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Type 2 Immunity Reflects Orchestrated Recruitment of Cells Committed to IL-4 Production

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Summary

Using IL-4 reporter mice we identified eosinophils, basophils, and Th2 cells as the three IL-4-producing cell types that appear in the lungs of mice infected with the migrating intestinal helminth, *Nippostrongylus brasiliensis*. Eosinophils were most prevalent, peaking by ~1000-fold on day 9 after infection, with Th2 cells and basophils at 3- and 10-fold lower numbers, respectively. Eosinophil and basophil expansion in blood in response to parasites and their capacity for IL-4 expression required neither Stat6 nor T cells. Th2 induction and expansion in draining lymph nodes was also Stat6 independent. In contrast, eosinophil (and Th2 cell) recruitment to the lung was dependent on Stat6 expression by a bone marrow-derived tissue resident cell, whereas basophil recruitment was Stat6 and IL-4/IL-13 independent but T cell dependent. Primary type 2 immune responses in the lung represent the focal recruitment and activation of discrete cell populations from the blood that have previously committed to express IL-4.

Introduction

Type 2 immunity is characteristic of host responses to pathogen invasion at cutaneous or mucosal sites and provides protection from intestinal helminths and biting arthropods. When dysregulated, these responses contribute to allergic diseases such as asthma and atopy. Considerable progress over the past few years has been made in cataloging the different cell types and effector molecules that contribute to type 2 immunity. In general, type 2 immunity is characterized by accumulation of the Th2 subset of CD4 T cells and orchestrated by the cytokines these cells produce, particularly IL-4, IL-13, IL-5, and IL-9. Infiltration of blood eosinophils and basophils, activation of tissue resident mast cells, and elevations of serum IgE contribute to alterations in the cutaneous or mucosal epithelial barrier that are thought to mediate expulsion of parasitic worms or insects, but also the pathology attributed to atopic diseases.

Current understanding of how these elements interact to coordinate this complex host response remains incomplete, in part due to interactions among rare cells with overlapping functions. IL-4, produced mainly by Th2 cells, can also be produced by NK T cells, γδ T cells, eosinophils, basophils, and mast cells (Brown et al., 1987; Howard et al., 1982; Moqbel et al., 1995; Seder et al., 1991; Tentori et al., 1988; Yoshimoto and Paul, 1994). IL-13 is likely produced by most of these cells as well. Both cytokines share interactions with the IL-4Rα chain and transmit signals via Stat6, which translocates to the nucleus and regulates expression of target genes. Mice deficient in IL-4/IL-13, IL-4Rα, or Stat6 develop inefficient type 2 immune responses against gastrointestinal parasites (McKenzie et al., 1999; Urban et al., 1998) and fail to display the pathologic features of experimental asthma (Akimoto et al., 1998; Kuperman et al., 1998).

Type 2 immunity is characterized by inflammation mediated in part by the release of toxic constituents from blood-borne myeloid cells recruited to the site of infection or allergen contact. The recruitment of eosinophils and Th2 cells to the lung has been shown to be Stat6 dependent (Akimoto et al., 1998), although the cell type that requires expression of Stat6 to mediate recruitment to the site of inflammation has not been identified. Further, control of basophil recruitment and activation remains poorly characterized, in part due to the lack of basophil-specific surface markers and their relatively low numbers. These cells can play an important role as an initial source of IL-4 and other factors that polarize the early type 2 immune response (Genovese et al., 2003; Luccioli et al., 2002; MacGlashan et al., 1994).

To examine the orchestration of type 2 immunity in the lung, we used experimental infection of mice with the gastrointestinal nematode, *Nippostrongylus brasiliensis*. After inoculation of larvae under the skin, organisms invade the venules, become trapped in the lung capillaries, translocate into the alveoli, ascend the trachea, are swallowed, and mature to adults in the small intestines (Ogilvie and Hockley, 1968). Naturally a pathogen of rats, *N. brasiliensis* is expelled from the mouse intestine after 10 days by a process dependent on CD4 T cells, IL-4/IL-13, the IL-4Rα chain, and Stat6 (Finkelman et al., 1997; Urban et al., 1998). IL-4-deficient mice can expel worms, consistent with the critical role for IL-13, although exogenous IL-4 can alone confer immunity to infected SCID mice (Lawrence et al., 1996). We used mice containing a bicistronic knock-in IL-4 gene linked via an internal ribosomal entry site (IRES) with enhanced green fluorescent protein (eGFP) (Mohrs et al., 2001) to characterize IL-4-producing cells recruited to the lungs of mice during infection with *N. brasiliensis*. In prior experiments, we were able to show a major role for eosinophils among innate IL-4-producing cells (Shinkai et al., 2002). Here, we use flow cytometry, microarray, and histologic analysis to identify, quantitate, and characterize all IL-4-producing cells that accumulate during a tissue type 2 immune response. We use this information to define distinct requirements for assembling the various cellular elements that comprise the host response involved in mucosal defense and allergy.

Results

Three Populations of IL-4-Expressing Cells Are Recruited to the Lung after *N. brasiliensis* Infection

Infection with *N. brasiliensis* results in potent type 2 immune responses during migration through the lung.

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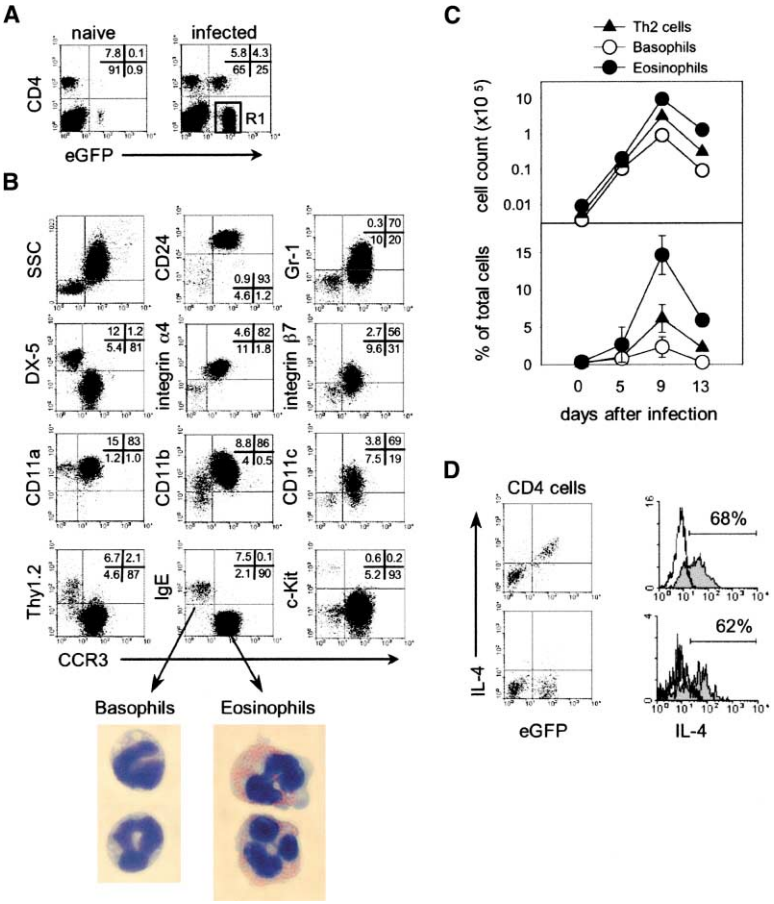

Figure 1. Flow Cytometric Analysis of Surface Markers on Eosinophils and Basophils

(A) Total lung cells of 4get mice before (left) and 9 days after (right) *N. brasiliensis* infection were stained for CD4 and analyzed by flow cytometry.

(B) Total lung cells from day 9 *N. brasiliensis*-infected 4get mice were stained for CD4, CCR3, and indicated surface markers. Dot plots are gated on $CD4^{-}GFP^{+}$ cells from R1 (indicated in [A]). Eosinophils ($CCR3^{+}IgE^{-}$) and basophils ($CCR3^{-}IgE^{+}$) were sorted from *N. brasiliensis*-infected lungs and stained with DiffQuick.

(C) Kinetics of IL-4-expressing cells in the lung after *N. brasiliensis* infection. Total and relative cell numbers of the indicated populations were determined by flow cytometry.

