paralleled by decreased GFP fluorescence. Based on both the frequency and the expression levels, we determined the half-life of surface huCD2 to be approximately 24 hr. Upon restimulation of resting Th2 cells, the vast majority of IL-4-secreting cells also expressed surface huCD2 at all time points analyzed (Figure 2C). Of note, the 24 hr half-life of huCD2 leaves a footprint on all cells that have been producing IL-4 at any period during the culture, while the cytokine secretion assay only marks cells that were actively secreting IL-4 during the last 45 min. Thus, the huCD2 reporter reflects IL-4 protein

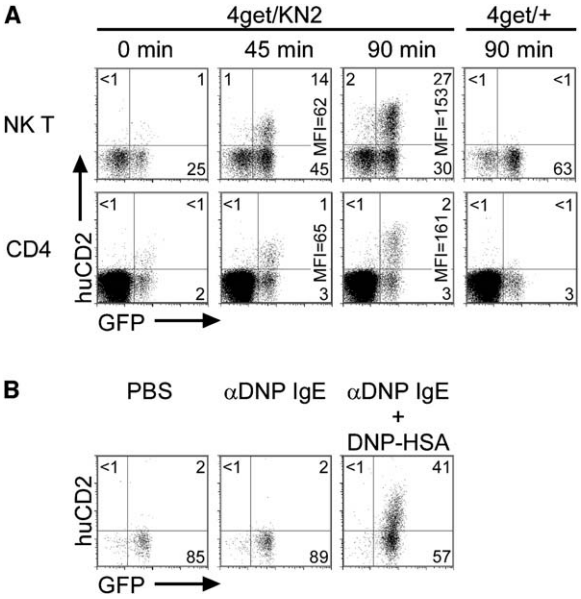

Figure 3. huCD2 Expression Reflects IL-4 Protein Secretion by Lymphoid and Nonlymphoid Cells In Vivo

(A) Naive 4get/KN2 mice or 4get/+ littermate controls were injected i.v. with either PBS (0 min) or anti-CD3ε. Splenic NK T cells (NK1.1$^+$/CD4$^+$) or conventional (NK1.1$^-$/CD4$^+$) CD4$^+$ T cells were analyzed 45 and 90 min later for the expression of GFP and huCD2.

(B) Naive 4get/KN2 mice were injected with either PBS or anti-DNP mouse IgE. 24 hr later, the sensitized animals were injected i.p. with either PBS or DNP_{30-40}-HSA. Basophils (FcεRI$^+$/CD4$^-$/SSC^{lo}) in the livers of all groups were analyzed 2 hr later for the expression of GFP and huCD2.

secretion with a half-life of 24 hr, while expression of the bicistronic IL-4-GFP reporter indicates the competence for rapid IL-4 production.

huCD2 Expression Reflects IL-4 Protein Secretion by Lymphoid and Nonlymphoid Cells In Vivo

NK T cells, which are constitutively GFP$^+$ in untreated 4get mice, but do not spontaneously secrete IL-4 protein, rapidly produce IL-4 upon injection of anti-CD3 (Min et al., 2004; Stetson et al., 2003; Yoshimoto and Paul, 1994). To analyze the expression of huCD2 on CD4$^+$/NK1.1$^+$ NK T cells, we crossed KN2 BALB/c mice to NK1.1-bearing C57BL/6 4get mice. Splenic CD4$^+$/NK1.1$^+$/GFP$^+$ NK T cells in naive mice did not express huCD2 (Figure 3A). Within 45 min of injection of anti-CD3, NK T cells expressed huCD2, and both the frequency and the MFI_{huCD2} increased between 45 and 90 min. A low frequency of conventional CD4$^+$ T cells (CD4$^+$/NK1.1$^-$) was also GFP$^+$ in the spleen of untreated mice (Figure 3A). These cells were predominantly huCD2$^-$ but rapidly upregulated huCD2 upon injection of anti-CD3. Similar to NK T cells, the frequency and the MFI_{huCD2} of huCD2 expression increased between 45 and 90 min after injection. The expression of huCD2 by NK T and conventional CD4$^+$ T cells was restricted to GFP$^+$ cells. No huCD2 staining was detected in 4get/+ littermate controls despite a similar frequency of GFP$^+$ cells.

Basophils express the high-affinity IgE receptor (FcεRI) and are a major source of IL-4 produced by non-B, non-T cells (Gessner et al., 2005; Khodoun et al., 2004; Min et al., 2004; Seder et al., 1991; Voehringer et al., 2004). To study the expression of huCD2 by basophils in vivo, we sensitized naive 4get/KN2 mice and 4get/+ controls by injection of mouse anti-DNP IgE. FcεRI-bearing cells were then activated by injection of the DNP_{30-40}-HSA antigen (Dombrowicz et al., 1993). Basophils in the livers of untreated mice were almost exclusively GFP$^+$ (Gessner et al., 2005) but did not express huCD2 (Figure 3B). Upon sensitization, basophils upregulated FcεRI expression as expected (Yamaguchi et al., 1997), but remained huCD2$^-$ (Figure 3B and data not shown). Injection of the antigen induced huCD2 expression within 1 hr (data not shown), and by 4 hr almost half of the basophils in the liver were huCD2$^+$ (Figure 3B). These data show that the IL-4-replacing huCD2 reporter is rapidly expressed on conventional CD4$^+$/GFP$^+$ T cells, NK T cells, and nonlymphoid IL-4 producers under conditions known to induce IL-4 protein production in vivo.

GFP and huCD2 Expression in Response to Infection with *H. polygyrus*

We next analyzed the IL-4 response to infection with the strictly enteric nematode, *H. polygyrus* (Finkelman et al., 1997). As expected (Mohrs et al., 2001b), naive mice had a very low frequency of GFP$^+$ cells within the CD4$^+$ population and huCD2 expression was minimal in all tissues analyzed (Figure 3A and data not shown). Two weeks after infection, high frequencies of CD4$^+$/GFP$^+$ Th2 cells were found in the draining mesLN and all other tissues analyzed (Figure 4A). Despite the wide dissemination of CD4$^+$/GFP$^+$ Th2 cells, the expression of huCD2 was largely restricted to the draining mesLN (44% of CD4$^+$/GFP$^+$), PP (49% of CD4$^+$/GFP$^+$), IEL (52% of CD4$^+$/GFP$^+$), and LP (16% of CD4$^+$/GFP$^+$). In contrast, less than 5% of CD4$^+$/GFP$^+$ cells were huCD2$^+$ in the liver, PEC, and BAL, although these tissues were routinely among those with the highest frequencies of GFP$^+$ cells within the CD4$^+$ population (Figure 4A).

In addition to CD4$^+$ T cells, a substantial fraction of non-CD4 cells contributed to the GFP$^+$ population in various tissues including the liver, spleen, PBL, and PEC (Gessner et al., 2005). We have previously identified these constitutively GFP$^+$ cells as basophils and eosinophils (Gessner et al., 2005; Voehringer et al., 2004). Despite their abundance and robust GFP fluorescence, huCD2$^+$ basophils and eosinophils were in the liver (Figure 4B), spleen, blood, or the PEC (data not shown) of *Hp*-infected 4get/KN2 mice infrequently.

