Pathogen-Related Differences in the Abundance of Presented Antigen Are Reflected in CD4⁺ T Cell Dynamic Behavior and Effector Function in the Lung

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Pathogen-Related Differences in the Abundance of Presented Antigen Are Reflected in CD4+ T Cell Dynamic Behavior and Effector Function in the Lung

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Exposure to pathogens in the periphery elicits effector T cell differentiation in local lymph nodes followed by migration of activated T cells to and within the infected site. However, the relationships among pathogen abundance, Ag display on MHC molecules, effector T cell dynamics, and functional responses at the infected sites are incompletely characterized. In this study, we compared CD4+ T cell effector dynamics and responses during pulmonary mycobacterial infection versus acute influenza infection. Two-photon imaging together with in situ as well as ex vivo analysis of cytokine production revealed that the proportion of migration-arrested, cytokine-producing effector T cells was dramatically higher in the influenza-infected lungs due to substantial differences in Ag abundance in the two infectious states. Despite the marked inflammatory conditions associated with influenza infection, histocytometric analysis showed that cytokine production was focal, with a restriction to areas of significant Ag burden. Optimal effector function is thus constrained by the availability of TCR ligands, pointing to the value of increasing Ag stimulation rather than effector numbers in harnessing CD4+ T cells for therapeutic purposes in such conditions. The Journal of Immunology, 2014, 192: 000–000.

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Cellular adaptive immunity is initiated in secondary lymphoid organs, where naive recirculating T cells encounter presenting cells (APCs) bearing cognate Ag. These interactions lead to TCR engagement, T cell activation, proliferation, and acquisition of an effector phenotype. The stimulated T cells are then poised to exit secondary lymphoid organs, migrate to inflamed/infected sites, and carry out their effector functions, which, in the case of infectious agents, are aimed at eliminating the pathogen. Although lymphocyte dynamic behavior during the early stages of T cell activation within lymph nodes has been well described (1–4), there are only limited quantitative data on the spatiotemporal aspects of T cell function in peripheral sites. Most but not all studies of effector T cell dynamics in tissues have found that these cells exhibit reduced migration and/or arrest upon recognizing their cognate ligand (pMHC) presented by tissue APCs (5–14). Unfortunately, only a few reports link the assessment of cell motility to Ag-induced activation and local effector responses such as cytokine production by the T cells at the infectious site (5, 14), events that are central to host defense. Indeed, the most commonly used method to measure effector responses is assessment of cytokine production following restimulation of isolated effector T cells ex vivo with Ag or chemical stimuli, an approach that prevents developing an understanding of the extent to which these same T cells are activated to a functional level in vivo, or where in the tissue such function occurs. In considering how adaptive immunity can be harnessed for more effective eradication of infection, especially in organs such as the lungs where the pathogen-induced disturbance of physiological homeostasis can have severe consequences, it is critical to better understand how effector function is delivered, how pathogens evade such responses, and what approaches might be used to improve antimicrobial activity.

We have previously shown that only a very small fraction of Ag-specific CD4+ effector T cells within hepatic granulomas of mice infected i.v. with Mycobacterium tuberculosis or bacillus Calmette–Guérin (BCG) actively produced IFN-γ or TNF-α within the infected liver at a given time. Likewise, only a correspondingly small proportion of the Ag-specific T cells showed migration arrest (14). However, arrest of nearly all Ag-specific effector CD4+ T cells within granulomas could be seen when a substantial amount of mycobacteria-derived antigenic peptide was introduced systematically into the infected animal, and this in turn was accompanied by a parallel increase in the frequency of cytokine-producing effector CD4+ T cells and the magnitude of per cell cytokine synthesis. This implies that there is no intrinsic effector CD4+ T cell deficiency or insurmountable suppressive activity in this infectious setting, but rather that Ag presentation in mycobacterial lesions is limiting (14). Bold et al. (15) used this method of providing extra synthetic specific Ag to examine the potential therapeutic benefits of increased Ag presentation and subsequent increased cytokine production by effector CD4+ T cells in M. tuberculosis–infected mice, documenting greater CD4+ T cell effector function and reduced bacterial burden with such treatment. Thus, for mycobacterial...
infections, low levels of Ag presentation constrain effector activity, and providing additional Ag at the infection site can be used as a strategy for treatment in experimental animal settings.

