The initial response of CD4⁺ IL-4-producing cells

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Abstract

Naive CD4⁺ T cells were reported to produce small amounts of IL-4 in vitro, which are implicated to be sufficient to initiate Th2 response in vivo. However, IL-4-producing naive CD4⁺ T cells are difficult to study in vivo because they are present in low numbers shortly after the first antigen exposure. Here, we used IL-4/green fluorescence protein (GFP) reporter mice (G4 mice) to track the initial response of CD4⁺ IL-4-producing cells. We first established a flow cytometry method to estimate the number of GFP⁺ cells. We demonstrated the effectiveness of this method by showing that the responding CD4⁺ GFP⁺ cells exhibited an activated phenotype, possessed the capacity to express IL-5 and IL-13, but not IFN-γ mRNA, and showed enhanced levels of GATA3 and c-maf mRNA expression. More importantly, we showed that the cell lines derived from FACS-sorted CD4⁺ GFP⁺ cells were antigen specific. By using this newly established method, we showed that the majority of responding GFP⁺ cells were CD4⁺ T cells. Our study provides direct ex vivo evidence to show that a small percent of CD4⁺ T cells that have no previous experience of antigenic stimulation might produce IL-4 to initiate Th2 response.

Introduction

Th1-mediated immune responses play critical roles in health and disease states. Th1 can be divided into two major types. Th1,1 principally produce IFN-γ but not IL-4, whereas Th1,2 produce IL-4, IL-5 and IL-13 but not IFN-γ. Naive CD4⁺ T cells—defined as no prior experience of antigenic stimulation—can differentiate into either Th1,1 or Th1,2, primarily depending on the cytokine environments where they first encounter antigens. IL-12 drives naive CD4⁺ T cells to differentiate into Th1,1, whereas IL-4 directs naive CD4⁺ T cells to differentiate into Th1,2. While the cellular source for initial IL-12 production has become reasonably well established, the cellular source for initial IL-4 production remains less studied (1, 2).

Cellular sources for initial IL-4 production have been reported to include NKT cells (3, 4), basophils (5, 6), eosinophils (7–9), mast cells (10, 11) and CD4⁺ T cells (12–15). These cells possess the capacity to produce IL-4 promptly in various experimental systems without a requirement for priming. Among these initial IL-4-producing cells, CD4⁺ T cells as initial IL-4-producing cells offer several advantages. First, in contrast to NKT cells and γδ-T cells, which use restricted TCRs that can only recognize a limited number of antigens, CD4⁺ T cells provide a wider spectrum of TCRs that recognize a variety of allergens and parasitic antigens. Second, CD4⁺ T cells, unlike basophils, eosinophils and mast cells, are in the right place at the right time; they are located in the lymph node where they recognize antigens to initiate immune response.

However, CD4⁺ T cells that can produce initial IL-4 are difficult to study in vivo because these cells are present in low numbers shortly after antigen stimulation. Furthermore, the small amounts of IL-4 produced could be quickly consumed by surrounding cells, making it even more difficult for detection. Here, we used IL-4/green fluorescence protein (GFP) reporter mice (G4 mice) to overcome those problems and demonstrate that directly ex vivo the majority of antigen-responding IL-4-producing cells were CD4⁺ T cells.

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Methods

Mice
IL-4/GFP reporter mice (G4 mice) were described previously (16, 17). A breeding pair of the G4 mice (129 backcrossed to C57BL/6 mice for 12 generations) was provided to us by William E. Paul of the National Institutes of Health (NIH) (Bethesda, MD, USA). These mice were bred into gfp-Il4/ gfp-Il4 homozygous [homozygous for a knock-in gfp gene in the place of exon 1 of the Il4 gene (G4hom mice)] in our pathogen-free facility and fed a protein-free diet (Harlan 7012). Handling of animals complied with the animal protocols approved by either the Loyola University Medical School or National Jewish Medical and Research Institutional Animal Care and Use Committee.

Immunization protocols and cell sample preparations
For preparing GFP+ immunization protocols and cell sample preparations School or National Jewish Medical and Research Institutional cols approved by either the Loyola University Medical Louis, MO, USA) in 100 l of PBS 1:1 mixed with 100 l of Imject aluminum hydroxide and magnesium (alum) (Pierce, Rockford, IL, USA) on day 0. Mesenteric lymph nodes (MLNs) were taken out at the time points specified.

Flow cytometric analysis
Single-cell suspension of lymph node cells was stained with allophycocyanin (APC)-labeled anti-CD4 antibody (BD PharMingen, San Diego, CA, USA). PE-labeled antibodies to all TCRxβ, NK1.1 and CD62L were purchased from BD PharMingen. The stained cells were analyzed with a FACScan (Becton-Dickinson, Mountain View, CA, USA).