To test whether the lack of huCD2 expression was due to a general inability to express the reporter or to cell-intrinsic nonresponsiveness, blood-borne cells, which are almost exclusively huCD2$^-$ ex vivo (Figure 4A and Figure S2), were cultured for 4 hr in the absence or presence of various stimuli. The activation of basophils either with anti-IgE, with IL-3, plus IL-18 (Yoshimoto et al., 1999), or with ionomycin induced robust huCD2 expression (Figure S2). Similarly, blood-borne CD4$^+$/GFP$^+$/huCD2$^-$ T cells (Figure 4A and Figure S2) expressed huCD2 after stimulation with anti-CD3. Unexpectedly,

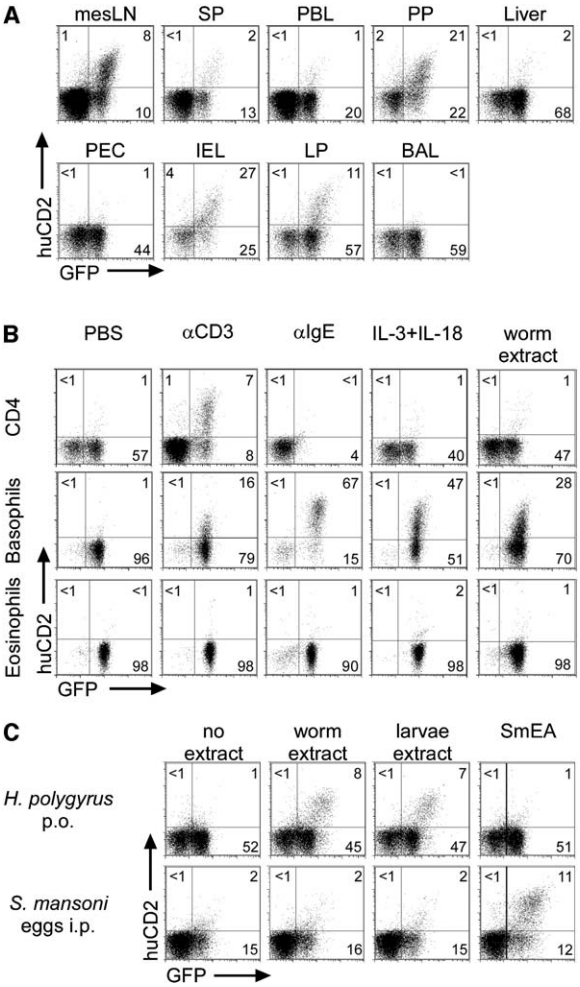

Figure 4. GFP and huCD2 Expression in Response to Infection with *H. polygyrus*

4get/KN2 mice were infected with *Hp* and analyzed 2 weeks later.

(A) CD4+ T cells in the indicated organs were analyzed ex vivo for the expression of GFP and huCD2.

(B) *Hp*-infected animals were injected with PBS (PBS), anti-CD3ε (αCD3), anti-IgE (αIgE), IL-3 + IL-18, or worm extract and sacrificed 2–4 hr later. Hepatic CD4+ T cells, basophils (FcεRI+/CD4−/SSC^{lo}), and eosinophils (CCR3+/CD4−/SSC^{hi}) were analyzed for the expression of GFP and huCD2.

(C) PEC from *Hp*-infected or *S. mansoni* egg-immunized mice were cultured in the absence (no extract) or presence of extracts prepared from adult worm (worm extract), larvae, or *S. mansoni* egg antigen (SmEA) (top). The next day, CD4+ T cells were analyzed for the expression of GFP and huCD2.

anti-CD3 stimulation also resulted in huCD2 expression on basophils, presumably induced indirectly by T cell-derived factors. Eosinophils did not respond to any of the specific stimuli, but expressed huCD2 in response to ionomycin stimulation.

To test whether these cells were rendered nonresponsive in vivo by an immunosuppressive environment, these stimuli were delivered to *Hp*-infected mice. As observed in naive mice (Figure 3A), the injection of anti-CD3 induced high levels of huCD2 on CD4+/GFP+ Th2 cells in the liver (Figure 4B), the PEC, and the blood (data not shown). Similar to the ex vivo stimulation of T cells (Figure S2), the injection of anti-CD3 also induced huCD2 expression on basophils. Injection of anti-IgE, of IL-3 plus IL-18, or of worm antigen induced basophils in the liver (Figure 4B), spleen, and the blood (data not shown) to express huCD2. Although eosinophils can be induced to express huCD2 (Figure S2) and produce IL-4 ex vivo (Gessner et al., 2005; Voehringer et al., 2004), neither acute infection nor any of the stimuli tested resulted in huCD2 expression in vivo (Figure 4B).

Finally, to demonstrate that the CD4+/GFP+/huCD2− Th2 population contains antigen-specific cells, PEC, which contain large numbers of MHC class II-bearing macrophages and B cells (data not shown), were cultured in the presence or absence of worm or larvae extracts. Stimulation with either extract induced huCD2 expression within the CD4+/GFP+ population (Figure 4C). The elicited response was antigen specific, because the extracts did not induce huCD2 expression on peritoneal CD4+ T cells from mice immunized i.p. 1 week earlier with Th2-inducing *S. mansoni* eggs (Figure 4C; MacDonald et al., 2001). Conversely, soluble *S. mansoni* egg antigen (MacDonald et al., 2001) did not induce huCD2 expression on peritoneal CD4+ T cells from *Hp*-infected mice, but did induce expression on CD4+ T cells from *S. mansoni* egg-injected mice. These data show that IL-4 secretion is largely restricted to specific antigen-stimulated CD4+/GFP+ Th2 cells in certain tissues, although GFP+/huCD2− cells are fully functional and can be induced to secrete IL-4 in situ.

huCD2+ and huCD2− GFP+ Th2 Cells Express Different Patterns of Surface Molecules

We next compared CD4+ T cells with a GFP+/huCD2+ (IL-4-secreting), a GFP+/huCD2− (IL-4 competent but nonsecreting), and GFP−/huCD2− (not competent for IL-4 production) phenotype for the expression of various surface molecules (Figure 5). CD4+/GFP+ cells expressed high levels of CD43, CD44, and CD11a and were low for CD45RB and CD62L irrespective of huCD2 expression, consistent with prior activation, while GFP− cells displayed a naive phenotype (Figure 5; Mohrs et al., 2001b). Both huCD2− and huCD2+ GFP+ T cells shared a blasting phenotype (FSC^{hi}), had a comparable frequency of CD27− cells (Hendriks et al., 2000), and had downregulated the IL-4Rα chain (CD124). The huCD2+ population contained a higher frequency of CD69+ cells, consistent with acute antigen-induced activation, and expressed higher levels of CD95, although huCD2+ and huCD2− cells appear to have a similar apoptotic potential as indicated by Annexin V staining. The expression of CD162 (PSGL-1) and the integrins α2 (CD49d), α_V (CD51), and α4β7, which is required for intestinal homing (Hamann et al., 1994), were lower on GFP+/huCD2+ cells than on GFP+/huCD2− cells. Thus, IL-4-secreting Th2 cells display a highly activated phenotype but express lower levels of integrins associated with tissue homing.

