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The Functional Heterogeneity of Type 1 Effector T Cells in Response to Infection Is Related to the Potential for IFN-γ Production

Katrin D. Mayer,* Katja Mohrs,* Sherry R. Crowe,* Lawrence L. Johnson,* Paul Rhyne,† David L. Woodland,* and Markus Mohrs*2*

The expression of IFN-γ is a hallmark of Th1 cells and CD8+ effector T cells and is the signature cytokine of type 1 responses. However, it is not known whether T cells are homogeneous in their capacity to produce IFN-γ, whether this potential varies between tissues, and how it relates to the production of other effector molecules. In the present study we used bicistronic IFN-γ-enhanced yellow fluorescent protein (IFN-γ-eYFP) reporter mice (Yeti) and MHC class I tetramers to directly quantify IFN-γ expression at the single cell level. The eYFP fluorescence of Th1 cells and CD8+ effector T cells was broadly heterogeneous even before cell division and correlated with both the abundance of IFN-γ transcripts and the secretion of IFN-γ upon stimulation. CD4+ and CD8+ T cells of influenza-infected mice revealed a similarly heterogeneous IFN-γ expression, and eYFP[high] cells were only found in the infected lung. Ag-specific T cells were in all examined tissues eYFP+, but also heterogeneous in their reporter fluorescence, and eYFP[high] cells were also restricted to the infected lung. A similar heterogeneity was observed in Toxoplasma gondii-infected animals, but eYFP[low] cells were restricted to different tissues. Highly eYFP fluorescent cells produced elevated levels of proinflammatory cytokines and chemokines in addition to IFN-γ, suggesting their coregulated expression as a functional unit in highly differentiated effector T cells. The Journal of Immunology, 2005, 174: 7732–7739.

The expression of IFN-γ is a hallmark of Th1-polarized CD4+ T cells and effector CD8+ T cells and is the signature cytokine of type 1 responses induced by infection with intracellular bacteria, protozoa, and viruses (1, 2). In fact, IFN-γ was originally described as a soluble factor protecting cells from viral infection (2). Mice deficient for IFN-γ (3), its receptor (4), or the downstream Stat-1 (5, 6) have severely impaired type 1 immunity. CD4+ and CD8+ T cells are major sources of IFN-γ during adaptive immune responses (2), and recent data suggest that Th1 cells express IFN-γ monoaecially (7). The diverse biological functions of IFN-γ include the up-regulation of Ag processing and presentation by MHC class I and II, the induction of class switch recombination in B cells, the priming of macrophages for NO production, the induction of immunomodulatory and proinflammatory cytokines, as well as the recruitment and death of activated T cells (2, 8).

Infection with the respiratory influenza virus or the protozoan parasite Toxoplasma gondii elicits a canonical type 1 response, with the production of IFN-γ as a signature cytokine (9–13). Numerous studies have analyzed the cellular response to infection, and the production of IFN-γ in response to in vitro peptide stimulation has been used to indirectly identify Ag-specific cells (9, 10, 14). The development of Ag-specific MHC class I tetramer reagents has contributed substantially to our understanding of the cellular response (15, 16). Despite the clear association of IFN-γ with these infectious disease models, none of the studies was tailored to specifically analyze the cells that elaborate the cytokine response. Thus, we know little about the phenotype of IFN-γ-expressing cells in vivo; whether these cells have a homogeneous potential to produce IFN-γ, other cytokines, or chemokines; and whether these parameters vary with their anatomical locations. Commonly used methods to identify IFN-γ expression at the single cell level require in vitro restimulation, thereby altering the in situ phenotype (17–19). Recently generated bicistronic IFN-γ reporter mice (IFN-γ-eYFP)5 protein reporter mice (Yeti) were specifically designed to overcome these limitations and allow the noninvasive detection and isolation of live, cytokine-expressing cells (20, 21). Yeti mice were generated by the targeted integration of an internal ribosomal entry site linked to an eYFP-encoding sequence into the 3’-untranslated region of the Il10 gene, thereby linking IFN-γ expression to eYFP fluorescence in any cell (20, 21). Using Yeti mice, we previously observed a substantial heterogeneity in the level of eYFP fluorescence in CD4+ T cells (20, 21).