(D) IL-4 secretion was assessed by the IL-4 capture assay as described in Experimental Procedures. Gated CD4 T cells were analyzed for eGFP expression and surface bound IL-4 protein. The upper dot plot shows cells that were stimulated with PMA/ionomycin and the lower dot plot shows unstimulated cells. The histograms on the right show IL-4 secretion from stimulated (filled histogram) and unstimulated (open histogram) total lung cells gated on eosinophils ($CD4^{-}eGFP^{+}SSC^{hi}$; upper histogram) and basophils ($CD4^{-}eGFP^{+}SSC^{lo}$; lower histogram).

To visualize IL-4-expressing cells, we used 4get mice, which express IL-4 and eGFP from a bicistronic mRNA and thus allow the detection of IL-4-expressing cells directly by flow cytometry without the need for restimulation ex vivo (Mohrs et al., 2001). Prior to infection, very few cells, of which 90% were non-CD4 T cells, expressed eGFP among dispersed lung cells. By 9 days after infection, however, approximately half of the lung CD4 T cells expressed eGFP (Figure 1A). Even larger numbers of eGFP-positive cells were CD4-negative, encompassing the eosinophil population we have previously described (Shinkai et al., 2002). Using forward- and side-scatter (SSC) characteristics along with additional surface markers, we could clearly delineate a population distinct from the small numbers of SSC^{lo} eosinophils. Eosinophils expressed the chemokine receptor CCR3 (Ponath et al., 1996), whereas the major SSC^{lo} population did not (Figure 1B). In contrast, the high-affinity receptor for IgE, which is expressed on basophils and mast cells (Kinet, 1999), was expressed by the $SSC^{lo}CCR3^{-}$, but not the $SSC^{hi}CCR3^{+}$, population. Other surface markers, including CD24, CD11c, CD11b, Gr-1, α_4- and β_7-integrin, were also expressed at high levels on the $CCR3^{+}$ eosinophils, whereas only the $CCR3^{-}$ population was positive for IgE, CD69, DX-5, and Thy1.2 (Figure 1B). Both populations were negative for CD3, $\gamma\delta$ TCR, and $\alpha\beta$ TCR (data not shown). Unlike mast cells, which express the high-affinity IgE receptor and are generally FSC^{hi}/SSC^{hi} and $c\text{-}kit^{+}$, the $eGFP^{+}CCR3^{-}$ cells were largely FSC^{lo}/SSC^{lo} and $c\text{-}kit^{-}$, consistent with their identification as basophils. Cytospin preparations of $CCR3^{-}IgE^{+}$ and $CCR3^{+}IgE^{-}$ cells revealed cells with histologic and morphologic criteria consistent with their identification as basophils and eosinophils, respectively (Figure 1B).

Approximately 1000 eosinophils and slightly fewer basophils could be recovered from the lung of uninfected mice. These cells were spontaneously fluorescent, indicating constitutive activation of the IL-4 gene (Figure 1A and data not shown). After infection, eosinophils increased about 1000-fold ($9.7 \pm 2.3 \times 10^5$ cells), and remained the most prevalent $eGFP^{+}$ cells throughout infection (Figure 1C). Each of the three $eGFP^{+}$ populations displayed similar kinetics, with Th2 cells and basophils peaking at $3.2 \pm 0.6 \times 10^5$ and $0.9 \pm 0.18 \times 10^5$ cells, respectively (Figure 1C). Maximum recovery occurred on day 9, by which time adult worms were expelled from the intestines (data not shown), and then declined.

To confirm that eGFP expression was accurately marking cells with the capacity to secrete IL-4, we collected lung cells at day 9 of infection during the peak of the response and examined IL-4 protein production using surface antibody capture (Figure 1D). Without stimulation, none of the $eGFP^{+}$ populations showed significant IL-4 secretion, indicating that cytokine secretion is tightly regulated. The discordance between eGFP and IL-4 protein production, despite translation from a single

bicistronic mRNA, likely reflects the presence of the IRES upstream of eGFP, which establishes permissive translation otherwise constrained at the 5′-cap in resting cells (Stetson et al., 2003). After stimulation with PMA/ionomycin, however, essentially all of the eGFP$^+$ CD4 T cells secreted IL-4 protein in direct proportion to their fluorescence intensity (Figure 1D). The majority of eGFP$^+$ eosinophils and basophils also secreted IL-4 protein after stimulation. Notably, very few eGFP$^-$ CD4 T cells (or other eGFP$^-$ cells among dispersed lung cells) stained with the IL-4 capture reagent after stimulation (Figure 1D), indicating that secreted IL-4 did not contaminate neighboring cells. Thus, eGFP fluorescence reliably identifies both innate and adaptive cells capable of IL-4 expression and secretion during a type 2 immune response in the lung.

NK T Cells, γδ T Cells, or Mast Cells Do Not Contribute to the Primary Th2 Response in the Lung

NK T cells comprise a specialized population of CD1d-restricted T cells poised for the rapid production of cytokines, including IL-4 (Yoshimoto and Paul, 1994). Despite their role in early cytokine production in other systems, we could find no evidence for the activation of NK T cells after *N. brasiliensis* infection. First, there was no increase in the small numbers of cells that stained with CD1d tetramers loaded with α-galactosylceramide in the lung on day 9 after infection. Second, the numbers and phenotypes of eGFP$^+$ cells were the same in 4get/CD1d-deficient mice, in which canonical NK T cells are absent (data not shown). In addition, γδ T cells were present only at a low frequency (~1% of total cells on day 9 after infection), but did not express eGFP (data not shown). Thus, in this infection model, canonical Th2 cells comprise the only IL-4-producing T cells recruited to the lung.

Mast cells comprise a second population of resident tissue cells that can produce IL-4 in other systems (Bradding et al., 1992). As resident tissue cells, mast cells may not have been reliably dispersed from tissues to allow their detection. Using histochemical methods sufficient to detect mast cells in intestines, however, we were unable to find significant numbers of mast cells in the lung, either at rest or 9 days after infection with *N. brasiliensis* (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/20/2/267/DC1). Similar observations have been made previously in the mouse lung, demonstrating that chronic antigenic stimulation is required to induce mast cell populations in this tissue (Ikeda et al., 2003). Thus, in this infection model, eosinophils and basophils appear to be the only major innate IL-4-producing cells and, like Th2 cells, are recruited to the lung.

Microarray Analysis Supports the Identification of Eosinophils and Basophils

Despite our characterization using multiple surface markers and histochemistry, the identification of basophils remains incomplete. Mast cell protease 8 is one of few putative basophil-specific markers described in the mouse (Lutzelschwab et al., 1998). To confirm their identity and extend our ability to define eosinophils and basophils, we isolated highly purified populations of eGFP$^+$ eosinophils and basophils from the lungs of infected 4get mice and analyzed their gene expression profiles by microarray. Eosinophils were compared directly to basophils (E/B), eosinophils were compared to a general reference pool (E/R), and basophils were compared to the reference pool (R/B) (Figure 2). Hybridization in this way allowed the detection of transcripts expressed differentially in eosinophils and basophils, but also transcripts expressed at similar levels in eosinophils and basophils but at different levels compared to the reference pool. The direct comparison of eosinophil and basophil RNA revealed that 868 genes were more than 2-fold, and 285 genes were more than 3-fold, differentially expressed between these populations.

The group I (eosinophil) subset contained known eosinophil-specific genes, including major basic protein, eosinophil peroxidase, and eosinophil-associated ribonuclease 11 (Figure 2). Expression of some of the genes in this group, including CCR3, FIRE, CD11c, and CD24, was confirmed at the protein level using flow cytometry (Figures 1 and 3). The transcription factor GATA-1, an obligatory lineage-specific element in eosinophil development (McNagny and Graf, 2002), was highly expressed, as were several inflammatory proteins associated with type 2 immune responses, such as the EBI3 component of IL-27 (Nieuwenhuis et al., 2002), IL-1 receptor antagonist (IL-1Ra), and resistin-like α/FIZZ1 (Arend, 2002; Holcomb et al., 2000).

The group II (basophil) subset contained the high-affinity IgE receptor α chain and mast cell protease 8, consistent with their identification as basophils. The transcription factor GATA-2, required for mast cell progenitors, was also expressed (Walsh et al., 2002). CCR2 appeared to be the only chemokine receptor that was expressed highly in basophils but not in eosinophils or the reference pool. The activating receptor 2B4 and the H4 histamine receptor, shown to be expressed in human basophils and mast cells (Hofstra et al., 2003; Nakajima et al., 1999), were present in mouse basophils, as was the IL-18 receptor, which can induce the release of IL-4 and histamine from basophils (Yoshimoto et al., 1999). Although IL-4 expression was higher in basophils than in eosinophils, IL-4 transcripts were readily apparent in both populations, consistent with the functional studies (Figures 1B and 1D).