Impaired Cytokine Production by huCD2+ T Cells Despite the Abundance of Cytokine Transcripts

To analyze the transcriptional profile of CD4+ T cells with the GFP−/huCD2−, GFP+/huCD2−, or GFP+/huCD2+

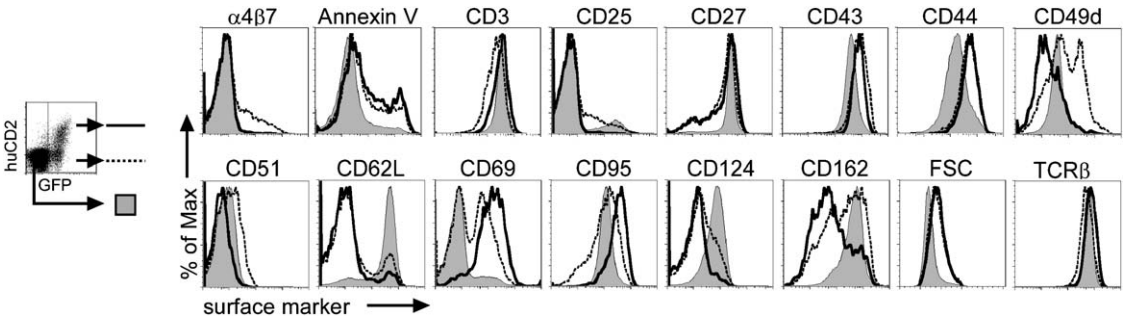

Figure 5. Phenotype of huCD2$^+$ and huCD2$^-$ GFP$^+$ Th2 Cells

4get/KN2 mice were infected with *Hp* and the mesLN were analyzed 2 weeks later. CD4$^+$ T cells with a GFP$^-$/huCD2$^-$, GFP$^+$/huCD2$^-$, or GFP$^+$/huCD2$^+$ phenotype (as indicated on the small dot plot to the left) were analyzed for the expression of the indicated surface markers.

phenotypes, the respective populations were sorted from the mesLN of *Hp*-infected mice (Figure 6A). Transcripts for IL-2 were only moderately increased in both GFP$^+$ populations as compared to GFP$^-$ cells, whereas the type 2 effector cytokines IL-4, IL-5, and IL-13 were substantially more abundant, irrespective of huCD2 expression (Figure 6B). GFP$^+$/huCD2$^+$ cells expressed higher levels of IL-4 transcripts than GFP$^+$/huCD2$^-$ cells, consistent with their enhanced GFP fluorescence intensity.

To determine the capacity of the respective populations to produce these effector cytokines, the sorted cells were cultured in the absence or presence of plate bound anti-CD3 (Figure 6C). CD4$^+$/GFP$^-$ cells produced some IL-2 (116 ± 29 pg/ml) but none of the type 2 cytokines over 24 hr. In contrast, both CD4$^+$/GFP$^+$ populations produced IL-2 (huCD2$^-$ 1732 ± 17 pg/ml; huCD2$^+$ 325 ± 17 pg/ml), IL-4 (huCD2$^-$ 1333 ± 82 pg/ml; huCD2$^+$ 377 ± 32 pg/ml), IL-5 (huCD2$^-$ 1059 ± 69 pg/ml; huCD2$^+$ <40 pg/ml), and IL-13 (huCD2$^-$ 2729 ± 193 pg/ml; huCD2$^+$ 481 ± 21 pg/ml) within 4 hr of stimulation. Unexpectedly, GFP$^+$/huCD2$^-$ cells produced substantially larger amounts of all cytokines at 4 and 24 hr than GFP$^+$/huCD2$^+$ cells. Consistent with the increased IL-4 production, cells that were GFP$^+$/huCD2$^-$ in vivo (Figure 6A) induced huCD2 within 4 hr of stimulation, and after 24 hr, almost 50% of these cells expressed high levels of huCD2 and GFP (Figures 6D and 6E). Because the GFP fluorescence correlates with IL-4 mRNA abundance (Figures 6A and 6B), these cells induced high IL-4 transcript levels upon stimulation. In contrast, GFP$^+$ cells that also expressed huCD2 in vivo (Figure 6A) did not upregulate huCD2 within 4 hr, and only 10% expressed high levels of huCD2 and GFP after 24 hr, revealing a failure to induce IL-4 transcripts and produce IL-4. Cytokine production and the increase in GFP and huCD2 expression remained compromised even upon stimulation with PMA/ionomycin, thereby bypassing proximal TCR signaling events, or after an 18 hr rest period prior to plate bound anti-CD3 stimulation (Figure 6F and data not shown). No cytokines were detectable when any of the subsets were cultured in the absence of stimulation (Figure 6C) and GFP$^+$/huCD2$^+$ cells lost huCD2 expression with similar kinetics as determined in Figure 2B. Thus, CD4$^+$/GFP$^+$ Th2 cells express high transcript levels of canonical type 2 cytokines, but huCD2$^+$ cells that had recently secreted IL-4 in vivo were impaired in their capacity to upregulate IL-4 mRNA and produce cytokines upon restimulation.

huCD2 Expression Is Highly Regulated during the Course of a *H. polygyrus* Infection

Although primary challenge with *Hp* results in chronic infection (Finkelman et al., 1997; Robinson et al., 1989), it is apparent that the immune response, particularly the production of IL-4, is modulated during the course of infection (Finkelman et al., 2000). Because IL-4 mRNA is found mainly in CD4$^+$ T cells and the depletion of CD4$^+$ T cell ablates the IL-4 response (Svetic et al., 1993), CD4$^+$ T cells are the likely source of the observed modulation. To follow the IL-4 response over time, CD4$^+$ T cells in the mesLN of *Hp*-infected 4get/KN2 mice were analyzed after various periods for the expression of GFP and huCD2. Both GFP$^+$ and huCD2$^+$ cells increased approximately 100-fold in frequency and total number within the first week of a primary infection (Figure 7A). While the total number of GFP$^+$ and huCD2$^+$ cells increased further during the second week, the frequencies of both populations actually decreased. To follow the transition to memory, the infection was terminated by drug treatment in one group after 2 weeks, while the control group remained infected (Figure 7A), and both groups were analyzed 5 weeks later. Although the frequency of GFP$^+$ was comparable between both groups, the drug-cured mice contained less than 10% of the huCD2$^+$ cells as compared to the untreated animals. The observation that GFP$^+$ cells persisted while huCD2 expression declined was also apparent in the spleen, PP, and the LP (Figure S3). Moreover, the reduced frequency of huCD2$^+$ cells in drug-cured mice was accompanied by lower levels of huCD2 staining and reduced MFI_{GFP} among GFP$^+$ cells (Figure S3), consistent with diminished IL-4 protein and transcript levels.

As compared to naive mice, drug-cured animals mount a protective, CD4$^+$ T cell- and IL-4-dependent memory response against a second *Hp* challenge (Finkelman et al., 1997; Urban et al., 1991a, 1991b). Indeed, when drug-cured 4get/KN2 mice were reinfected with *Hp*, both the frequency and the number of GFP$^+$ and

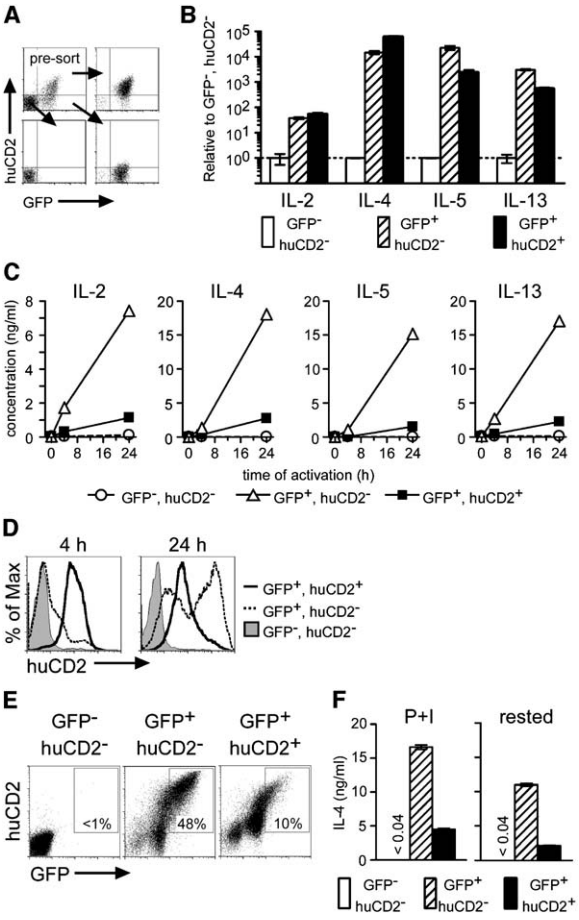

Figure 6. Cytokine Transcripts and Protein Production by huCD2$^+$ and huCD2$^-$ GFP$^+$ Th2 Cells

(A) 4get/KN2 mice were infected with *Hp*, and CD4$^+$ T cells with a GFP$^-$/huCD2$^-$, GFP$^+$/huCD2$^-$, or GFP$^+$/huCD2$^+$ phenotype were sorted 2 weeks later from the mesLN.