There are many reasons to wonder whether this striking limitation in Ag-dependent tissue activation of anti-pathogen effector T cells is generally the case or characteristic of only a subset of infections or specific tissue sites. Aerosol mycobacterial infection leads to a protracted immune response culminating in the formation of lung granulomas, which are agglomerations of macrophages and other immune cells, including effector lymphocytes. The formation of granulomas is dependent on MHC class II (MHCII) and IFN-γ, which is mainly produced by effector CD4+ T cells (16, 17). Mycobacteria-derived peptides are presented on MHCII molecules, and these peptide/MHCII complexes can subsequently activate CD4+ T cells (16). The inflammatory cytokines IFN-γ and TNF-α produced by Ag-specific CD4+ T cells then augment the antimicrobial activity of infected macrophages (16, 18–20). It is therefore evident why mycobacteria have developed mechanisms to modulate MHCII presentation to limit such effector CD4+ T cell responses (21, 22). Additionally, mycobacteria are slowly growing organisms, which might itself result in relatively low levels of Ag presentation. For these reasons, it is important to understand whether the limited CD4+ T cell activation at the effector sites is a phenomenon restricted to liver mycobacterial granulomas, or whether it applies to other tissues and pathogens.

To address this issue, we have examined effector CD4+ T cell dynamic behavior and cytokine responses in mycobacteria-infected lungs, which is more physiologically relevant than the liver in areas of substantial Ag burden. Taken as a whole, this study reveals in response to influenza infection was largely focal and restricted to underused capacity for effector molecule production. Interestingly, BCG-infected lungs, indicating that this population has a large, edly increase the very small fraction of cytokine-producing cells in transferred into a BCG-infected host. These differences in cell CD4+ T cells recovered from influenza-infected lungs actively producing IFN-γ as compared with Ag-specific CD4+ T cells recovered from BCG-infected lungs. Peptide Ag administration could markedly increase the very small fraction of cytokine-producing cells in BCG-infected lungs, indicating that this population has a large, underused capacity for effector molecule production. Interestingly, despite the highly inflammatory environment, cytokine production in response to influenza infection was largely focal and restricted to areas of substantial Ag burden. Taken as a whole, this study reveals markedly different efficiencies of effector CD4+ T cell activation in the infected lung in the presence of these two divergent pathogens, driven by differences in the extent of Ag presentation. Our findings have implications for how one might manipulate the adaptive immune response to better deal with mycobacteria and for understanding the contribution of effector responses to immunopathology when Ag reaches high levels and leads to high rate contemporaneous cytokine production by a large population of recruited effector T cells.

Materials and Methods

Mice

C57BL/6 mice were from The Jackson Laboratory, C57BL/6 OTII TCR transgenic (23) RAG1-deficient (24) mice and C57BL/6 OTII TCR transgenic mice were from Taconic. C57BL/6 P25 TCR transgenic RAG1-deficient mice (25) were provided by J. Ernst (New York University School of Medicine, New York, NY). These animals were also crossed to C57BL/6 mice expressing enhanced GFP (EGFP) under the control of the human ubiquitin promoter (UBsEGFP) (26) from The Jackson Laboratory. C57BL/6 Ly5.1+EGFP knock-in animals (27) and C57BL/6 CD11c-EYFP (28) animals were obtained through the National Institute of Allergy and Infectious Diseases/Taconic exchange program. All animals were maintained in specific pathogen-free facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal procedures were approved by and carried out in accordance with National Institute of Allergy and Infectious Diseases Animal Care and Use Committee guidelines under approved animal study protocols.