Although basophils preferentially expressed the chemokines CCL3 (MIP1α) and CCL4 (MIP1β), which are chemotactic for activated T cells (Taub et al., 1993), both eosinophils and basophils expressed high levels of CCL6 (C10) and CCL17 (TARC), which have been associated previously with type 2 immune responses (Hogaboam et al., 1999; Mathew et al., 2001) (Figure 2, group III). Taken together, the gene expression data support identification of these innate IL-4-producing cells as eosinophils and basophils, and extend information regarding surface markers and transcripts present in these cells during type 2 immune responses in vivo.

Eosinophils and Basophils Are Constitutively Fluorescent in Blood of Uninfected 4get Mice and Modulate Their Membrane Protein Expression in Inflammatory Tissue

Prior analysis in 4get mice demonstrated that NK T cells in spleen, in contrast to conventional naive T cells, con-

I

unigene symbol - name	unigeneID	E/B M	E/B A	E/R M	E/R A	R/B M	R/B A
Adam19	Mm.89940	2.94	11.69	4.19	10.68	-1.88	9.89
Ccr3 - Chemokine receptor 3	Mm.57050	1.05	10.87	1.14	9.96	-0.12	9.84
Cd24a - CD24a antigen	Mm.6417	1.84	13.94	1.71	15.01	-0.76	14.39
Clec2-pending - C-type lectin-like rec. 2	Mm.30700	1.88	9.69	1.20	8.29	-0.05	8.04
Clecsf6 - DCIR	Mm.47384	1.60	9.88	2.41	8.55	-0.60	8.71
Csf1r - M-CSF receptor	Mm.22574	3.04	12.40	4.01	11.64	-0.85	9.40
Csf2ra - GM-CSF receptor alpha	Mm.156264	1.47	9.10	1.70	8.06	-0.20	8.35
Ctsh - Cathepsin H	Mm.2277	3.55	11.90	5.35	10.96	-3.12	9.90
Cxcl2 - MIP-2	Mm.4979	2.00	13.22	5.02	10.98	-4.40	10.66
Cysltr2 - cys leukotriene receptor 2	Mm.158324	1.60	9.95	1.92	8.88	-0.56	8.34
Dok2 - Downstream of tyrosine kinase 2	Mm.10761	1.73	11.95	3.70	10.29	-1.73	9.83
Ear11 - eosinophil-ass. ribonuclease 11	Mm.153688	1.90	13.88	4.93	11.79	-2.83	9.70
Ebi3 - Epstein-Barr virus induced gene 3	Mm.7254	2.21	11.55	2.91	10.28	-1.08	8.73
Emr4 - D17Ertd479e - FIRE	Mm.210497	2.03	9.75	3.70	9.35	-0.85	8.10
Epx - Eosinophil peroxidase	Mm.1315	2.79	10.11	2.99	10.53	-1.00	9.03
F5 - Coagulation factor V	Mm.12900	1.95	9.03	2.55	8.43	-0.38	7.94
GATA-1	Mm.1344	1.33	12.91	3.19	10.70	-1.77	9.95
H2-Ea - MHC class II, E of d alpha chain	Mm.15680	1.10	10.09	2.65	9.85	-1.75	9.38
H2-Q8 - Histocompatibility 2, Q8	Mm.234168	1.54	9.11	1.50	8.64	-0.35	8.29
Hck - Hemopoietic cell kinase	Mm.715	3.67	14.07	4.75	12.96	-2.91	10.70
Hspg2 - Perlecan	Mm.7257	2.77	12.39	4.02	10.25	-1.59	9.05
Il10rb - Interleukin 10 receptor, beta	Mm.4154	1.94	14.02	2.64	13.21	-0.70	13.55
Il1rn - Interleukin 1 receptor antagonist	Mm.882	1.70	14.87	4.71	13.75	-3.47	12.36
Irf1 - Interferon regulatory factor 1	Mm.1246	1.35	12.68	1.50	12.07	-0.67	12.91
Irf5 - Interferon regulatory factor 5	Mm.6479	1.77	10.48	1.58	10.15	0.07	8.94
Itgax - integrin alpha X (CD11c)	Mm.22378	3.10	13.22	4.09	10.95	-0.50	8.51
Marco	Mm.1856	1.80	9.51	3.40	8.87	-0.45	7.78
Mmp15 - Matrix metalloproteinase 15	Mm.7283	2.88	13.16	4.27	11.19	-1.63	9.34
Ncf2 - Neutrophil cytos. factor 2 (p67phox)	Mm.10729	1.35	13.19	4.67	10.27	-3.42	10.44
Ncf4 - Neutrophil cytos. factor 4 (p40phox)	Mm.2068	1.48	13.73	3.81	11.18	-2.47	10.77
Nos2 - Nitric oxide synthase 2, inducible	Mm.2893	1.62	9.42	1.12	8.54	0.15	7.90
Oas1a - 2'-5' oligoadenylate synthetase 1A	Mm.14301	1.80	11.48	1.53	10.67	0.11	9.87
Pira1 - Paired-Ig-like receptor A1	Mm.193462	1.24	10.00	3.31	8.62	-1.40	8.62
Prg2 - Proteoglycan 2 (major basic protein)	Mm.142727	3.75	11.01	4.99	10.57	-0.33	8.42
Ptpns1 - SHP-1	Mm.1682	1.94	12.56	2.36	10.78	-0.68	9.69
Retnla - Resistin like alpha (FIZZ1)	Mm.33772	2.49	14.36	5.09	12.64	-3.07	10.12
S100a9 - Calgranulin B	Mm.2128	2.34	10.34	3.05	9.47	-1.06	8.99
Sla - Src-like adaptor protein	Mm.7601	1.65	11.49	2.35	9.89	-0.42	9.20
Slc26a2 - Sulfate transporter	Mm.24803	2.97	11.79	3.64	11.91	-0.94	9.03