(B) mRNA from the respective populations was analyzed by real-time RT-PCR for the indicated transcripts normalized to GAPDH. Depicted are the mean ± SD from triplicate samples relative to GFP$^-$ cells.

(C–E) The sorted populations (A) were cultured in the absence (0 hr) or presence of plate bound anti-CD3ϵ. Supernatants were harvested after 4 and 24 hr and analyzed by ELISA for the indicated cytokines. Depicted are the mean ± SD. The stimulated CD4$^+$ T cells (C) were analyzed after 4 and 24 hr for the expression of huCD2 (D) and GFP versus huCD2 after 24 hr (E).

(F) The sorted populations (A) were either directly stimulated for 4 hr with PMA + ionomycin (P+I, left) or rested for 18 hr prior to 24 hr stimulation on plate bound anti-CD3 (rested, right), and the supernatants were analyzed by ELISA for IL-4. Data represent the mean ± SD.

huCD2$^+$ cells in the mesLN increased within 4 days (Figure 7A), while primary infection of naive mice did not result in any increase over the same time period. Although the frequency of GFP$^+$ and huCD2$^+$ cells was comparable by 1 week after a primary or recall infection, the total numbers of GFP$^+$ and huCD2$^+$ cells were significantly ($p < 0.05$ for both populations in a Student's t test) greater in the rechallenged mice. To compare the IL-4 production by basophils and eosinophils

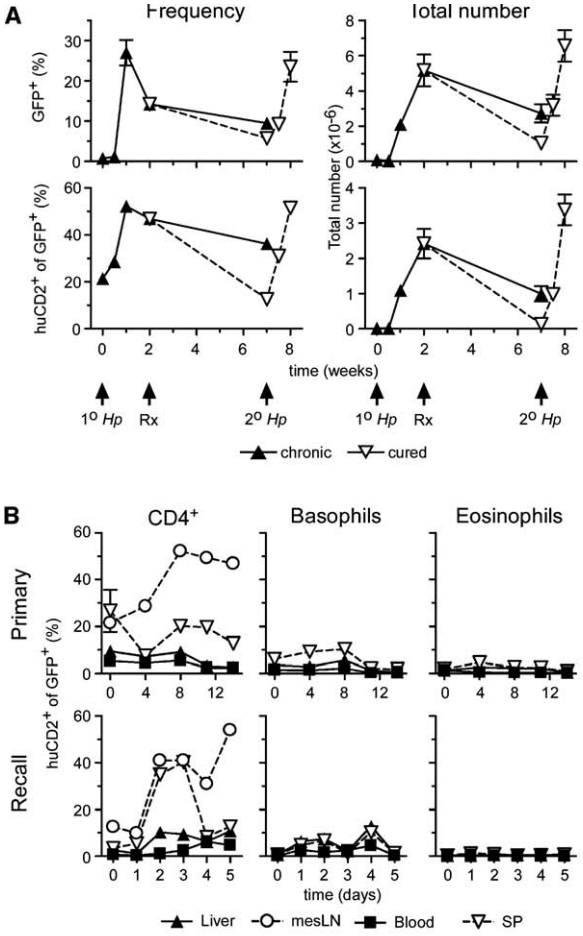

Figure 7. huCD2 Expression Is Highly Regulated during the Course of a Primary and Challenge *Hp* Infection

(A) 4get/KN2 mice were infected with *Hp* (1° *Hp*), and CD4$^+$ T cells in the mesLN were analyzed at the indicated time points for GFP (top) and huCD2 (bottom) expression. One cohort was drug-cured (Rx) after 2 weeks, while the control group remained untreated. Animals from both groups were analyzed 5 weeks after drug treatment. The remaining drug-cured animals were rechallenged (2° *Hp*) 5 weeks after drug treatment and analyzed at the indicated time points. Depicted are the mean ± SD.

(B) Naive (primary) or drug-cured (recall) 4get/KN2 mice were infected with *Hp*, and CD4$^+$ Th2 cells (CD4$^+$/FcϵRI$^-$/GFP$^+$), basophils (FcϵRI$^+$/CD4$^-$/GFP$^+$), and eosinophils (CCR3$^+$/GFP$^+$) were analyzed at various time points in the indicated organs for huCD2 expression. Data represent the mean ± SD.

(C) Naive (primary) or drug-cured (recall) 4get/KN2 mice were infected with *Hp* and CD4$^+$ T cells in the LP were analyzed for the expression of GFP and huCD2 at the indicated days.

to those of CD4+/GFP+ Th2 cells, we analyzed the frequency of huCD2+ cells in the respective populations over the course of a primary and recall infection in various organs. While up to 50% of the CD4+/GFP+ Th2 cells were huCD2+, basophils or eosinophils revealed a substantially lower frequency of huCD2+ at any time point in any organ (Figure 7B; Figure S4).

In striking contrast to naive animals, a substantial fraction of CD4+ T cells taken from the site of infection at the intestinal lamina propria was GFP+ in the drug-cured mice, but these cells did not express huCD2 (Figure 7C). Within 4 days after infection, however, robust huCD2 expression was apparent in the rechallenged mice, while neither huCD2+ nor GFP+ CD4+ T cells were detected at this time following primary infection. Even 1 week after infection, GFP+ and GFP+/huCD2+ CD4+ T cells were more frequent in the intestinal walls of the rechallenged mice and huCD2 was expressed at higher levels.

Discussion

We generated dual reporter mice to analyze the relationship between effector cells competent to secrete IL-4 with effector cells actually secreting IL-4 protein. In agreement with prior findings (Gessner et al., 2005; Mohrs et al., 2001b; Voehringer et al., 2004), the latter subset contained all of the cells in the former subset, a finding that suggests that the capacity for IL-4 secretion is gained only after cells have fully committed to their discrete effector function. The competence of Th2 cells for IL-4 production was associated with their capacity to disseminate widely throughout body tissues. Using a strictly enteric helminth, we demonstrated that active IL-4 secretion was limited to sites of presumptive antigen presentation in intestinal tissues and draining lymph nodes. Comparisons between IL-4-secreting and nonsecreting effector cells demonstrated additional levels of regulation affecting activation thresholds and the potential for the secretion of other cytokines. Finally, memory responses reflected the capacity of cytokine-competent effector cells, which persisted in tissues, to respond rapidly to reinfection. These findings extend prior observations regarding CD4+ and CD8+ IFN-γ-producing effector cells generated during systemic infections (Masopust et al., 2001; Reinhardt et al., 2001, 2003), but add previously unsuspected detail to the dynamic nature of immune effector function.