FACS sorting, reverse transcriptase–PCR and real-time PCR
GFP+ cells were electronically sorted into a test tube by using a BD FACStar plus instrument. Over 99% of sorted cells were GFP+ as examined under a fluorescence microscope. Naive CD4+ cells were prepared from MLNs of unimmunized G4hom mice by using the method as described previously (18). mRNA was isolated using MicroPoly (A) PureTM kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. cdna was synthesized and PCR was subsequently performed with the following primer pairs—IL-5 primer sequences: forward, 5’-CAATGAGACGATGAGGCT TC-3’ and reverse, 5’-CCACTCTGTACTCATCACAAC-3’; IL-13: forward, 5’-ACAGCTCCCTGTTCTCTCA-3’ and reverse, 5’-CGTGGGCAAAAAGTGTGTTTGTG-3’; T-bet: forward, 5’-CTTGTGGTGTTCTCAAGTT and reverse, TTTCACACTGCA CCCACTT, and IFN-γ: forward, ATTGAAGGCTAGAAAGTCGTG and reverse, CTCATGAAATGCACTCCTTTTGCG. PCR for amplifying IL-5, IL-13 and GAPDH consisted of 30 cycles at 95°C for 20 s, 58°C for 30 s and 72°C for 65 s, followed by 7 min at 72°C. For amplification of IFN-γ and T-bet, we performed 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 60 s.

For real-time PCR analysis, the following primers were used—GATA3: forward, CCTACCGGTTCTCGGATGATA and reverse, TCACACACTTCTCGGCTCT; c-maf: forward, AGC AGTGGGTGACAGATGTCG and reverse, TGGGATCTCCTGCTTGAG, and Jun B: forward, CCTGCTCTACACGCACACACGAG, and CTTGAG. Real-time PCR was performed in an ABI PRISM™ 7700 Sequence Detection System. The cycling conditions were 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min for 40 cycles. The amounts of mRNA were expressed as a relative fold of induction to GAPDH (relative fold = 2ΔCt, where ΔCt = Ctすごくsample – CtすごくGAPDH).

Testing of antigen specificity of CD4+GFP+ cell lines
Fifty of the CD4+GFP+ cells prepared from G4hom mice that received peritoneal injection of OVA plus alum 24 h prior to sorting were deposited onto one well of a 96-well plate containing 0.1 × 10⁶ irradiated APC, anti-CD3 (3 μg ml⁻¹), anti-CD28 (3 μg ml⁻¹) and IL-2 (10 U ml⁻¹) (PeproTech Inc., Rocky Hill, NJ, USA). Colonies were screened 2 weeks after the initial stimulation. Colonies were re-stimulated with 1 × 10⁶ irradiated APC plus anti-CD3 (3 μg ml⁻¹), anti-CD28 (3 μg ml⁻¹) and IL-2 (10 U ml⁻¹). APC plus OVA peptide (prepared by Alpha Diagnostic International, San Antonio, TX, USA) and IL-2 or APC plus cytochrome c peptide (prepared by National Institute of Allergy and Infectious Disease, Biological Resource Branch, Bethesda, MD, USA) and IL-2 for 36 h. Supernatants were collected and used for the measurement of IL-5 protein by ELISA (BD Pharamingen).

Results

Establishment of a method to analyze initial responding GFP+ cells
To analyze GFP+ cells, we first developed a method to estimate a small number of GFP+ cells (Fig. 1). We used a live-gate that was defined by using the FL1 (GFP) and the FL3 plots (Fig. 1A). FL1 and FL3 plots allowed us to differentiate auto-fluorescence from green fluorescence. Auto-fluorescence can be detected by both FL1 and FL3, whereas green fluorescence can only be detected by FL1. After GFP+ cells were collected (Fig. 1B), we used a combinatorial analysis gate consisting of a more stringently defined GFP+ gate and a size gate to analyze multiple-color-labeled GFP+ cells. The GFP+ gate was determined by comparing the background mean fluorescence intensity (MFI) shown by lymphocytes prepared from unimmunized G4hom mice to MFI displayed by lymphocytes prepared from immunized G4hom mice (Fig. 1C). The size gate was determined by using a ‘backgating’ technique (Fig. 1C). First, we gated on GFP+ cells prepared from immunized G4hom mice using the FL3 and the FL1 plot. Then we used a forward scatter and a side scatter plot to determine the size of GFP+ cells. This gate was useful when multiple-color analysis was involved; it gated out cell aggregates that bind to antibodies non-specifically.