In the present study we used Yeti mice (20, 21) in combination with MHC class I tetramers (15, 16) to visualize, phenotype, and functionally characterize IFN-γ-expressing cells in various tissues in vitro and in response to infection with the respiratory influenza virus and the protozoan parasite T. gondii.

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2 Address correspondence and reprint requests to Dr. Markus Mohrs, Trudeau Institute, 154 Algonquin Avenue, Saranac Lake, NY 12983. E-mail address: mmohrs@trudeauinstitute.org

3 Abbreviations used in this paper: eYFP, enhanced yellow fluorescent protein; BAL, bronchoalveolar lavage; cRPMI, complete RPMI 1640; i.n., intranasal; p.i., postinfection; mediLN, mediastinal lymph node; MFI, median fluorescence intensity; NP, nucleoprotein; PA, acid polymerase protein; SDF, stromal cell-derived factor; wt, wild type; Tc1, CTL producing type-1 cytokines; Tc2, CTL producing type-2 cytokines.
Materials and Methods

**Mice and viral infections**

Bicistronic IFN-γ reporter (Yeti) mice (20) were backcrossed to C57BL/6 for a minimum of five generations. All Yeti mice were heterozygous for the bicistronic reporter knockin, and wild-type (wt) littermates were used as controls. Experimental animals were kept under specific pathogen-free conditions in filter-top cages at the animal facility of Trudeau Institute. Mice, 8–12 wk of age, were anesthetized with 2.2-tribromoethanol (i.p.) and infected intranasally (i.n.) with 300,000 egg infectious doses of the A/HK-x31 (x31, H3N2) (22) influenza A virus in 10 μl of PBS. The T. gondii strain ME49 was obtained from brains of chronically infected mice, and infections were initiated by oral gavage of 10 cysts in 0.1 ml of diluted brain suspension (23). All experimental procedures involving mice were approved by the institutional animal care and use committee at Trudeau Institute.

**Tissue sampling and preparation**

Peripheral blood was collected into heparin before pleural exudates cells were isolated by lavage through the diaphragm. Next, bronchoalveolar lavage (BAL) cells were collected by five consecutive washes with 1 ml each. Mice were perfused through the heart after the portal vein was cut for drainage. Perfused lung tissue was dispersed by passage through a 70-μm pore size strainer and subsequently digested with collagenase IV (100 U/ml; Sigma-Aldrich) and DNase I (10 U/ml; Sigma-Aldrich). Lymphocytes were enriched in the interphase of a discontinuous 60%/40% Percoll (Amersham Biosciences) gradient spun at 1200 g at 4°C for 20 min at room temperature. Single cell suspensions were prepared from spleen and mesenchymal lymph nodes (medLN) by mechanical dissection through a 70-μm pore size cell strainer. Erythrocytes were removed from blood and spleen by lysis with ACK buffer. Adherent cells were depleted from BAL, pleural exudates cells, and spleen by incubation in complete RPMI 1640 (cRPMI) 100 μl in a 10 μl Percoll gradient for 20 min at room temperature, followed by washing. T cells sorted from influenza-infected animals were cultured at 5 × 10^6/ml in flat-bottom, 96-well plates for 24 h in cRPMI at 37°C in 5% CO₂. Cells were activated by plate-bound anti-CD3e (10 μg/ml) where indicated.

**Cell culture**

Purified T cells (1 × 10^6/ml) were primed as previously described (25) in cRPMI with anti-CD3e (145-2C11; 2 μg/ml) and anti-CD28 (37.51; 5 μg/ml) in the presence of IL-2 (5 ng/ml) and irradiated (3000 rad) APC (5 × 10^5/ml). Cultures were supplemented with IL-12 (5 ng/ml) and anti-IL-4 (11B11; 20 μg/ml) or IL-4 (50 ng/ml) and anti-IFN-γ (XM11.2; 20 μg/ml) where indicated. Some cells were labeled with the vital red fluorescent dye SNARF-1 (Molecular Probes). T cells sorted from influenza-infected animals were cultured at 5 × 10^6/ml in flat-bottom, 96-well plates for 24 h in cRPMI at 37°C in 5% CO₂. Cells were activated by plate-bound anti-CD3e (10 μg/ml) where indicated.