We have previously demonstrated that IL-4-producing cells—including Th2 cells, NK T cells, eosinophils, basophils, and mast cells—from 4get mice are constitutively GFP+ but do not secrete IL-4 protein unless stimulated (Gessner et al., 2005; Stetson et al., 2003; Voehringer et al., 2004). In each of these cells, the presence of IL-4 transcripts is mirrored by GFP fluorescence, suggesting that the EMCV IRES relieves translation that is otherwise blocked or undetectable from the canonical 5′-cap of the bicistronic mRNA. IRES elements confer competency for translation even under conditions where 5′-cap-dependent translation is disabled (Fernandez et al., 2002; Sachs et al., 1997). Thus, the 4get mice reliably identify cells competent for IL-4 secretion (and marked by constitutive IL-4 transcripts), but actual IL-4 secretion is observed only after activation and is accompanied by increased transcript levels and GFP fluorescence (Figure 2A; Gessner et al., 2005; Voehringer et al., 2004). Compared to the bicistronic 4get mice, the frequency of reporter-positive cells was very low in IL-4-replacing reporter mice during immune activation in vivo (Min et al., 2004; Mohrs et al., 2001b; Voehringer et al., 2004). In order to combine the advantages of both reporter strategies, we generated heterozygous, dual-reporter mice, thus enabling us to identify IL-4-secreting cells from among the population of IL-4-competent cells. We emphasize that the IL-4-substituting reporter mice we made used knockin strategies that leave intact all of the known regulatory sites of the *Il4* locus while also deleting the neomycin selection cassette (Ansel et al., 2003, 2004; Guo et al., 2004; Lee et al., 2003; Mohrs et al., 2001a). Our present data (Figures 2, 3, 4, and 7) and prior studies (Gessner et al., 2005; Mohrs et al., 2001b) do not support the frequent monoallelic IL-4 expression; however, we cannot formally exclude the infrequent occurrence of monoallelic expression. Thus, the two reporter alleles provide a unique reagent for following cytokine transcription and protein production over relevant time frames.

The ability to identify huCD2− and huCD2+ Th2 cells enabled us to compare IL-4-producing and -nonproducing Th2 cells from the same animal. Unexpectedly, both GFP+ populations had downregulated the IL-4Rα chain (CD124) (Figure 5) and were presumably no longer receptive to the Th2-promoting effects of IL-4 (Abbas et al., 1996; Mohrs et al., 2001b). Thus, *Il4* gene-expressing cells have apparently committed to the Th2 lineage and do not require further signals mediated through the IL-4Rα. Further, both GFP+ populations had downregulated CD27 (Hendriks et al., 2000), indicating a similar frequency of terminally differentiated cells as assessed by this marker, and arguing against the preferential memory cell generation from either population. Both GFP+ populations were CD43hi, CD44hi, and CD62Llo (Figure 5) and therefore would no longer constitutively home to secondary lymphoid organs (Bradley et al., 1994; DeGrendele et al., 1997; Stockton et al., 1998). In contrast, while GFP+/huCD2− cells upregulated the expression of integrins required for their emigration into tissues (CD49d, CD51), IL-4 protein-producing cells expressed strikingly lower levels of most integrins, including α4β7, which confers intestinal homing (Hamann et al., 1994). These data suggest that IL-4-competent Th2 cells develop an increased potential to traffic into tissues while it is downregulated in cells that were additionally stimulated to produce IL-4. This mechanism may serve to confine effector functions to sites of antigen exposure.

In agreement with prior findings, we identified basophils and eosinophils as the primary innate cells in the blood and tissues in response to helminth infection (Gessner et al., 2005; Min et al., 2004; Shinkai et al., 2002; Voehringer et al., 2004). Despite the wide distribution of eosinophils and basophils, the frequency of huCD2 expression, and hence IL-4 secretion, from these cells was low as compared to CD4+/GFP+ Th2 cells during a primary or recall *Hp* infection. Of note, the huCD2 reporter is capable of recording even the most rapid and transient IL-4 responses, because

huCD2 expression can be induced within minutes (Figure 3A) and is maintained on the cell surface with a half-life of 24 hr (Figure 2B). Consequently, huCD2 expression does not precisely parallel IL-4 secretion but accurately identifies cells that have secreted IL-4 within the biological half-life of surface huCD2.

Although the low frequency of huCD2$^+$ basophils contrasts with recent reports that helminth-induced hepatic basophils may be actively secreting IL-4 and that basophils initiate IL-4 production during a memory T-dependent response (Khodoun et al., 2004; Min et al., 2004), we demonstrate that huCD2 expression can be induced on basophils in naive or *Hp*-infected animals in response to FcεRI cross-linkage, IL-3- and IL-18-induced activation (Yoshimoto et al., 1999), the injection of *Hp* extracts (presumably by antigen-specific cross-linkage of the FcεRI), and in response to indirect mechanisms mediated by the activation of T cells (Figures 3B and 4B; Figure S2). One explanation for this apparent discrepancy might be that *Hp* is a strictly enteric nematode parasite, whereas *N. brasiliensis* migrates systemically after s.c. infection before eventually reaching the intestine (Finkelman et al., 1997; Min et al., 2004; Mohrs et al., 2001b; Voehringer et al., 2004).

Based on the present data, we propose a sequential two-step model for the production of IL-4, which is likely applicable to all known IL-4-producing cells (Brown and Hural, 1997). First, the *Il4* gene is rendered accessible by chromatin alterations as suggested by prior studies (Ansel et al., 2003; Grogan et al., 2003). As proposed here and elsewhere (Gessner et al., 2005; Grogan et al., 2001; Stetson et al., 2003; Voehringer et al., 2004), these chromatin alterations correlate with the constitutive presence of IL-4 transcripts. Cells that have accomplished this initial step are poised for rapid cytokine production but do not necessarily secrete IL-4 protein. This first step is linked with lineage differentiation of innate IL-4 producers, such as basophils, eosinophils, mast cells, and NK T cells (Gessner et al., 2005; Stetson et al., 2003; Voehringer et al., 2004). In contrast, CD4$^+$ T cells defer activation of the *Il4* gene until stimulated under appropriate conditions in secondary lymphoid organs. In a distinct second step, IL-4-competent cells can rapidly make and secrete IL-4 protein upon activation. This step is reversible, and IL-4-producing cells can revert to a nonproducing state (Figure 2B). Under the conditions used here, such cells remain IL-4 competent and constitutively express cytokine mRNAs as revealed by GFP fluorescence. Importantly, CD4$^+$ T cells that recently had been producing IL-4 in vivo, as assessed by the presence of surface huCD2, were impaired in their capacity to upregulate IL-4 mRNA levels and produce effector cytokines in response to TCR-mediated stimulation (Figures 6C–6F). This impairment is not due to reduced CD3 or TCR expression (Figure 5; Corbin and Harty, 2005) and cannot be overcome by a rest period prior to stimulation or bypassing critical TCR signaling events by stimulation with PMA+ ionomycin (Figure 6F). This impaired responsiveness might be an important mechanism to prevent immunopathology by limiting the production of effector cytokines at sites of frequent antigenic stimulation.

Because some CD4$^+$ T cells remain GFP$^+$ weeks after elimination of the infection (Figures 7A and 7C), memory T cells become functionally equivalent to innate IL-4-producing cells but impart exquisite antigen specificity to the repertoire of rapid immune responses. Further studies will be required to assess whether and under which conditions IL-4-competent Th2 cells can revert to a state where constitutive cytokine transcripts are no longer maintained.