The majority of CD4+GFP+ cells in response to OVA stimulation are antigen-specific conventional CD4+ T cells
To identify the cells that promptly expressed IL-4 after antigenic stimulation, we used two criteria to describe an initial IL-4 producer. First, initial IL-4 producers must produce IL-4 independent of IL-4. Second, they must produce IL-4 without requiring a priming period. To meet these requirements, we used G4hom mice, which we confirmed previously did not
produce IL-4 (9). And we measured GFP+ cells at early time points (24 h) after single i.p. injection of OVA. We found that the majority of GFP+ cells 24 h after a single injection of OVA plus alum were CD4+ T cells (CD4+GFP+: 84.2 ± 5.4% and CD4+GFP−: 14.4 ± 6.3%) (Fig. 1D).

CD4+ NKT cells have been reported to produce IL-4 promptly in response to anti-CD3 treatment in vivo (3, 4). To determine what percentage of CD4+GFP+ cells were conventional CD4+ T cells and what percentage of CD4+GFP+ cells were NKT cells, we stained the CD4+GFP+ cells with PE-labeled anti-TCRβ antibody or anti-NK1.1 antibody. We showed that the majority of CD4+GFP+ cells expressed TCRβ and β chains, and ~16% of CD4+GFP+ cells expressed NK1.1 (Fig. 2). Our data suggest that although NKT cells were enriched in CD4+GFP+ cell population, the majority of CD4+GFP+ cells detected in our system were conventional CD4+ T cells.

To compare these IL-4-independent CD4+GFP+ cells with T12, we FACS sorted CD4+GFP+ cells from mice that received OVA injection 24 h prior to assay and stimulated them with phorbol myristate acetate–ionomycin for 4 h. We examined T12 and T11 cytokine mRNA expression and found that CD4+GFP+ cells possessed the capacity to express IL-5 and IL-13, but not IFN-γ mRNA (Fig. 3A). We further found that these CD4+GFP+ cells expressed enhanced GATA3 mRNA, c-maf and Jun B mRNA were also enhanced but to a less degree compared with that of T12 (Fig. 3B).

Fig. 1. A live-gate and two analysis gates used for collecting and analyzing GFP+ cells are shown. G4.5 None mice were not injected (Unim) or injected i.p. with 100 µg OVA plus alum (OVA). Single cells were prepared from MLNs and stained with APC-labeled anti-CD4 antibody, PE-labeled isotype control, PE-labeled anti-all Vβ antibody or PE-labeled anti-NK1.1 antibody. GFP+ cells were collected through the live-gate. GFP− cells were collected without the live-gate. CD4+GFP+ cells were analyzed by using the combinational analysis gate consisting of the size gate, the CD4+ gate and the GFP+ gate, whereas CD4+GFP− cells were analyzed by using the combinational gate consisting of the size gate, the CD4+ gate and the GFP− gate shown in Fig. 1.

Fig. 2. The majority of CD4+GFP+ cells are conventional CD4+ T cells. Single cells were prepared and pooled from MLNs of seven G4.5 mice 24 h after OVA injection. Cells were stained with APC-labeled anti-CD4 antibody, PE-labeled isotype control, PE-labeled anti-all Vβ antibody or PE-labeled anti-NK1.1 antibody. GFP+ cells were collected through the live-gate. GFP− cells were collected without the live-gate. CD4+GFP+ cells were analyzed by using the combinational analysis gate consisting of the size gate, the CD4+ gate and the GFP+ gate, whereas CD4+GFP− cells were analyzed by using the combinational gate consisting of the size gate, the CD4+ gate and the GFP− gate shown in Fig. 1.
CD4^+GFP^+ cells did not express the Th1-specific transcription factor T-bet (Fig. 3A). These results suggest that the CD4^+GFP^+ cells exhibit a phenotype that resembles that of CD4^+Th2 memory cells.

Because the frequency of CD4^+GFP^+ cells in response to OVA stimulation was low, we examined whether CD4^+GFP^+ cells can become activated by OVA stimulation in vivo. We found that ~95% of CD4^+GFP^+ cells, compared with 16.3% of CD4^+GFP^- cells, expressed low levels of CD62L, an activation marker, 24 h after i.p. injection of OVA (Fig. 4). To demonstrate whether CD4^+GFP^+ cells were antigen specific, we FACS sorted CD4^+GFP^+ cells from mice that received an OVA injection 24 h prior to assay and expanded them in vitro to establish cell lines. We showed that two cell lines derived from CD4^+GFP^+ cells produced IL-5 in response to OVA peptide but not cytochrome c peptide stimulation (Fig. 5). These data demonstrated that the CD4^+GFP^+ cells detected in our system were OVA specific.