**IFN-γ secretion assay**

Splenocytes from day 9 influenza-infected mice were cultured at 5 × 10^6/ml in the presence or the absence of PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in cRPMI for 4 h. The IFN-γ secretion assay was performed according to the manufacturer’s instructions (Milltenyi Biotech). A high control (26) was included by the addition of mouse rIFN-γ (100 ng/ml; ebioscience), and the staining specificity was confirmed using a PE-labeled rat IgG1 isotype control (R3-34).

**Cytokine and chemokine quantification**

For RT-PCR, RNA was extracted using the RNAqueous-4PCR kit (Ambion) and reverse transcribed with the SuperScript II RNase H⁻ kit (Invitrogen Life Technologies) using oligo(dT)₁₄ priming. Quantitative real-time RT-PCR was performed using gene-specific primers and probes (23) and the ABI PRISM 7700 Sequence BioDetector (Applied Biosystems) according to the manufacturer’s instructions (TaqaMan; PerkinElmer). Threshold cycle values for GAPDH were routinely between 15 and 18 cycles, and normalization to β₂-microglobulin gave similar results.

Cytokines and chemokines in culture supernatants were quantified using a multiplex flow cytometry suspension microbead array (27) according to the manufacturer’s instructions (Beadleyt MultiCytokine/Chemokine Flex; Kit; Upstate Biotechnology). The following mouse Beadmate sets were combined in a multiplex analysis: IL-2, IFN-γ, GM-CSF, TNF-α, RANTES (CCL5), stromal cell-derived factor (SDF) (CXCL12), and MIP-1β (CCL4). The median fluorescence intensity (MFI) was measured from at least 50 beads/set using the LumineX eMAP system. The concentrations of cytokines and chemokines were determined using a five-parameter curve-fit generated from the MFIs of the respective mouse cytokine and chemokine standards.

**Statistical analysis**

Statistical analysis was performed using PRISM 3.0c (GraphPad). Asterisks indicate statistical differences with p < 0.05, by Student’s t test.

**Results**

**Heterogeneous IFN-γ expression of CD4⁺ and CD8⁺ T in vitro**

To analyze the expression of IFN-γ during the activation of T cells, CD4⁺ and CD8⁺ T cells were purified from naive Yeti mice or wt littermates and stimulated with anti-CD3e and anti-CD28 in the presence of irradiated APC. The cultures were supplemented with IL-12 plus anti-IL-4 (Th1/CTL producing type-1 cytokines (Tc1) conditions) or IL-4 plus anti-IFN-γ (Th2/CTL producing type-2 cytokines (Tc2) conditions) as indicated. To assess cell division, some cells were labeled with the vital red-fluorescent dye SNARF-1 and cultured under the same conditions. As shown in Fig. 1A, CD8⁺ T cells expressed high levels of eYFP within 24 h of priming, although they had not yet divided. The eYFP fluorescence was broadly heterogeneous, and Tc1 conditions clearly increased IFN-γ expression, whereas Tc2 conditions reduced the frequency and brightness. Both effects were more apparent after 3 days of culture. In contrast to CD8⁺ T cells, CD4⁺ T cells required 2 days of priming to express IFN-γ and were only induced to do so under Th1 conditions (Fig. 2B). Although one cell division was apparent, even undivided cells were eYFP⁺, indicating that the...
time in culture, rather than cytokines, is the limiting factor for IFN-γ expression. Heterogeneous eYFP expression was maintained in Th1 cultures over the 5-day culture period. To confirm that eYFP fluorescence correlates with the expression of IFN-γ, we sorted CD8+ and CD4+ T cells into eYFPneg, eYFPint, and eYFPhigh populations and determined the abundance of IFN-γ transcript by real-time RT-PCR. The eYFP brightness of both CD8+ (Fig. 1C) and CD4+ (Fig. 1D) T cells correlated directly with the abundance of IFN-γ transcripts of the respective population. Together, these data show that IFN-γ expression by CD4+ and CD8+ is broadly heterogeneous and can be directly quantified by the bicistronic eYFP reporter.