II

unigene symbol - name	unigeneID	E/B M	E/B A	E/R M	E/R A	R/B M	R/B A
C3ar1 - Complement 3a receptor 1	Mm.2408	-1.75	12.75	3.26	9.58	-3.62	12.19
Ccl3 - MIP-1alpha	Mm.1282	-1.85	15.12	2.17	13.24	-2.75	14.43
Ccl4 - MIP-1beta	Mm.1255	-3.22	11.39	-0.18	8.12	-2.43	10.67
Ccl9 - MIP-1gamma	Mm.2271	-4.27	12.73	1.48	11.61	-3.48	14.23
Ccr2 - Chemokine receptor 2	Mm.6272	-4.18	12.83	0.71	7.83	-5.14	11.19
Cpa3 - Carboxypeptidase A3, mast cell	Mm.1135	-4.16	11.27	0.02	8.23	-5.55	12.82
Csf1 - Colony stimulating factor 1	Mm.795	-1.34	11.16	-0.36	9.21	-1.75	10.40
Csf2rb2 - IL-3 receptor class II beta chain	Mm.1940	-2.16	10.50	-0.02	8.63	-2.03	10.26
Ctsg - Cathepsin G	Mm.4858	-3.87	11.06	0.04	7.30	-3.06	10.72
Cyp11a - CytP450, 11a	Mm.108678	-4.85	11.10	0.15	7.34	-5.30	12.58
Emb - Embigin	Mm.89123	-2.80	11.52	-0.06	9.56	-1.50	10.47
Fcer1a - Fce receptor I alpha	Mm.5266	-3.00	9.77	0.64	8.52	-3.76	11.40
Fyb - FYN binding protein	Mm.803	-1.03	12.61	2.79	10.34	-3.04	11.77
GATA-2	Mm.1391	-3.99	13.63	0.56	8.98	-3.10	13.96
Gp49a/b - Glycoprotein 49 A/B	Mm.34408	-2.73	14.34	2.52	11.76	-3.83	13.48
Gpr34 - G protein-coupled receptor 34	Mm.89979	-1.06	12.22	1.91	9.02	-3.12	9.97
Gpr97 - G protein-coupled receptor 97	Mm.27995	-2.34	14.74	3.97	9.57	-5.06	12.29
Gzmb - Granzyme B	Mm.14874	-2.10	9.72	0.08	7.62	-1.23	8.48
H2-Q7 - Histocompatibility 2, Q7	Mm.34421	-1.33	12.74	-1.47	10.47	-2.14	12.20
Hcst - DAP10	Mm.24026	-1.49	14.48	3.03	10.56	-3.01	10.73
Hrh4 - Histamine H4 receptor	Mm.207073	-3.57	9.51	0.06	7.34	-3.32	9.97
Il4 - Interleukin 4	Mm.371	-1.40	12.27	1.87	9.09	-2.40	10.24
Il5 - Interleukin 5	Mm.4461	-2.07	8.67	0.16	7.27	-1.49	8.48
Il6 - Interleukin 6	Mm.1019	-3.18	10.03	0.07	9.21	-3.15	10.76
Il7r - Interleukin 7 receptor	Mm.389	-2.19	9.55	0.41	7.99	-2.73	9.15
Il15 - Interleukin 15	Mm.4392	-2.01	10.58	-0.08	8.41	-1.63	9.14
Il18r1 - Interleukin 18 receptor 1	Mm.4773	-4.25	11.35	1.06	7.95	-4.56	11.60
Il18rap - Interleukin 18 rec. accessory prot.	Mm.20466	-2.37	10.55	0.17	8.17	-2.29	9.35
Il1rl1 - Interleukin 1 receptor-like 1	Mm.35692	-1.29	13.60	2.83	10.86	-2.70	11.52
Isg20 - Interferon-stimulated protein (20kD)	Mm.19029	-1.80	9.90	-0.13	8.89	-1.66	9.69
Itk - IL2-inducible T-cell kinase	Mm.16009	-1.94	9.94	0.34	8.40	-1.64	10.03
Jak2 - Janus kinase 2	Mm.809	-2.67	10.79	-0.55	9.51	-1.50	9.29
Mcpt8 - Mast cell protease 8	Mm.41979	-4.35	10.92	0.01	7.25	-4.29	11.54
Mox2r - OX2 receptor	Mm.143773	-1.37	9.92	0.99	9.03	-2.63	9.79
Nmrk - 2B4	Mm.2299	-1.90	9.38	1.20	7.90	-1.50	8.29
P2ry1 - Purinergic receptor P2Y	Mm.3556	-3.88	10.14	-0.13	8.53	-3.19	10.61
Pglyrlp-pending - PGRPL	Mm.86752	-2.59	9.87	0.50	7.81	-1.40	8.36
RGS1 - Regulator of G-protein signaling 1	Mm.103701	-2.18	13.13	2.38	10.84	-3.77	12.37
Serpina3g - Serine protease inhibitor 2-1	Mm.15085	-3.53	10.77	-0.08	7.77	-1.87	8.77
Serpinb1a - Serine proteinase inhibitor	Mm.46316	-3.13	14.17	-0.33	11.08	-3.38	14.23
Tec - Cytoplasmic tyrosine kinase	Mm.2350	-1.22	14.25	2.04	10.48	-3.58	12.01
Tlr1 - Toll-like receptor 1	Mm.33922	-2.48	10.19	-0.22	8.93	-1.54	10.30
Tnfrsf18 - GITR	Mm.3180	-1.36	12.25	0.61	10.08	-1.73	10.23
Wbscr5 - Williams-Beuren syndrome 5	Mm.23955	-3.67	13.17	1.08	10.65	-2.20	14.05

III

unigene symbol - name	unigeneID	E/B M	E/B A	E/R M	E/R A	R/B M	R/B A
Abcg1 - ATP-binding cassette	Mm.15691	0.92	12.06	3.01	10.70	-1.97	10.67
Adam8	Mm.15969	0.33	12.72	3.59	10.56	-2.14	9.39
Alox5ap - Arach. 5-lipoxyg. act. protein	Mm.19844	0.03	15.31	5.37	12.39	-4.38	13.05
Btk - Bruton tyrosine kinase	Mm.4475	0.83	13.55	2.94	10.62	-2.02	10.00
Ccl17 - TARC	Mm.41988	0.48	10.39	2.22	9.05	-1.89	10.04
Ccl6 - C10	Mm.137	-0.61	15.31	4.58	12.13	-3.73	12.59
Cd52 - CD52 antigen	Mm.24130	0.79	14.50	4.47	11.84	-3.35	11.82
Crlf3 - Cytokine rec.-like factor 3 (Crm-2)	Mm.33962	-0.30	15.26	2.31	13.47	-2.52	13.76
Ctse - Cathepsin E	Mm.33671	0.81	14.21	3.72	11.62	-2.86	12.77
Fcer1g - Fce receptor I gamma	Mm.22673	-0.30	16.00	3.42	12.94	-3.55	13.16
Fcgr2b - Fcg receptor IIb	Mm.10809	-0.54	11.66	2.29	10.74	-2.52	10.75
Fcgr3 - Fcg receptor III	Mm.22119	-0.18	14.88	5.34	12.29	-4.41	12.50
Gpr43 - G protein-coupled receptor 43	Mm.97338	0.90	11.58	3.97	9.52	-2.33	8.92
H2-T23 - Histocomp. 2, T region locus 23	Mm.35016	-0.57	15.21	2.65	12.31	-2.66	12.58
Ltb4r1 - Leukotriene B4 receptor 1	Mm.20853	-0.28	12.52	3.22	10.64	-2.51	10.80
Lyn - Protein tyrosine kinase	Mm.1834	0.60	15.16	4.97	13.17	-3.68	13.33
Prg - Proteoglycan, secretory granule	Mm.22194	-0.75	11.13	3.99	11.10	-3.48	10.88
Rgs14 - Regulator of G-protein signaling 14	Mm.1426	0.63	12.41	3.48	10.43	-2.94	10.93
Rgs2 - Regulator of G-protein signaling 2	Mm.28262	0.45	14.35	5.23	12.74	-4.04	12.15
Socs1 - suppressor of cytokine signaling 1	Mm.130	-0.58	12.68	1.75	10.89	-2.11	10.77
Sorl1 - Sortilin-related receptor	Mm.5396	0.55	12.97	5.00	11.38	-3.96	10.92
Tln - Talin	Mm.4053	0.44	14.74	4.37	12.73	-3.49	14.02
Tmsb4x - Thymosin, beta 4	Mm.142729	0.06	15.10	3.34	13.60	-3.00	11.78
Tslpr - Thym. strom.-der. lymphopoietin rec.	Mm.35771	-0.19	14.21	3.93	11.26	-3.84	12.56
Tyrobp - DAP12	Mm.46301	-0.74	14.25	3.25	11.15	-2.41	11.86

Figure 2. Gene expression profile of purified eosinophils and basophils.

Eosinophils and basophils were sorted from the lung 9 days after *N. brasiliensis* infection. Total RNA was extracted, amplified, labeled and hybridized to oligonucleotide-spotted microarray slides. Three hybridizations were performed: eosinophils vs. basophils (E/B), eosinophils vs. reference (E/R) and reference vs. basophils (R/B). The entire data set can be retrieved from the GEO database at NCBI (GSM15485, GSM15486, GSM15487). Due to space limitations, genes of known or potential immunological interest were selected that passed threshold levels for signal intensity (A>9 in at least one of the three arrays) and signal ratio (M>1 in E/B and E/R for group I, M<1 in E/B and R/B for group II, and M>1 in E/R and M<1 in R/B for group III). The definitions for A and M values are given in Experimental Procedures. This resulted in a set of genes that are relatively highly expressed in eosinophils (group I), in basophils (group II), or in both (group III). Orange, red, light green and dark green colors indicate M values >1, >2, <-1 and <-2, respectively. Genes are shown in alphabetic order of the Unigene symbol names.

stitutively express IL-4 mRNA but not IL-4 protein, revealing their capacity for rapid IL-4 secretion upon stimulation (Stetson et al., 2003). Similarly, eosinophils and basophils, which can be distinguished by forward- and side-scatter characteristics and expression of CCR3 and high-affinity FcεR, respectively, were constitutively

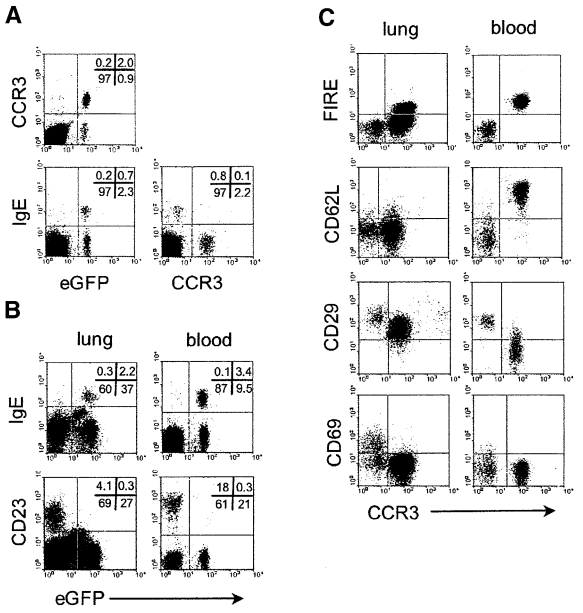

Figure 3. Activation Markers on Eosinophils and Basophils

(A) Total cells from the blood of noninfected 4get mice were analyzed for expression of IgE and CCR3.