The number of CD4^+GFP^+ but not CD4^+GFP^- cells increases in the draining lymph node

To determine the kinetics of the initial response of IL-4-producing cells to antigen stimulation in vivo, we estimated the number of CD4^+GFP^+ cells and CD4^+GFP^- cells in various treatment groups. We observed a significant increase in the number of CD4^+GFP^+ cells in the MLN starting at 24 h after injection. Such increase reached peak levels at 48 h.
and returned to near-background levels at 5 days after injection. In contrast, alum plus PBS did not cause an increase in the number of CD4*GFP* cells. The number of CD4*GFP* cells did not increase throughout the course of antigenic stimulation (Fig. 6). These results suggest that CD4 T cells may contribute to the majority of IL-4-independent IL-4 production shortly after antigen stimulation.

**Discussion**

Initial IL-4 producers are of great interest because they are thought to provide the first burst of IL-4 to prime naive CD4 T cells into Th2 effector cells and thus initiate a Th2 immune response. In this report, we took advantage of an enhanced GFP as a reporter expression, which, unlike IL-4 protein, does not turn over quickly and cannot be consumed by the surrounding cells. This feature enabled us to develop and to verify the flow cytometry method to detect small numbers of GFP* cells.

The frequencies of initial IL-4-producing CD4 T cells, as reported by GFP expression, were low but consistent with previous studies. McHeyzer-Williams et al. (19) demonstrated that the number of CD4 T cells that were specific for cytochrome c was ~1000 cells per draining lymph node 3 days after antigen stimulation. The number of antigen-specific CD4 T cells that also produced IL-4 directly ex vivo was even lower (20). In another study, Stetson et al. (21) showed that the number of Leishmania-specific T cells were also low ~200 cells per lymph node at 24 and 48 h after infection.

We observed that the number of CD4*GFP* cells increased 24 h after administration of OVA plus alum and reached peak levels at 48 h after administration of OVA plus alum. We think the increase could be the result of the activation of a small number of OVA-specific CD4 T cells that were pre-committed with the capacity to produce IL-4. This activation explanation is consistent with the activation kinetics of GFP reporter expression in vitro. We observed that high levels of GFP expression by CD4 T cells prepared from G4 mice required overnight TCR stimulation. It is reasonable to consider that OVA-specific CD4* IL-4-producing T cells could divide 24 h after OVA stimulation. Indeed, this division may explain the highest number of CD4*GFP* cells observed at 48 h after the initial antigenic stimulation. Because G4m mice have two copies of the knock-in gfp gene and lose the ability to produce IL-4 (9), we considered the development of CD4*GFP* cells to be IL-4 independent. It remains unclear whether a unique population of CD4 T cells produces IL-4 or if naive CD4 T cell possesses an equal ability to produce IL-4 stochastically. Further characterization of CD4*GFP* cells with unique cell-surface markers may reveal whether these cells belong to a separate lineage from conventional CD4 T cells.

The CD4*GFP* cells analyzed in this study resembled the phenotype of Th2 memory cells. These cells expressed IL-5, IL-13 mRNA and Th2-specific transcription factors but not IFN-γ mRNA or the Th1-specific transcription factor T-bet. The CD4*GFP* cells found in our study might include naive CD4 T cells and resting CD4 T cells. The resting CD4 T cells could have previously been exposed to environmental antigens that may prepare these cells to mount a recall response to an antigen that they have never encountered before, such as OVA that is a chicken protein and was unlikely to be present in the food or in the animal facilities. It is possible that the resting CD4*GFP* cells, although bearing a memory phenotype, have never encountered an antigenic stimulation. Although the exact contribution to initial IL-4 production by the type of resting CD4 T cells that are cross-primed by environmental antigens could be difficult to determine and require further study, inclusion of these cells in our study could provide a more realistic estimate of initial...
IL-4 production by CD4$^+$ T cells. Together, these two types of cells could be important cellular sources of initial IL-4 production because CD4$^+$ T cells, unlike NK cells, basophils or eosinophils, express TCRs that can recognize a broad spectrum of antigenic structures and become activated and produce cytokines soon after first antigenic stimulation. The small amounts of IL-4 production by the CD4$^+$ T cells could be instrumental in directing naive CD4$^+$ T cells to differentiate into Th2, especially under certain conditions where anti-CD4$^+$ adoptive transfer experiments, and our study may provide are authentic initial IL-4-producing cells require the finding of unique surface markers and confirmation provided by adoptive transfer experiments, and our study may provide a useful model for a detailed molecular analysis of how CD4$^+$ T cells produce initial IL-4 to initiate Th2 responses.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>alum</td>
<td>aluminum hydroxide and magnesium</td>
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<td>APC</td>
<td>allophycocyanin</td>
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<td>GFp</td>
<td>homzygous for a knock-in GFP gene in the place of exon 1 of the Il4 gene</td>
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<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
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<td>i.p.</td>
<td>intra-peritoneal</td>
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<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>MLN</td>
<td>mesenteric lymph node</td>
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<td>OVA</td>
<td>ovalbumin peptide</td>
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References