**Expression of IFN-γ in influenza virus-infected Yeti mice is broadly heterogeneous, and highly fluorescent cells are restricted to the infected lung**

To investigate the expression of IFN-γ during infection, we analyzed the response to i.n. challenge with influenza virus. Yeti mice and wt littermates were infected i.n. with influenza virus and analyzed 9 days later at the peak of the cellular response by FACS for eYFP fluorescence. Consistent with published data, noninfected Yeti controls revealed only a background of eYFP fluorescence. In contrast, the frequency of eYFP+ T lymphocytes remained low in all secondary lymphoid organs, including the draining mediLN, and in other peripheral tissues besides lung (Fig. 2B and data not shown).

The eYFP fluorescence intensity of eYFP+ CD4+ and CD8+ T cells was remarkably heterogeneous in all examined tissues, and highly fluorescent cells (eYFPhigh) were present only in the lung airways and parenchyma of infected mice (Fig. 2B). Even the pleural cavity, which harbors the infected lung and had a similar increase and frequency of eYFP+ cell as the lung, did not contain cells of comparable brightness.

We next used NP366–374 and PA224–233 MHC class I tetramers to analyze IFN-γ expression in Ag-specific cells (15, 16). Both NP366–374+ and PA224–233+ specific CD8+ T cells disseminated into all examined tissues and were almost exclusively eYFP+, but, nonetheless, were highly heterogeneous in their fluorescence intensity (Fig. 2C and data not shown). Moreover, highly fluorescent Ag-specific cells were also only present in the lung airways and parenchyma. The fluorescence heterogeneity and anatomical restriction of eYFPhigh cells were observed in both NP366–374+ and PA224–233+ specific cells and therefore are not due to differences in the Ag specificity of IFN-γ-expressing cells (data not shown). The frequency of NP366–374+ or PA224–233+ specific cells was comparable between Yeti and wt littermate controls in all organs, demonstrating that insertion of the bicistronic reporter does not affect the cellular response to infection with the influenza virus (Fig. 2C).

**eYFP fluorescence correlates directly with the expression of acute activation markers**

Next, we investigated whether heterogeneous eYFP fluorescence intensity correlates with the expression of activation markers. Conventional methods to identify cytokine-producing cells at the single cell level require in vitro restimulation (17, 18), which alters...
the expression patterns of surface Ags and prevents their assessment ex vivo (28, 29). In contrast, bicistronic cytokine reporter mice allow direct phenotypic analysis of cytokine-expressing cells ex vivo (20, 25, 30, 31). Yeti mice were infected with influenza virus and analyzed 9 days later by FACS. T lymphocytes with a naive phenotype (CD62L<sup>high</sup>, CD44<sup>low</sup>, CD45RB<sup>high</sup>, and CD11a<sup>low</sup>) were eYFP-negative (Fig. 3A and data not shown). Conversely, eYFPhigh CD4<sup>+</sup> and CD8<sup>+</sup> T cells were CD62L<sup>low</sup>, CD44<sup>high</sup>, CD45RB<sup>low</sup>, and CD11a<sup>high</sup>, consistent with an activated/memory phenotype. The activated/memory phenotype of eYFPhigh cells was displayed on lymphocytes derived from either secondary lymphoid tissues or peripheral sites and was independent of eYFP brightness. In contrast, the surface expression of acute activation markers such as CD69, CD25, and CD122 on the majority of eYFP<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells was clearly heterogeneous (Fig. 3B and data not shown). The expression of these markers on CD4<sup>+</sup> and CD8<sup>+</sup> T cells correlated positively with eYFP fluorescence. For example, eYFP<sup>high</sup> cells in the lung expressed higher levels of CD69 on the surface than eYFP<sup>int</sup> or eYFP<sup>neg</sup> cells, suggesting a higher level of acute activation.