(B) Total cells from lung and blood samples of *N. brasiliensis*-infected 4get mice were analyzed for surface expression of IgE and CD23.

(C) Gated $CD4^{-}GFP^{+}$ cells from lung and blood of day 9 *N. brasiliensis*-infected mice were analyzed for surface expression of the indicated markers.

fluorescent in the blood of uninfected 4get mice, comprising populations of ~2% and ~0.8% of total blood cells (Figure 3A). At the peak of the tissue response on day 9 after infection, eosinophils and basophils comprised up to 10% and 3%, respectively, of circulating blood cells (Figure 3B). To distinguish whether surface IgE on basophils was bound to the high-affinity or the low-affinity (CD23) IgE receptor, cells from the blood and lung of *N. brasiliensis*-infected mice were stained with anti-IgE and anti-CD23. All of the IgE-positive cells in both sites appeared to be eGFP-positive, whereas all of the CD23-positive cells were eGFP-negative (Figure 3B). Thus, the detectable cell-surface IgE is bound to the high-affinity IgE receptor and basophils are the predominant cells that express this receptor in the blood in this infection model.

Comparison of eosinophils and basophils in the blood and the lung on day 9 after infection revealed modulation of surface markers consistent with either the local activation of these innate cells within inflammatory tissues or the selective recruitment of activated cells to sites of inflammation. Eosinophils in blood expressed high levels of FIRE, an F4/80-like transmembrane receptor, but apparently downregulated this receptor after recruitment to the lung, similar to the downregulation of F4/80 on activated macrophages (Figure 3C) (Caminschi et al., 2001). CD62L (L-selectin) was similarly present on eosinophils in the blood and downregulated in the lung. In contrast, eosinophils expressed low levels of CD29 (integrin β1) in the blood but upregulated CD29 in the lung; β2 and β7 integrin expression was not different (data not shown). As compared to basophils in blood, basophils in the lung upregulated CD69, consistent with their local activation (Figure 3C). The apparent modulation of the cell surface phenotypes suggests that activation of these innate effector cells is tightly restricted to sites of inflammation.

Recruitment of Basophils and Eosinophils to the Lung

The finding that eosinophils and basophils were poised for IL-4 secretion, as revealed by their constitutive fluorescence in 4get mice, suggested that recruitment of these discrete cell types from blood might be the critical step in orchestrating type 2 immunity. Efficient type 2 immunity does not develop in mice deficient in Stat6, perhaps reflecting the role of Stat6 in mediating the expression of chemokines, like CCL11 (eotaxin), CCL24 (eotaxin 2), and CCL17 (TARC), that have been associated with tissue recruitment of effector cells (Hoeck and Woisetschlager, 2001; Mathew et al., 2001).

To analyze the role of Stat6 and the adaptive immune system in the recruitment of eosinophils and basophils to the lung, we infected 4get, 4get/$Stat6^{-/-}$, and 4get/$Rag^{-/-}$ mice with *N. brasiliensis* and analyzed the blood and lung on day 9 after infection. At this time, infected 4get mice, as noted previously, demonstrated marked expansion of eosinophils and basophils in the blood and lung, as identified primarily through discrimination of SSC, CCR3, and high-affinity IgE receptor expression among $eGFP^{+}$ cells (Figure 4). In contrast, infected 4get/$Stat6^{-/-}$ and 4get/$Rag^{-/-}$ mice had greatly reduced numbers of eosinophils in the lung, although expansion of eosinophils in blood was comparable to wild-type mice (Figures 4A and 4C). 4get/$Stat6^{-/-}$ mice showed no defect in basophil recruitment to the lung, which indicates that eosinophil and basophil recruitment are regulated differently. In these latter mice, basophils did not stain for surface IgE, due to the defect in class switch recombination in Stat6-deficient mice (Figure 4A) (Shimoda et al., 1996; Takeda et al., 1996). Strikingly, 4get/$Rag^{-/-}$ mice showed a complete block in basophil recruitment. This was not due to a defect in mobilization of basophils from the bone marrow, as these cells were present in the blood at levels similar to infected wild-type mice (Figure 4A). Thus, the recruitment of basophils appears to be independent of Stat6 but requires the adaptive immune system, whereas the efficient recruitment of eosinophils requires both Stat6 and adaptive immunity.

Stat6 in the Recruitment of Type 2 Immune Cells

As shown previously, Stat6 deficiency has a greater effect on the recruitment of IL-4-expressing Th2 cells to the lung than on their induction in draining lymph nodes (Mohrs et al., 2001). In agreement with these studies, infected 4get/$Stat6^{-/-}$ mice had a 2-fold reduction in numbers of Th2 cells in the tracheal lymph nodes, but recruitment to the lungs was reduced over 80% compared to wild-type 4get mice (Figure 4D). This was not due to reduced proliferation of T cells from 4get/$Stat6^{-/-}$ mice, since T cells in the lung and lymph nodes from both mice incorporated bromodeoxyuridine (BrdU) with similar efficiency during an 18 hr pulse period be-

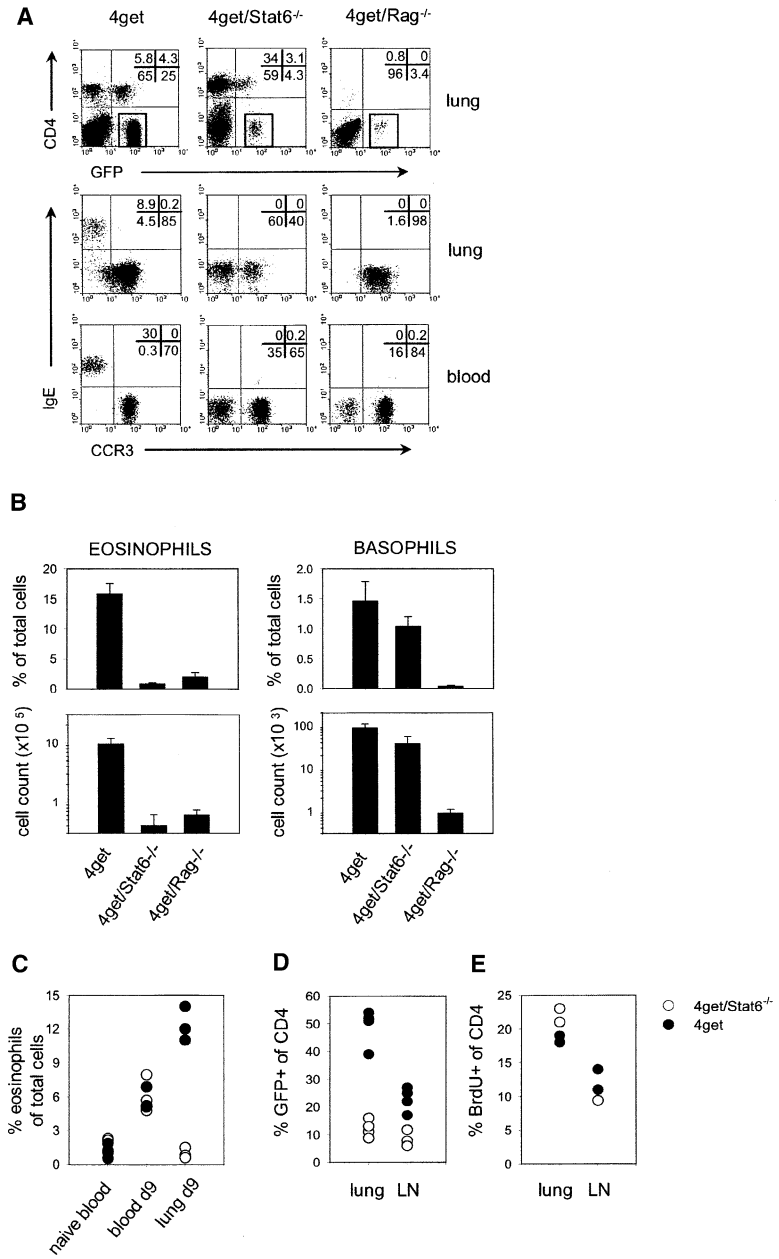

Figure 4. Eosinophil and Basophil Recruitment to the Lung of *N. brasiliensis*-Infected Mice