The systemic dissemination of Th2 cells poised for rapid IL-4 production has important implications for type 2 immunity and immunopathology. As we show here, CD4$^+$/GFP$^+$ Th2 cells persist at high frequencies in the intestinal wall of drug-cured mice and are ideally positioned to respond to recall infections. Indeed, these cells rapidly produce high levels of IL-4 upon reinfection well before Th2 cells appear in the intestine during a primary infection. The accelerated IL-4 response by CD4$^+$ T cells at the site of infection likely underlies the immunity of *Hp*-primed mice to reinfection (Morimoto et al., 2004; Urban et al., 1991a, 1991b). Due to the wide dissemination of CD4$^+$/GFP$^+$ Th2 cells, the production of IL-4 would presumably be similarly accelerated in other tissues if the appropriate reactive antigens were introduced at those sites. However, the production of type 2 cytokines by inappropriately activated Th2 cells resident in tissues, such as the lung (Figure 4A), might contribute to immunopathologies such as asthma (Wills-Karp, 1999). An accelerated immune response is not only the hallmark of immune memory, but also the goal of successful vaccines. Further work will be needed to address the mechanisms by which effector cells disseminate widely to tissues in the apparent absence of antigen and how their longevity, turnover, and effector function is regulated.

Experimental Procedures

Mice and Parasites

HuCD2/IL-4 reporter mice, designated KN2 (knockin CD2), were generated by replacing exons 1 and 2 of the *Il4* locus by a previously described huCD2 reporter cassette (Sawada et al., 1994). The reporter cassette was organized by using the following elements beginning at the 5′ end: the noncoding first and beginning of the second exon of the murine *Cd4* gene with the first intron containing deletions of the silencer elements suppressing CD4 expression in CD8 cells, the human CD2 cDNA cloned in-frame into the ATG start codon in exon 2 of the *Cd4* gene, a SV40 polyadenylation sequence, and a neomycin selection cassette flanked by loxP sites (Mohrs et al., 2001b; Sawada et al., 1994). This reporter cassette was flanked with 4.8 kb of the 5′ *Il4* sequence ending at the IL-4 start codon and on the 3′ end with the 0.7 kb of the second *Il4* intron. The construct was electroporated into 129/SvJ embryonic stem cells (Mohrs et al., 2001b) and G418-resistant clones were screened by Southern blotting. The neo cassette was excised by transient Cre transfection (Mohrs et al., 1999), and ES cells were injected into C57BL/6 blastocysts to generate chimeric mice. After germline transmission, heterozygous offspring were backcrossed to BALB/c for 10 generations. Offspring were genotyped by PCR using the following primers: A, 5′-AGAGAGGTGCTGATTGGCCCAG-3′; B, 5′-CTATCACAGGCATTTCTCATTCAG-3′; C, 5′-ATGGCAGGC AAAGATGAGAAGGGC-3′.

4get mice, in which the *Il4* gene is linked via an internal ribosomal entry site (IRES) to GFP (Mohrs et al., 2001b), were backcrossed to BALB/c mice and C57BL/6 mice for 10 and 8 generations, respectively. Homozygous 4get and KN2 mice were interbred to generate heterozygous 4get/KN2 dual-reporter mice. Animals were kept under specific pathogen-free conditions in filter top cages at the animal facility of the Trudeau Institute and were used at 8 to 12 weeks of age. Infective third-stage larvae of *H. polygyrus* (*Hp*) were pre-

pared as described (Gessner et al., 2005). Mice were inoculated by oral gavage with 200 larvae. Where indicated, the animals were treated twice orally with the antihelminthic drug pyrantel pamoate (2 mg). Parasite extracts were prepared from adult worms by mechanical disruption using a glass tissue grinder (Fernando Monroy et al., 1985). *Hp* larvae were killed by repeated freeze-thaw cycles prior to homogenization. *S. mansoni* eggs were purified as published and mice were injected i.p. with 2500 eggs (MacDonald et al., 2001). All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee at Trudeau Institute and the University of California, San Francisco.

Tissue Sampling and Preparation

Single-cell suspensions were prepared from the mesLN, PP, and the SP by mechanical disruption. BAL cells were collected by five consecutive washes of the respiratory tract with 1 ml PBS/1% BSA each. Adherent cells were depleted from BAL and PEC by incubation in complete RPMI 1640 (cRPMI, 10% heat-inactivated fetal calf serum, 50 μM 2-ME, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin) at 37°C, 5% CO_2 for 2 hr. Intrahepatic lymphocytes were isolated as described (Huang et al., 1994). Briefly, livers were perfused, forced through a cell strainer, and digested for 40 min at 37°C with collagenase IV (100 U/ml; Sigma) and DNaseI (10 U/ml; Sigma) in RPMI 1640 medium without serum. Hepatocytes were sedimented at 30 × g. IEL and cells from the LP were isolated from the small intestine after removal of the PP (Laky et al., 1997). The small intestine was cut longitudinally, washed with CMF, stirred with cRPMI medium for 30 min at 37°C, and finally briefly vortexed. This step was repeated and the IEL fractions were pooled. LP lymphocytes were obtained by repeated digestion with collagenase VIII (100 U/ml; Sigma) in cRPMI for 60 min at 37°C. Intrahepatic lymphocytes, IELs, and LPs were enriched in the interphase of a discontinuous 60%/40% Percoll (Amersham Biosciences) gradient centrifuged at 1200 × g for 20 min at room temperature.

Flow Cytometry

Flow cytometry reagents were purchased from BD Biosciences, Caltag Laboratories, or eBioscience, unless otherwise stated, and clone designations are given in parentheses. All samples were first incubated with anti-CD16/32 (2.4G2) to block nonspecific binding of antibodies to Fcγ III/II receptors. The following mAbs were used as PE-, APC-, APC-Cy7-, or biotin conjugates: α4β7 integrin (DATK32), CCR3 (83101; R&D Systems), CD3ε (145-2C11), CD4 (RM4-5), CD11a (M17/4), CD25 (PC61 5.3), CD27 (LG.3A10), CD43 (1B11), CD44 (IM7.8.1), CD45RB (16A), CD49d (9C10), CD51 (RMV-7), CD62L (MEL-14), CD69 (H1.2F3), CD95 (Jo2), CD117 (c-Kit, 2B8), CD124 (IL-4Rα, M1), CD162 (PSGL-1, 2PH1), IgE (R35-72), NK1.1 (PK136), TCRβ (H57-597). Additional reagents included anti-human CD2 (RPA-2.10), streptavidin-APC, and streptavidin-PE. Annexin V staining (BD Biosciences) and the IL-4 cytokine secretion assay (Hu-Li et al., 2001) (Miltenyi Biotec) were performed according to the manufacturer's instructions. Dead cells were discriminated by the addition of propidium iodide (PI, 3 μg/ml, Sigma) and excluded from the analyses.

Samples were acquired on a FACSCalibur (Beckton Dickinson) flow cytometer or a FACScan (Beckton Dickinson) flow cytometer equipped with a Multicolor upgrade (Cytek Development). Data were analyzed using FlowJo (Tree Star) software. Numbers on flow cytometry plots indicate the percentage of cells in the respective quadrant and fractions were rounded to the nearest full digit. Median fluorescence intensities (MFI) were only directly compared when samples were acquired in the same experiment with the same instrument settings.