**FIGURE 3.** Surface phenotype of T lymphocytes in correlation with eYFP fluorescence. Yeti mice were infected i.n. with influenza virus. BAL and mediLN were analyzed by flow cytometry 9 days later. The depicted dot plots were gated on CD4<sup>+</sup> or CD8<sup>+</sup> cells. Shown are eYFP fluorescence vs CD44 (A) or CD69 (B). The depicted plots are representative of three individual mice. Similar results were obtained in three independent experiments.

**FIGURE 2.** Expression of the bicistronic IFN-γ-eYFP reporter in naive and influenza-infected Yeti mice. A. Indicated organs from naive Yeti mice were analyzed by FACS for eYFP fluorescence of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. B, Yeti mice were infected i.n. with influenza virus, and FACS was performed 9 days later together with the analysis of naive mice in A. C. Yeti mice and wt littermate controls were infected as described in B, and CD8<sup>+</sup>-gated T cells from the indicated organs were analyzed by flow cytometry for the expression of eYFP and NP<sub>366-374</sub> tetramer staining. The vertical dashed line demarcates eYFP<sup>int</sup> and eYFPhigh cells. Data are representative of multiple independent experiments. In some of these experiments, three mice per cohort were analyzed with comparable results.

*IFN-γ production correlates with eYFP fluorescence, but is only induced after stimulation*

As described above, we observed remarkable fluorescence heterogeneity in eYFP<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figs. 1–3). We next asked whether the brightness of reporter expression correlated with the secretion of IFN-γ protein. Yeti mice and wt controls were infected, and the secretion of IFN-γ was analyzed 9 days later by cytokine secretion assay (26). As shown in Fig. 4A, the vast majority of eYFPhigh, but not eYFP<sup>+</sup>, cells secreted IFN-γ upon short-term stimulation. The secretion of IFN-γ by CD4<sup>+</sup>, total CD8<sup>+</sup>, and Ag-specific CD8<sup>+</sup> T cells correlated directly with the eYFP fluorescence. In fact, eYFP<sup>high</sup> cells secreted the maximum amount of IFN-γ that can be measured by this assay, as determined by the addition of rIFN-γ as a High Control (26) (data not shown). However, the frequency of IFN-γ-secreting cells was very low when the same cells were cultured in the absence of stimulation with plate-bound anti-CD3ε despite robust eYFP fluorescence. Similar results were obtained by intracellular cytokine staining and Ag-specific stimulation with the NP<sub>366-374</sub> peptide (data not shown).