Pathogens

PR8/05AgA3 (variant of influenza genetically engineered to express the MHCII-restricted peptide from OVA [OVA257-264] presented in H-2Kb) virus stock was provided by Drs. P. Thomas and P. Doherty (St. Jude’s Children’s Hospital) (29). To grow the PR8/05AgA3 virus stock, Madin–Darby canine kidney cells (American Type Culture Collection) were cultured to confluence in DMEM (Life Technologies) supplemented with 10% non-heat–inactivated FCS (Atlanta Biologicals), 1-glutamine, penicillin-streptomycin (Lonza), and nonessential amino acids (Life Technologies). One ml of virus stock was added to the Madin–Darby canine kidney cell layer at 1:10 dilution in DMEM with 7.5% IgG-free BSA (Sigma-Aldrich), further supplemented with 2.5 μg/ml of recombinant bovine trypsin (Tryp2Zean; Sigma-Aldrich). After ~48 h, when ~75% of the cells displayed cytopathic changes, the supernatant was harvested, aliquoted, and stored at ~80°C until used. Mice were inoculated with 50 μl of this supernatant intranasally after a brief period of anesthesia, achieved with inhalational isoflurane.

Wild-type BCG strain Pasteur was provided by Dr. Alan Sher (National Institute of Allergy and Infectious Diseases, National Institutes of Health). Low-dose (5 × 103) or high-dose (5 × 104) CFUs were used for intranasal infection of the animals.

In vitro T cell stimulation and adoptive transfer

To generate in vitro effectors, lymph node and spleens of donor TCR transgenic mice (OTII or P25) were harvested and homogenized through a 70-μm cell strainer. Following RBC lysis, cells underwent CD4 negative T cell selection using magnetic beads (Miltenyi Biotec). Three to 4 × 106 cells were plated with 1 × 104 mitomycin C–treated (Sigma-Aldrich) C57BL/6 splenocytes in RPMI 1640 (Lonza) supplemented with 10% non–heat–inactivated FCS (Atlanta Biologicals), 1-glutamine, penicillin-streptomycin (Lonza), nonessential amino acids (Life Technologies), and 0.1-g/ml L-glutamine (Roche) at a concentration of 1.66 Wuensch units/ml and DNAseI (Roche) at 10 μg/ml. The tissue was then disrupted and processed further into a digestion mixture containing Liberase (Roche) at 10 μg/ml anti-mouse IL-4 (eBioscience), and 10 ng/ml recombinant human IL-12 (R&D Systems). On day 2, 10 ng/ml recombinant human IL-2 (R&D Systems) was also added to the culture medium. On day 6, cells were harvested and for FACS experiments 106 to 107 cells were transferred into recipient mice, whereas for imaging experiments, ~6–8 × 106 T cells were transferred. Transferred populations were either congenically marked, genetically labeled with GFP, or labeled with intracellular dyes (CMTMR, CMFDA, TAMRA, or CF2HSC, all from Invitrogen) for further identification by flow cytometry or imaging.

Cell isolation

For isolation of lymphocytes from lung tissue, mice were sacrificed and the lungs were perfused via the right ventricle. Following RBC lysis, cells underwent CD4 negative T cell selection using magnetic beads (Miltenyi Biotec). Three to 4 × 106 mitomycin C–treated (Sigma-Aldrich) C57BL/6 splenocytes in RPMI 1640 (Lonza) supplemented with 10% non–heat–inactivated FCS (Atlanta Biologicals), 1-glutamine, penicillin-streptomycin (Lonza), nonessential amino acids (Life Technologies), and 0.1-g/ml L-glutamine (Roche) at 10 μg/ml anti-mouse IL-4 (eBioscience), and 10 ng/ml recombinant human IL-12 (R&D Systems). On day 2, 10 ng/ml recombinant human IL-2 (R&D Systems) was also added to the culture medium. On day 6, cells were harvested and for FACS experiments 106 to 107 cells were transferred into recipient mice, whereas for imaging experiments, ~6–8 × 106 T cells were transferred. Transferred populations were either congenically marked, genetically labeled with GFP, or labeled with intracellular dyes (CMTMR, CMFDA, TAMRA, or CF2HSC, all from Invitrogen) for further identification by flow cytometry or imaging.