Cell Culture

$CD4^+$ T cells were purified from lymph nodes by using magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Purified $CD4^+$ T cells (1×10^6/ml) were stimulated as described (Mohrs et al., 2001b) in cRPMI with anti-CD3ε (145-2C11, 2 μg/ml) and anti-CD28 (37.51, 5 μg/ml) in the presence of IL-2 (5 ng/ml) and irradiated splenic APC (5×10^6/ml) for 5–7 days. Th1 cultures were supplemented with IL-12 (5 ng/ml) and anti-IL-4 (11B11, 20 μg/ml), and Th2 cultures with IL-4 (50 ng/ml) and anti-IFN-γ (XMG1.2, 20 μg/ml). For restimulation, tissue culture plates were coated with anti-CD3ε (10 μg/ml) and anti-CD28 (5 μg/ml) or the cells were cultured for 4 hr in the presence of PMA (50 ng/ml) and ionomycin (500 ng/ml). For intracellular cytokine staining, Brefeldin A was added during the last 2 hr. Stimulation ex vivo was done either with plate bound anti-CD3ε (10 μg/ml), soluble anti-IgE (2 μg/ml), IL-3 (30 ng/ml) plus IL-18 (30 ng/ml), or ionomycin with or without PMA. Larvae and worm extracts were used at 20 μg/ml. Soluble *S. mansoni* egg antigen was used at 50 μg/ml (MacDonald et al., 2001).

In Vivo Activation

NK T cells were activated in vivo by the i.v. injection of anti-CD3ε (2 μg) (Min et al., 2004; Yoshimoto and Paul, 1994). FcεRI-bearing basophils were sensitized in vivo by i.p. injection of 20 μg murine anti-DNP IgE (SPE-7, Sigma). After 24 hr, mice were injected i.p. with PBS or 1 mg $DNP_{30\text{-}40}$-HSA (Sigma) and analyzed 2–4 hr later (Dombrowicz et al., 1993). *Hp*-infected mice were injected i.v. either with PBS, anti-CD3ε (2 μg), anti-IgE (2 μg), with IL-3 (1 μg) plus IL-18 (1 μg), or with 50 μg of crude worm extracts and analyzed 2–4 hr later.

Cell Sorting

Two weeks after *Hp* infection, $CD4^+$ T cells were purified by negative selection from the mesLN by using magnetic beads (Miltenyi Biotec), subsequently stained with anti-CD4-APC and anti-huCD2-PE, and sorted with a FACSVantage flow cytometer (Beckton Dickinson) equipped with DiVa electronics.

RT-PCR

RNA was extracted by using the RNAqueous-4PCR kit (Ambion) and reverse transcribed with the Superscript II RNase H^- kit (Invitrogen) using oligo$(dT)_{18}$ priming. Quantitative real-time RT-PCR with specific primers and probes (Gessner et al., 2005) was performed by using an ABI Prism 7700 Sequence BioDetector (PE Biosystems) according to the manufacturer's instructions (TaqMan, Perkin Elmer). All cytokines were analyzed in triplicate. Ct values for GAPDH were routinely between 15 and 18 cycles and normalization to β2m gave similar results.

ELISA

Cytokines in culture supernatants were quantified by ELISA via paired antibodies for IL-2 (JES6-1A12, JES6-5H4), IL-4 (11B11, BVD6-24G2), IL-5 (TRFK5, TRFK4), and IL-13 (38213, polyclonal goat anti-mouse IL-13). Recombinant mouse IL-2 (BD), IL-4, IL-5, and IL-13 (R&D Systems) were used as standards. The detection limits were typically 20 pg/ml for IL-4, 40 pg/ml for IL-2 and IL-5, and 160 pg/ml for IL-13.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.immunity.com/cgi/content/full/23/4/419/DC1/.

Acknowledgments

This work was supported by funds from the Trudeau Institute (M.M.) and the National Institutes of Health grants AI45666 (M.M.), AI046530 (M.M.), and AI30663 (R.M.L.). R.M.L. is a Senior Scholar of the Ellison Medical Foundation for Global Infectious Disease. N.K. is a Scholar of the Leukemia and Lymphoma Society. A.E.W. was supported by a Career Development Award from The Crohn's and Colitis Foundation of America. We thank Dr. Edward Pearce for providing us with critical reagents and advice, Simon Monard and Brandon Sells for cell sorting, and Z.E. Wang for technical support. We thank Drs. Frances Lund and Lewis Lanier for critical review of the manuscript. The authors declare that they have no competing financial interests.

Received: May 11, 2005
Revised: September 1, 2005
Accepted: September 14, 2005
Published: October 18, 2005

References

Abbas, A.K., Murphy, K.M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. Nature *383*, 787–793.

Ansel, K.M., Lee, D.U., and Rao, A. (2003). An epigenetic view of helper T cell differentiation. Nat. Immunol. *4*, 616–623.

Ansel, K.M., Greenwald, R.J., Agarwal, S., Bassing, C.H., Monticelli, S., Interlandi, J., Djuretic, I.M., Lee, D.U., Sharpe, A.H., Alt, F.W., and Rao, A. (2004). Deletion of a conserved Il4 silencer impairs T helper type 1-mediated immunity. Nat. Immunol. *5*, 1251–1259.

Bradley, L.M., Watson, S.R., and Swain, S.L. (1994). Entry of naive CD4 T cells into peripheral lymph nodes requires L-selectin. J. Exp. Med. *180*, 2401–2406.

Brown, M.A., and Hural, J. (1997). Functions of IL-4 and control of its expression. Crit. Rev. Immunol. *17*, 1–32.

Corbin, G.A., and Harty, J.T. (2005). T cells undergo rapid ON/OFF but not ON/OFF/ON cycling of cytokine production in response to antigen. J. Immunol. *174*, 718–726.

DeGrendele, H.C., Estess, P., and Siegelman, M.H. (1997). Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science *278*, 672–675.

Dombrowicz, D., Flamand, V., Brigman, K.K., Koller, B.H., and Kinet, J.P. (1993). Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. Cell *75*, 969–976.

Fernandez, J., Yaman, I., Sarnow, P., Snider, M.D., and Hatzoglou, M. (2002). Regulation of internal ribosomal entry site-mediated translation by phosphorylation of the translation initiation factor eIF2alpha. J. Biol. Chem. *277*, 19198–19205.

Fernando Monroy, G., Brindley, P.J., and Dobson, C. (1985). Vaccination against *Nematospiroides dubius* in mice using adult worm extracts as antigens. Aust. J. Exp. Biol. Med. Sci. *63*, 423–430.

Finkelman, F.D., Shea-Donohue, T., Goldhill, J., Sullivan, C.A., Morris, S.C., Madden, K.B., Gause, W.C., and Urban, J.F., Jr. (1997). Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. Annu. Rev. Immunol. *15*, 505–533.

Finkelman, F.D., Morris, S.C., Orekhova, T., Mori, M., Donaldson, D., Reiner, S.L., Reilly, N.L., Schopf, L., and Urban, J.F., Jr. (2000). Stat6 regulation of in vivo IL-4 responses. J. Immunol. *164*, 2303–2310.

Gause, W.C., Urban, J.F., Jr., and Stadecker, M.J. (2003). The immune response to parasitic helminths: insights from murine models. Trends Immunol. *24*, 269–277.

Gessner, A., Mohrs, K., and Mohrs, M. (2005). Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. J. Immunol. *174*, 1063–1072.

Grogan, J.L., Mohrs, M., Harmon, B., Lacy, D.A., Sedat, J.W., and Locksley, R.M. (2001). Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. Immunity *14*, 205–215.

Grogan, J.L., Wang, Z.E., Stanley, S., Harmon, B., Loots, G.G., Rubin, E.M., and Locksley, R.M. (2003). Basal chromatin modification at the IL-4 gene in helper T cells. J. Immunol. *171*, 6672–6679.