Although the cytokine secretion assay is ideal to analyze the secretion of IFN-γ at the single cell level, it is limited to the detection of one cytokine at a time, and it is unclear whether the brightness of staining reflects the secreted amount. To corroborate the positive correlation between eYFP fluorescence and IFN-γ secretion and assay for additional cytokines and chemokines, we measured the accumulation of effector molecules in culture supernatants of cells that were isolated based on different levels of eYFP fluorescence. Yeti mice were infected with influenza virus, and CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were sorted from pooled BAL/lung and mediLN into eYFP<sup>neg</sup>, eYFP<sup>int</sup>, and eYFP<sup>high</sup> cells (Fig. 4B). We gated on CD62L<sup>low</sup> cells, because the vast majority of eYFP<sup>neg</sup> cells in the lymph node have a naive phenotype (CD44<sup>low</sup>, CD62L<sup>high</sup>, CD45RB<sup>high</sup>, and CD11a<sup>low</sup>), whereas lymphocytes in the periphery display an effector/memory phenotype (CD44<sup>high</sup>, CD62L<sup>low</sup>, CD45RB<sup>low</sup>, and CD11a<sup>low</sup>). As mentioned above, eYFP<sup>high</sup> cells were present only in the pooled BAL and lung, whereas eYFP<sup>neg</sup> could not be obtained in sufficient numbers.
We speculated that increased reporter fluorescence might correlate not only with the secretion of IFN-γ, but also with the enhanced secretion of additional effector cytokines and chemokines. To test this hypothesis, we analyzed the culture supernatants from cells sorted by different levels of eYFP fluorescence, as described in Fig. 4B for additional effector cytokines and chemokines. Indeed, the secretion of the type 1 effector cytokines, TNF-α and GM-CSF (Fig. 5A), and the proinflammatory chemokines, CCL5 (RANTES) and CCL4 (MIP-1β; Fig. 5B), was largely restricted to eYFP" cells and correlated positively with the eYFP brightness of CD4" and CD8" from medLN or BAL/lung. In contrast, the secretion of IL-2 (Fig. 5A) and the chemokine CXCL12 (SDF-1; Fig. 5B) was not restricted to eYFP" cells and did not correlate with eYFP fluorescence intensity. As seen for IFN-γ, tissue-derived CD4" T lymphocytes secreted greater amounts of these soluble effector molecules than cells from the lymph node. In fact, among the CD4" T population, GM-CSF was only detectable in the supernatants of stimulated cells derived from BAL/lung. As seen for the secretion of IFN-γ, the secretion of these cytokines and chemokines was also largely dependent on activation of the CD3 complex.

These data show that the brightness of the bicistronic eYFP reporter correlates positively with the potential to coordinately secrete a select set of effector cytokines and chemokines in addition to IFN-γ. The secretion of these cytokines and chemokines by CD4" and CD8" T cells ex vivo is largely restricted to eYFP" cells and is dramatically enhanced by stimulation. These data suggest that eYFP fluorescence reflects the progressive effector differentiation of activated CD4" and CD8" T cells and that highly differentiated cells are restricted to the site of infection.

Anatomical restriction of eYFP" cells, not their heterogeneity, depends on the pathogen

Finally we wanted to study whether the heterogeneity of eYFP fluorescence or the anatomical restriction of eYFP" cells is specific for a given pathogen. To this end, Yeti mice were infected orally with the protozoan parasite T. gondii, and various organs were analyzed 1 wk later by FACS (Fig. 6). T. gondii-infected mice revealed a similar heterogeneity of eYFP fluorescence of CD4" and CD8" T cells as influenza-infected animals. In contrast to influenza-infected mice, however, eYFP" cells were restricted to other organs, such as mesLN or blood. In fact, blood-borne CD4" T cells from T. gondii-infected animals had substantially higher MIeYFP values than cells in any organ of influenza virus-infected animals (Figs. 2 and 6). Consistent with this observation, T. gondii-infected mice have high serum IFN-γ concentrations during acute infection (13, 23) (data not shown). Thus, the heterogeneous expression of IFN-γ by CD4" and CD8" T cells and the selective accumulation of eYFP" cells in certain tissues are not limited to viral infection. In contrast, in which tissue highly differentiated cells accumulate is dependent on the pathogen despite the wide dissemination of eYFP" cells.