(A) 4get, 4get/Stat6$^{-/-}$, and 4get/Rag$^{-/-}$ mice were infected with *N. brasiliensis* 9 days before analysis of lung and blood samples. Cells were stained for CD4, CCR3, and IgE and analyzed by flow cytometry. Plots in the first row are gated on total live cells from the lung and show expression of CD4 and eGFP. Plots in the second row are gated on CD4$^-$eGFP$^+$ cells as indicated in the first row and show the relative abundance of eosinophils (CCR3$^+$) and basophils (CCR3$^-$) in this gate. Plots in the third row are gated on CD4$^-$eGFP$^+$ from the blood.

(B) Bar graphs show the percentage among total lung cells and absolute numbers of eosinophils and basophils in the lung 9 days after *N. brasiliensis* infection of the indicated mice. 7–9 mice per group were analyzed and results were pooled from independent experiments. $P < 0.001$ for 4get versus 4get/Stat6$^{-/-}$ or 4get/Rag$^{-/-}$ eosinophils and 4get versus 4get/Rag$^{-/-}$ basophils. $P = 0.026$ for 4get/Stat6$^{-/-}$ versus 4get/Rag$^{-/-}$ basophils.

(C) The dot plot shows a comparison of blood and lung eosinophilia in naive versus infected 4get (filled circles) or 4get/Stat6$^{-/-}$ (open circles) mice.

(D and E) Recruitment of Th2 cells to the lung depends on Stat6. 4get (filled circles) and 4get/Stat6$^{-/-}$ (open circles) mice were infected with *N. brasiliensis*. BrdU was injected i.p. on day 6.5 and mice were analyzed on day 7 after infection. (D) and (E) show the percentage of GFP$^+$ and BrdU$^+$ cells among total CD4 cells in the tracheal lymph node (LN) and lung, respectively.

tween day 6 and 7 after infection (Figure 4E). Thus, the defect in type 2 immunity in Stat6-deficient mice is due to a recruitment defect of Th2 cells and eosinophils and not due to failure either to expand their precursor numbers or to initiate IL-4 expression in any of the key cells in the response—eosinophils, basophils, and Th2 cells—at least as assessed by surrogate expression of eGFP.

The deficiency in Th2 and eosinophil recruitment in the absence of Stat6 could be due to requirements for Stat6 expression in either hematopoietic or nonhematopoietic cells, or both. To address this issue, we lethally irradiated BALB/c or Stat6$^{-/-}$ mice and reconstituted them with bone marrow cells from 4get or 4get/Stat6$^{-/-}$ mice. Ten weeks later, chimeric mice were infected with *N. brasiliensis* and analyzed 9 days later for intestinal worms, serum IgE, and eosinophil and Th2 cell recruitment to the lungs (Figure 5). Worm expulsion from the small intestine was dependent on Stat6 expression in nonhematopoietic cells, consistent with a previous report showing the requirement for IL-4Rα on nonhematopoietic cells for worm expulsion (Urban et al., 2001). In contrast, IgE responses and effector cell recruitment to the lung were dependent on Stat6 expression in bone marrow-derived cells.

The finding that tissue recruitment of Th2 cells and eosinophils requires Stat6 expression by bone marrow-derived cells suggested the possibility that T cells themselves might provide a Stat6-dependent signal required for further recruitment. To assess whether Stat6- or IL-4/IL-13-expressing Th2 cells were required for tissue recruitment of eosinophils and basophils, 10^7 purified CD4

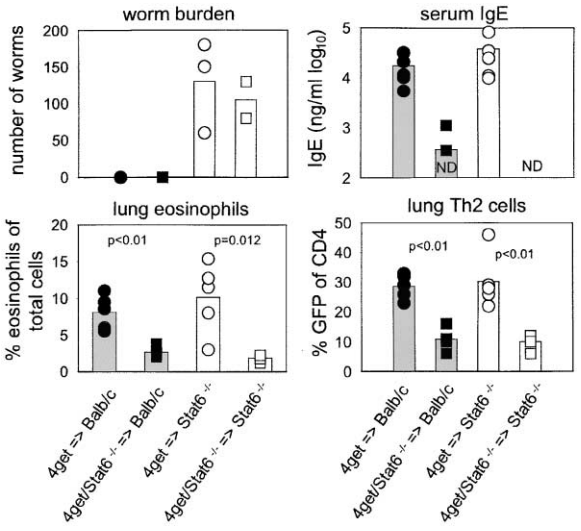

Figure 5. Eosinophil and Th2 Cell Recruitment Depends on Stat6 from a Bone Marrow-Derived Cell

Four sets of bone marrow chimeras were generated. Bone marrow from either wild-type 4get mice (circles) or 4get/Stat6$^{-/-}$ mice (squares) was transferred into lethally irradiated BALB/c mice (filled symbols) or Stat6$^{-/-}$ mice (open symbols). Ten weeks after reconstitution mice were infected with *N. brasiliensis* and analyzed 10 days later for worm burden in the intestine, serum IgE levels, and eosinophil and Th2 cell recruitment to the lung. ND = none detected. The indicated p-values were calculated by Student's t test.

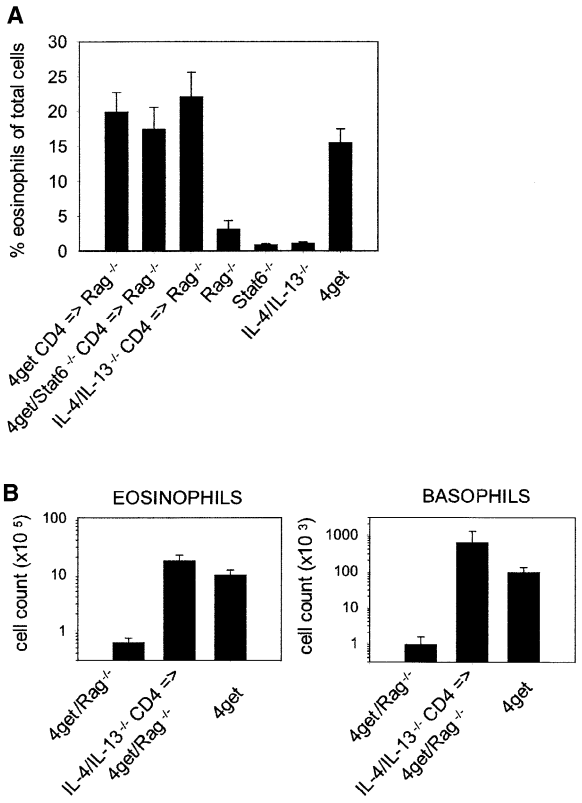

Figure 6. Stat6- or IL-4/IL-13-Deficient CD4 T Cells Induce Eosinophil and Basophil Recruitment

(A) 10^7 purified CD4 T cells from 4get, 4get/Stat6$^{-/-}$, or IL-4/IL-13$^{-/-}$ mice were transferred into Rag$^{-/-}$ recipients. Four days later mice were infected with *N. brasiliensis*. Normal 4get mice or Rag$^{-/-}$, Stat6$^{-/-}$, and IL-4/IL-13$^{-/-}$ mice that had not received T cell transfers were infected as controls. Lung eosinophils were quantitated 9 days later by flow cytometry. 6–13 mice per group were analyzed and results were pooled from different experiments. The p-values, calculated by Student's t test, were $p < 0.0001$ for all transfers versus Rag$^{-/-}$ mice.

(B) 4get/Rag$^{-/-}$ mice were reconstituted with 10^7 purified CD4 cells from IL-4/IL-13$^{-/-}$ mice and analyzed 9 days after *N. brasiliensis* infection for the total number of eosinophils and basophils in the lung. Unreconstituted 4get/Rag$^{-/-}$ mice and 4get mice were used as controls.