Guo, L., Hu-Li, J., and Paul, W.E. (2004). Probabilistic regulation of IL-4 production in Th2 cells: accessibility at the Il4 locus. Immunity *20*, 193–203.

Hamann, A., Andrew, D.P., Jablonski-Westrich, D., Holzmann, B., and Butcher, E.C. (1994). Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. J. Immunol. *152*, 3282–3293.

Hendriks, J., Gravestein, L.A., Tesselaar, K., van Lier, R.A., Schumacher, T.N., and Borst, J. (2000). CD27 is required for generation and long-term maintenance of T cell immunity. Nat. Immunol. *1*, 433–440.

Ho, I.C., Kaplan, M.H., Jackson-Grusby, L., Glimcher, L.H., and Grusby, M.J. (1999). Marking IL-4-producing cells by knock-in of the IL-4 gene. Int. Immunol. *11*, 243–247.

Huang, L., Soldevila, G., Leeker, M., Flavell, R., and Crispe, I.N. (1994). The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. Immunity *1*, 741–749.

Hu-Li, J., Pannetier, C., Guo, L., Lohning, M., Gu, H., Watson, C., Assenmacher, M., Radbruch, A., and Paul, W.E. (2001). Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. Immunity *14*, 1–11.

Khodoun, M.V., Orekhova, T., Potter, C., Morris, S., and Finkelman, F.D. (2004). Basophils initiate IL-4 production during a memory T-dependent response. J. Exp. Med. *200*, 857–870.

Kinet, J.P. (2002). Allergy and hypersensitivity. Curr. Opin. Immunol. *14*, 685–687.

Laky, K., Lefrancois, L., and Puddington, L. (1997). Age-dependent intestinal lymphoproliferative disorder due to stem cell factor receptor deficiency: parameters in small and large intestine. J. Immunol. *158*, 1417–1427.

Lee, G.R., Fields, P.E., Griffin, T.J., and Flavell, R.A. (2003). Regulation of the Th2 cytokine locus by a locus control region. Immunity *19*, 145–153.

MacDonald, A.S., Straw, A.D., Bauman, B., and Pearce, E.J. (2001). $CD8^-$ dendritic cell activation status plays an integral role in influencing Th2 response development. J. Immunol. *167*, 1982–1988.

Masopust, D., Vezys, V., Marzo, A.L., and Lefrancois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science *291*, 2413–2417.

Min, B., Prout, M., Hu-Li, J., Zhu, J., Jankovic, D., Morgan, E.S., Urban, J.F., Jr., Dvorak, A.M., Finkelman, F.D., LeGros, G., and Paul, W.E. (2004). Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. J. Exp. Med. *200*, 507–517.

Mohrs, M., Ledermann, B., Köhler, G., Dorfmüller, A., Gessner, A., and Brombacher, F. (1999). Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. J. Immunol. *162*, 7302–7308.

Mohrs, M., Blankespoor, C.M., Wang, Z.E., Loots, G.G., Afzal, V., Hadeiba, H., Shinkai, K., Rubin, E.M., and Locksley, R.M. (2001a). Deletion of a coordinate regulator of type 2 cytokine expression in mice. Nat. Immunol. *2*, 842–847.

Mohrs, M., Shinkai, K., Mohrs, K., and Locksley, R.M. (2001b). Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter. Immunity *15*, 303–311.

Mohrs, M., Lacy, D.A., and Locksley, R.M. (2003). Stat signals release activated naive th cells from an anergic checkpoint. J. Immunol. *170*, 1870–1876.

Morimoto, M., Whitmire, J., Xiao, S., Anthony, R.M., Mirakami, H., Star, R.A., Urban, J.F., Jr., and Gause, W.C. (2004). Peripheral CD4 T cells rapidly accumulate at the host: parasite interface during an inflammatory Th2 memory response. J. Immunol. *172*, 2424–2430.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M.K. (2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101–105.

Reinhardt, R.L., Bullard, D.C., Weaver, C.T., and Jenkins, M.K. (2003). Preferential accumulation of antigen-specific effector CD4 T cells at an antigen injection site involves CD62E-dependent migration but not local proliferation. J. Exp. Med. *197*, 751–762.

Rivière, I., Sunshine, M.J., and Littman, D.R. (1998). Regulation of IL-4 expression by activation of individual alleles. Immunity *9*, 217–228.

Robinson, M., Wahid, F., Behnke, J.M., and Gilbert, F.S. (1989). Immunological relationships during primary infection with *Heligmosomoides polygyrus* (*Nematospiroides dubius*): dose-dependent expulsion of adult worms. Parasitology *98*, 115–124.

Sachs, A.B., Sarnow, P., and Hentze, M.W. (1997). Starting at the

beginning, middle, and end: translation initiation in eukaryotes. Cell *89*, 831–838.

Sawada, S., Scarborough, J.D., Killeen, N., and Littman, D.R. (1994). A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell *77*, 917–929.

Seder, R.A., Paul, W.E., Dvorak, A.M., Sharkis, S.J., Kagey-Sobotka, A., Niv, Y., Finkelman, F.D., Barbieri, S.A., Galli, S.J., and Plaut, M. (1991). Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils. Proc. Natl. Acad. Sci. USA *88*, 2835–2839.

Shinkai, K., Mohrs, M., and Locksley, R.M. (2002). Helper T cells regulate type-2 innate immunity in vivo. Nature *420*, 825–829.

Stetson, D.B., Mohrs, M., Reinhardt, R.L., Baron, J.L., Wang, Z.E., Gapin, L., Kronenberg, M., and Locksley, R.M. (2003). Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J. Exp. Med. *198*, 1069–1076.

Stockton, B.M., Cheng, G., Manjunath, N., Ardman, B., and von Andrian, U.H. (1998). Negative regulation of T cell homing by CD43. Immunity *8*, 373–381.

Svetic, A., Madden, K.B., Zhou, X.D., Lu, P., Katona, I.M., Finkelman, F.D., Urban, J.F., Jr., and Gause, W.C. (1993). A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. J. Immunol. *150*, 3434–3441.

Urban, J.F., Jr., Katona, I.M., and Finkelman, F.D. (1991a). *Heligmosomoides polygyrus*: CD4+ but not CD8+ T cells regulate the IgE response and protective immunity in mice. Exp. Parasitol. *73*, 500–511.

Urban, J.F., Jr., Katona, I.M., Paul, W.E., and Finkelman, F.D. (1991b). Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proc. Natl. Acad. Sci. USA *88*, 5513–5517.

Voehringer, D., Shinkai, K., and Locksley, R.M. (2004). Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. Immunity *20*, 267–277.

Wills-Karp, M. (1999). Immunologic basis of antigen-induced airway hyperresponsiveness. Annu. Rev. Immunol. *17*, 255–281.

Yamaguchi, M., Lantz, C.S., Oettgen, H.C., Katona, I.M., Fleming, T., Miyajima, I., Kinet, J.P., and Galli, S.J. (1997). IgE enhances mouse mast cell Fc(epsilon)RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. J. Exp. Med. *185*, 663–672.

Yoshimoto, T., and Paul, W.E. (1994). CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. J. Exp. Med. *179*, 1285–1295.

Yoshimoto, T., Tsutsui, H., Tominaga, K., Hoshino, K., Okamura, H., Akira, S., Paul, W.E., and Nakanishi, K. (1999). IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. Proc. Natl. Acad. Sci. USA *96*, 13962–13966.