Discussion

Bicistronic cytokine reporter mice have been instrumental to visualize, quantify, and functionally characterize cytokine-expressing cells in response to infection (25, 30, 31). Using bicistronic IFN-γ reporter (Yeti) mice (20, 21), we show in this study that the expression of the IFN-γ-eYFP reporter by CD4" and CD8" T cells is broadly heterogeneous in vitro and upon infection. The
eYFP heterogeneity has important functional consequences, because it reflects both the abundance of IFN-γ transcripts and the potential for IFN-γ secretion upon stimulation (Figs. 1 and 4). Increased expression of the reporter also correlated with enhanced secretion of additional proinflammatory cytokines and chemokines. Highly eYFP-fluorescent CD4<sup>+</sup> and CD8<sup>+</sup> T cells were found only in certain tissues, and their restriction was dependent on the infectious agent. The expression of IFN-γ was broadly heterogeneous in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and occurred before cell division (Fig. 1). In contrast to CD4<sup>+</sup> T cells, which expressed IFN-γ only under Th1-polarizing conditions, CD8<sup>+</sup> T cells activated the *Ifng* gene more rapidly and regardless of a polarizing cytokine milieu. However, the expression levels of IFN-γ were substantially regulated by exogenous factors, such as the presence of polarizing cytokine conditions (Fig. 1). This mechanism might be important for the local modulation of CD8<sup>+</sup> effector functions in vivo. Indeed, a similar heterogeneity of IFN-γ expression by CD4<sup>+</sup> and CD8<sup>+</sup> T cells was observed after infection with the respiratory influenza virus. This heterogeneity represents a distinct functional adaptation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, because innate IFN-γ producers, such as NK and NK T cells, which are constitutively eYFP<sup>+</sup> (20, 21), do not reveal a similar heterogeneity in their reporter fluorescence in naive or infected mice (K. D. Mayer and M. Mohrs, unpublished observations). Despite the systemic dissemination of T cells with heterogeneous eYFP fluorescence, eYFP<sup>high</sup> cells were present only in the influenza-infected lung airways and parenchyma (Figs. 2 and 3). The broad spectrum of eYFP fluorescence was paralleled by the heterogeneous surface expression of acute activation markers, such as CD25, CD69, and CD122, indicating recent antigenic stimulation (28, 32–34). Consistent with this observation, the frequency of cells presenting MHC class I- and class II-restricted antigenic peptides is indeed highest in the infected lung (S. R. Crowe and D. L. Woodland, unpublished observations) (22, 35, 36). It is also conceivable that the highly inflammatory environment in the infected lung enhances IFN-γ expression, potentially synergizing with increased antigenic stimulation (37–39).

The fluorescence heterogeneity and anatomical restriction of eYFP<sup>high</sup> cells are not due to differences in Ag specificity, because identical patterns were observed in NP<sub>366–374</sub>- and PA<sub>224–233</sub>-specific T cells (data not shown). Moreover, recruitment into tertiary sites alone does not result in high levels of eYFP expression, because eYFP<sup>high</sup> cells were not present in the pleural cavity despite similar recruitment and frequency of Ag-specific cell as the infected lung (Fig. 2C). Because increased eYFP fluorescence correlates directly with the abundance of IFN-γ transcripts and the potential to secret larger amounts of IFN-γ (Fig. 4), the accumulation of eYFP<sup>high</sup> cells has a striking functional significance.
The potential for IFN-γ secretion is tightly linked to clonal expansion of Ag-specific CD8+ lymphocytes, because all NP366–374- or PA224–233-specific T cells were eYFP+ in all tissues (Fig. 2C and data not shown). This observation implies that both Ifnγ alleles are frequently expressed, because otherwise a substantial fraction of the cells would be eYFP−. The heterozygous reporter knockin mice that were used throughout the experiments. Expression of the Ifnγ gene in CD4+ T cells was also biallelic, because >95% of these cells in the lung airways and parenchyma were eYFP+ (Figs. 1B and 3A). Thus, the bicistronic IFN-γ reporter marks cells competent for rapid cytokine production, whereas the secretion of this effector cytokine is dependent on antigenic simulation. In fact, the rapid on/off cycling of IFN-γ production by CD4+ and CD8+ T cells has been demonstrated (40–42). As we show in this study, the amount of IFN-γ that can be secreted upon stimulation correlates directly with the expression of the bicistronic eYFP reporter. The accessible Ifnγ gene locus and the constitutive presence of transcripts may allow accelerated cytokine production (43), whereas the requirement for Ag-specific stimulation may limit collateral damage. The greatly enhanced secretion of cytokines upon TCR-mediated stimulation might also be important to focus Ag-specific Th functions to MHC class II-bearing cells (44–46). The accessible eYFP+ cells correlate positively with the enhanced secretion of a select set of cytokines and chemokines (Fig. 5), suggesting a generally functional in conventional CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. Immunity. 159: 105–117.