T cells from either 4get, 4get/Stat6$^{-/-}$, or IL-4/IL-13$^{-/-}$ mice were transferred into Rag$^{-/-}$ mice, which were then infected with *N. brasiliensis*. Unexpectedly, neither the recruitment of eosinophils nor basophils required expression of IL-4/IL-13 or of Stat6 by CD4 T cells (Figure 6). Thus, eosinophils and basophils are efficiently recruited to inflammatory sites by activated T cells in the absence of bona fide Th2 cells, but only if Stat6 is expressed in another hematopoietic cell.

Discussion

Using sensitive IL-4 reporter mice, we define the IL-4-expressing cells recruited to the lung during activation of a type 2 immune response to migrating intestinal parasites. Eosinophils were the most prevalent cell type, increasing up to 1000-fold, with Th2 cells and basophils comprising 3- and 10-fold lower cell numbers, respectively. Fluorescence consistent with IL-4 gene activation occurred in each of these populations prior to tissue recruitment, which was associated with modulation of cell surface markers indicating local activation. Hierarchical requirements for tissue recruitment were evident, with signals from activated T cells required for basophil recruitment and tissue-generated Stat6-dependent signals required for eosinophil and Th2 recruitment. Finally, and in agreement with prior observations, expression of Stat6 in nonhematopoietic tissue cells was required to transduce signals from the recruited type 2 immune cells to mediate biologic effector function, as assessed by worm expulsion. As revealed using this IL-4 reporter system, type 2 immunity represents the focal recruitment and activation of discrete cell populations from the blood that have been previously committed to express IL-4.

The identification of IL-4-expressing non-T, non-B cells with high-affinity IgE receptors in the lung and spleen of mice infected with *N. brasiliensis* was described initially over 10 years ago (Conrad et al., 1990). Eosinophils, however, were 10-fold more prevalent among these innate IL-4-expressing cells. Small numbers of SSC^{lo} eosinophils, consistent with degranulated cells, were present in tissues (Shinkai et al., 2002; Figure 1B), but the use of additional markers and rigorous genetic screening allowed us to identify two major nonlymphocyte IL-4-expressing populations. Morphologic criteria, display of distinct surface receptors, and expression of lineage-marking transcripts provide independently corroborating support for our identification of eosinophils and basophils as the innate cells mediating

type 2 immune responses, at least in the lung. Two unexpected findings, confirmed by cell surface staining, were the expression of CD11c, a prototypic dendritic cell marker, by eosinophils, and the expression of Thy1, previously believed restricted to T cells among mature blood elements, by basophils. Despite their capacity for rapid IL-4 secretion in other systems, NK T cells and mast cells did not appear to contribute substantially to tissue IL-4 production during type 2 immunity in the lung in this system. The few eGFP$^+$c-kit$^+$ cells in the lung (Figure 1B), possibly represent a common basophil/mast cell precursor as described in the human immune system (Kirshenbaum et al., 1992).

The microarray data suggest overlapping but distinct functions by eosinophils and basophils. This was supported by the demonstration of distinct requirements for their recruitment. Basophil recruitment, while Stat6 independent, was entirely T cell dependent. Eosinophil recruitment was greatly augmented by T cells, but required Stat6 expression by a bone marrow-derived cell other than CD4 T cells. A prior study, which used in vitro-generated Th2 cells transferred into Stat6-deficient mice in the setting of experimental asthma, suggested that Stat6 expression in lung fibroblasts might be required for Th2 recruitment (Mathew et al., 2001). By using bone marrow chimeric mice that expressed Stat6 in either tissue or bone marrow-derived cells, we could show that the recruitment of both Th2 cells and eosinophils was dependent on Stat6 expression by bone marrow-derived cells (Figure 6). We suspect that these cells are the source of eosinophil- and Th2-attracting chemokines (CCL11, CCL24, and CCL17) induced in wild-type but not Stat6-deficient mice during type 2 immunity (Mathew et al., 2001). Although we cannot exclude a population of fibroblasts that may have been reconstituted from bone marrow, we favor the hypothesis that a resident hematopoietic cell in the lung, such as a dendritic cell or alveolar macrophage, represents the Stat6-responsive cell required to initiate the first steps of immune cell recruitment.

Despite the role for Stat6 in the recruitment of type 2 immune cells and their capacity to mediate worm expulsion, Stat6 was not required for induction of IL-4 expression or for the expansion of IL-4-expressing cells in blood in response to helminth migration. Activation of IL-4 expression in naive T cells in lymph nodes draining the lung was only marginally affected by Stat6 deficiency. Surprisingly, eosinophils and basophils were spontaneously fluorescent in uninfected 4get mice, and this was unaffected by the absence of Stat6 (or IL-4Rα, data not shown), revealing activation of the IL-4 gene in both innate and adaptive immune cells to be a Stat6-independent pathway. Signals from tissue that mediate the induction of Th2 differentiation in lymphoid organs and the production and release of eosinophils and basophils from bone marrow remain the focus of further experiments. Intriguingly, however, the spontaneous eGFP fluorescence of eosinophils and basophils, as revealed using 4get mice, resembled the spontaneous expression of IFN-γ and IL-4/IFN-γ mRNA by NK and NK T cells, respectively. These cells reside in tissues poised with constitutive cytokine mRNA transcripts that correlated with their capacity for rapid activation and secretion of cytokines (Stetson et al., 2003). By analogy, eosinophils and basophils developmentally activate IL-4 expression, and this correlated with their capacity for rapid IL-4 secretion upon stimulation ex vivo. In contrast to NK and NK T cells, which reside primarily in tissues, however, eosinophils and basophils sustain a reservoir in blood that requires a second step for recruitment into tissues.

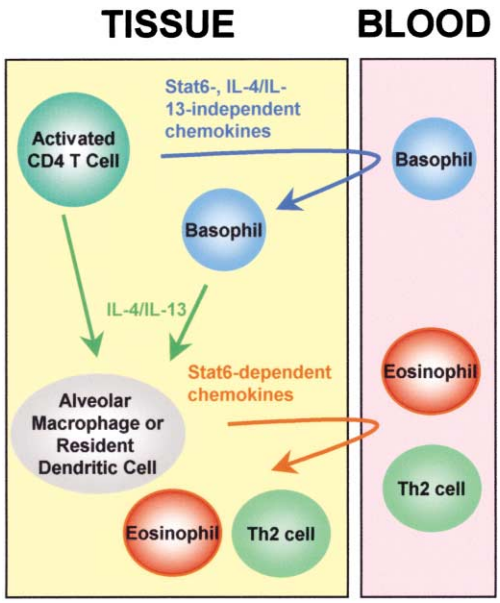

Figure 7. Model of Basophil, Eosinophil, and Th2 Cell Recruitment to the *N. brasiliensis*-Infected Lung

See text for details.

Unexpectedly, CD4 T cells incapable of producing IL-4 and IL-13, or CD4 T cells incapable of responding to IL-4/IL-13 signaling via Stat6, induced normal tissue recruitment of innate IL-4-producing cells (Figure 6). Thus, bona fide Th2 cells are not required, and other hematopoietic cells that produce IL-4 or IL-13 are involved. Indeed, a small number of eosinophils and basophils was already present in the lungs of uninfected mice (Figure 1A), indicating that a small, but identifiable, population of these innate cells exists in mucosal tissues and might contribute to early IL-4 release in response to worm migration. Indeed, secreted products from parasites or crosslinking of the IL-18 receptor can provoke IL-4 release from basophils (Phillips et al., 2003; Yoshimoto et al., 1999). In turn, IL-4/IL-13 could stimulate a resident bone marrow-derived cell, such as dendritic cells or alveolar macrophages, to express a set of Stat6-dependent products, including some with chemotactic activity for eosinophils (Gordon, 2003). Integration of these various observations into a model for induction of type 2 tissue responses in the lung is presented in Figure 7. Memory T cells, which reside in tissues, might generate Stat6-independent basophil-specific chemoattractants upon activation. IL-4, produced by basophils or other memory T cell populations, or IL-13 produced by other resident cell population(s), could induce eosinophil- and Th2-recruiting chemokines from a resident hematopoietic cell. The nature and source of systemic signals (apparently intact in *rag*-deficient mice) that mediate expansion of the blood reservoir of innate IL-4-

producing cells remain unknown. Identification of the early steps in the expansion and recruitment of these innate components of type 2 immunity will be important in defining novel therapeutic interventions for allergy, asthma, and atopic disease.