Flow cytometry

The following Abs were used for flow cytometry: CD3e (145-2C11); eBioscience and 500A2; BD Biosciences), CD4 (RM4-5; BD Biosciences), CD8 (53-6.7; BD Biosciences), and CD90.1 (HI1001; eBioscience). Intracellular cytokine staining was performed with anti–IFN-γ (XMG1.2; BD Biosciences) or anti–TNF-α (MP6-XT22; BD Biosciences) using the Cytofix/Cytoperm kit (BD Biosciences). Flow cytometric data were col-
lected on an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**Immunofluorescence staining and histochemistry**

Lungs were harvested and fixed using PLP buffer (0.05 M phosphate buffer containing 0.1 M l-lysin [pH 7.4], 2 mg/ml NaOAc, and 10 mg/ml paraformaldehyde) for 12 h, then dehydrated in 30% sucrose prior to embedding in OCT freezing media (Sakura Finetek). Thirty micrometer sections were cut on a CM3050S cryostat (Leica) and adhered to Superfrost Plus slides (VWR). Sections were then permeabilized and blocked in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and 10% normal mouse serum (Jackson ImmunoResearch Laboratories) followed by staining in the same blocking buffer. The following Abs were used for staining: anti–IFN-γ (XMG1.2; BD Biosciences), anti-Ag85b (rabbit polyclonal; Abcam), anti–PR8 (goat polyclonal serum; ViroStat), and anti-RSV (goat polyclonal serum; ViroStat). Unconjugated primary Abs were stained with Alexa Fluor–conjugated secondary Abs (Invitrogen). For IFN-γ staining, the signal was further amplified with a secondary goat anti-rat Alexa Fluor-conjugated Ab. Stained slides were mounted with Fluoromount-G (SouthernBiotech) and images were acquired on a 710 confocal microscope (Carl Zeiss Microimaging). Data were processed and analyzed with Imaris (Bitplane). For H&E stains, tissues were harvested in 4% paraformaldehyde and transferred into 70% ethanol 24 h later. Tissue was then processed and stained by Histoserv (Germantown, MD). Bright-field images were acquired on a Leica AF 6000 LX (Leica Systems).

Detailed quantitative analysis methods are described elsewhere (30). Briefly, cellular surfaces were three-dimensionally rendered via the Imaris surface creation module. Influenza stained regions were also analyzed via the Imaris spots creation module. Mean voxel fluorescence intensity statistics and positional information for each surface were exported into Excel. Minimal distances were measured using two-dimensional distance calculation formulas. Fluorescence intensity and distance data were then imported into FlowJo via the text to FCS Java file converter.

**Two-photon imaging of explanted lungs and image analysis**

Mice were euthanized by cervical dislocation, following a brief period of inhalational anesthesia with isoflurane. Lungs were taken out on bloc, rinsed in warm PBS, and the left lobe was then placed on a custom-made imaging platform (Supplemental Fig. 1). Continuous temperature monitoring of the setup was also performed to ensure that a narrow range of 36–38°C was maintained at all times. The images were then acquired on a Zeiss 510 system (Carl Zeiss Microimaging) equipped with a Chameleon laser (Coherent), tuned to 800, 840, or 860 nm, and a ×25 air lens (numerical aperture, 0.8), driven by LSM software. Z stacks of ~50 μm with 3-μm step size were acquired every 30 s. Data were processed and analyzed with Imaris (Bitplane). Through the surface and spot creation module in Imaris, spatial coordinates of objects of interest were obtained over time and data were further analyzed with MatLab.