Experimental Procedures

Mice

BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Interleukin 4 reporter mice, designated 4get (IL-4 green fluorescent protein-enhanced transcript), were described (Mohrs et al., 2001). These mice express an eGFP reporter gene under control of an IRES element that has been inserted after the stop codon of the IL-4 gene by homologous recombination. Stat6$^{-/-}$ mice (Kaplan et al., 1996), Rag$^{-/-}$ mice (Mombaerts et al., 1992), and 4get mice were backcrossed at least 10 generations to BALB/c. IL-4/IL-13 double-knockout mice were used on the BALB/c background (McKenzie et al., 1999). 4get/Rag$^{-/-}$, 4get/Stat6$^{-/-}$, and 4get/CD1d$^{-/-}$ mice (Mendiratta et al., 1997) were generated by intercrossing. Mice were maintained in the specific pathogen-free animal facility at UCSF according to institutional guidelines.

Nippostrongylus brasiliensis infection

Third-stage larvae (L3) of *N. brasiliensis* were recovered from the cultured feces of infected rats, washed extensively, and injected (500 organisms) into mice subcutaneously at the base of the tail. Infected mice were placed on antibiotic-containing water (2 mg/l neomycin sulfate, 100 mg/l chloramphenicol) for 5 days and killed for analysis on the indicated days.

Cell Preparations

To analyze the recruitment of cells into the lung, mice were killed and the lungs were perfused via the right cardiac ventricle with 10 ml cold PBS. Lungs were transferred into 5 ml cold RPMI 1640 supplemented with 10% heat-inactivated FCS, cut into small pieces, and thoroughly minced through a 74 μm nylon mesh (Netwell^{M}, Corning Costar). The mesh was washed with another 5 ml cold RPMI 1640/10% FCS. Erythrocytes were lysed in hypotonic lysis buffer and cells were washed once in RPMI 1640/10% FCS. No major differences were observed between collagenase-digested or -undigested samples (data not shown) and lung samples for these studies were used without collagenase digestion. Lymph nodes were collected in cold medium and minced as described for lung samples. Blood from the lateral tail vein was collected in heparin-containing FACS buffer (2% FCS, 1 mg/l NaN_3).

Flow Cytometry

Cell suspensions were washed with FACS buffer (PBS, 2% FCS, 1 mg/l NaN_3) and the resuspended cell pellets were incubated for 5 min with anti-CD16/CD32 mAb (2.4G2) before adding the appropriate antibody combinations. Cells were stained with Cy5.5-anti-CD4 (L3T4), PE-anti-CCR3 (R&D Systems, Minneapolis, MN), PE-anti-Ly6G/Gr-1 (Caltag, Burlingame, CA), the following biotinylated antibodies: anti-CD3 (145-2C11), anti-IgE (R35-72), anti-CD24 (M1/69), anti-α_4 integrin (R1-2), anti-β_7 integrin (M293), anti-β_1 integrin (Ha2/5), anti-CD11a (2D7), anti-CD11b (M1/70), anti-CD11c (HL3), anti-DX-5, anti-Thy1.2 (53-2.1), anti-CD69 (H1.2F3), anti-CD62L (MEL14), anti-FIRE (Caminschi et al., 2001), anti-c-kit (2B8), anti-CD23 (B3B4), and streptavidin-APC (Molecular Probes, Eugene, OR). mAbs were purchased from BD Pharmingen unless otherwise indicated. Samples were analyzed on a FACS Calibur flow cytometer with CellQuest II software (Becton Dickinson).

IL-4 Secretion Assay

Leukocytes from the lung were purified by Ficoll gradient from day 9 *N. brasiliensis* infected mice. 10^6 cells were incubated for 2 hr at 37°C in RPMI 1640/10%FCS in the presence or absence of 40 ng/ml PMA and 1 μg/ml ionomycin followed by IL-4 capture on the cell surface using the Mouse IL-4 Secretion Assay according to the manufacturer's instructions (Miltenyi Biotec, Germany).

Bromodeoxyuridine Staining

Mice were given 1 mg Bromodeoxyuridine (BrdU) i.p. in 250 μl PBS on day 6 after infection and killed after 18 hr. Single-cell suspensions from total lung tissue and tracheal lymph nodes were stained for surface CD4 and intracellular BrdU using the BrdU Flow Kit (BD Pharmingen).

Bone Marrow Chimeras

Recipient mice were lethally irradiated with 600 rad administered twice over 3 hr before animals were reconstituted intravenously with 2×10^6 bone marrow cells. Mice were kept on antibiotic-containing water and SCID MD tablets (Bioserv, Frenchtown, NJ) in the UCSF specific pathogen-free animal facility and used after 10 weeks.

Adoptive Transfers

CD4 T cells from spleen and mesenteric lymph nodes of 4get, 4get/Stat6$^{-/-}$, or IL-4/IL-13$^{-/-}$ mice were purified to >95% by negative selection with MACS CD4 T cell isolation kit (Miltenyi Biotech, Germany) and LS columns. 10^7 purified CD4 T cells were transferred i.v. into Rag$^{-/-}$ or 4get/Rag$^{-/-}$ recipient mice, which were infected with *N. brasiliensis* 4 days later. Mice were analyzed after 9 days.

ELISA

Serum IgE levels were determined by ELISA using the monoclonal antibody B1E3 for coating and the biotinylated monoclonal antibody EM95 for detection.

Histology

Tissue from lung and small intestine was collected 9 days after *N. brasiliensis* infection, frozen in OCT, cut into 8 μm thick sections, and stained with acidified Toluidine blue.

Microarrays

Ten male 4get mice were infected with *N. brasiliensis* and single-cell suspensions of total lung tissue were prepared 9 days after infection. Cells were stained for CD3, CD19, c-kit, and CCR3 and sorted for CD3$^-$CD19$^-$c-kit$^-$GFP$^+$CCR3$^+$SSC^{hi} (eosinophils) and CD3$^-$CD19$^-$c-kit$^-$ GFP$^+$CCR3$^-$SSC^{lo} (basophils) using a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO). Total RNA was isolated from 2×10^6 eosinophils and 1.75×10^5 basophils using the Total RNA Isolation Kit (Fluka, Buchs, Switzerland) and amplified by two rounds of in vitro transcription using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX). Universal Mouse Reference RNA (#740100; Stratagene, La Jolla, CA) was amplified in the same way. Aminoallyl-UTP was incorporated during the second round of amplification and 5 μg of amplified RNA was coupled to Cy3 and Cy5 fluorescent dyes (CyScribe dye labeling kit, Amersham Biosciences, Peapack, NJ). Probes were hybridized to spotted glass oligonucleotide (70-mer) arrays that cover just over 16,400 unique genes (Mouse Genome Set Version 2.0, Qiagen, Germany). The arrays were prehybridized in 1% BSA (Invitrogen), 5 × SSC, 0.1% SDS for 2 hr at 42°C, washed in water, and hybridized with the Cy3/Cy5-labeled probes in 2.8 × SSC, 0.2% SDS, 0.6 mg/ml Cot-1 DNA (Invitrogen), and 0.8 mg/ml yeast tRNA, at 63°C for 45 hr. Slides were washed sequentially in 0.03%SDS/1 × SSC, 0.2 × SSC, and 0.05 × SSC, dried, and scanned on an Axon 4000B scanner using Genepix 3.0 software (Axon Instruments, Inc.). Data were normalized using the R package Bioconductor software and "lowess" normalization on the pixel medians without background subtraction (Ihaka and Gentleman, 1996). Genes of interest were selected and grouped in Excel software (Microsoft). The complete data set has been deposited in the GEO database at NCBI (GSM15485, GSM15486, GSM15487). "A" values indicate the total signal intensity of a given spot on the microarray. This number is calculated as $A = 1/2*\log_2(R*G)$, where R and G give the intensity for the Cy5 and Cy3 channels, respectively. "M" values indicate the difference in gene expression on a $\log_2$ scale. This value is calculated as $M = \log_2(R/G)$ (e.g.: M=1 indicates a 2-fold higher expression in the sample that was labeled with the Cy5 dye compared to the sample that was labeled with the Cy3 dye, and M = 0 indicates equal expression in both samples).

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