**Statistical analysis**

A Student t test was used for statistical analysis of differences between groups.

**Results**

**Establishment of a robust in vivo system designed to study CD4+ T cell effector responses in lung tissue**

To compare the CD4+ T cell effector responses in chronic BCG versus acute influenza infection, we made use of two distinct TCR transgenic systems as the Ag-specific indicator populations, namely in vitro–generated effector CD4+ T cells derived from mycobacterial AgAg85b-specific (OVA-unrelated) P25 TCR transgenic RAG1-deficient mice (P25) and from OVA-specific (mycobacteria-unrelated) OTII TCR transgenic RAG1-deficient mice (OTII). Although it would be optimal to perform the studies as described using exactly the same TCR transgenic T cell reporter population, this would require either mycobacterial peptide–expressing influenza recombinants or recombinant mycobacteria expressing an Ag shared with wild-type or recombinant influenza. At present, such organisms with validated infectious behaviors have proved problematic to construct, as insertion of foreign peptides can markedly alter the infectivity of the virus and its virulence. We therefore obtained a PR8 strain expressing the MHCII-restricted peptide from OVA, OVA323 (29). This strain has been shown to elicit a CD4+ T cell response and to cause a viral pneumonia similar to, if slightly milder than, the parental strain (29), permitting us to use this organism for analysis with a well-characterized T cell population (OTII) in the context of a near normal infectious process. Unfortunately, BCG-expressing OVA has been very poorly immunogenic for OTII T cells in our hands (J.G. Egen and A.G. Rothfuchs, unpublished data). Given our expectation that influenza was likely not to limit MHCII Ag presentation in the same manner as BCG, we chose to use OTII T cells to analyze the response to influenza and the Ag85b-specific P25 transgenic T cells to assess the response to BCG infection, as we have done previously. This of course raises the possibility that observed differences in effector behavior might be attributable to intrinsic differences in sensitivity to cognate Ag rather than distinct biology associated with the two different pathogens.

To directly examine this critical issue of Ag sensitivity of the two transgenic T cell populations, wild-type splenocytes were pulsed with titrated amounts of either cognate peptide (Ag85b or OVA323) and these presenting cells were incubated for 5 h with their respective Ag-specific CD4+ T effector cells: either P25 or OTII day 7 effector cells generated in vitro employing a Th1-polarizing protocol. Intracellular cytokine staining was performed, along with surface staining for congenic markers to discriminate the transgenic T cell populations. More P25 T cells produced IFN-γ as compared with OTII T cells when exposed to APCs pulsed with the same molar concentration of cognate peptide (Fig. 1A). To examine this issue in vivo, we injected various equimolar amounts of OVA323 and Ag85b peptide i.v. after prior transfer of the respective effector cell populations. Two hours after peptide delivery, mice were sacrificed and spleens isolated. Single-cell suspensions were then stained for intracellular IFN-γ. Again, at each peptide dose, P25 T cells displayed a greater ability to produce cytokines, such as IFN-γ (Fig. 1B), recapitulating the in vitro findings. In combination with other studies (31) that reveal the same response hierarchy using methods independent of the capacity of the two peptides to bind MHCII molecules (i.e., basal levels of partial TCR ζ-chain tyrosine phosphorylation, levels of CD5 expression, and cognate tetramer binding), these data make a strong case that P25 T cells are intrinsically more sensitive to their cognate Ag as compared with OTII T cells and that if all, the present studies of effector responses are biased in favor of P25 T cells in terms of sensitivity to elicitation of effector cytokine production, even though it is this T cell population that shows limited responses to its cognate Ag under infection conditions in vivo (14). Thus, we anticipated that if we observed better effector responses from the OTII T cells in response to the recombinant PR8, this was unlikely to reflect a bias in this direction in our experimental design due to T cell–intrinsic factors.

Having established the Ag response hierarchy of OTII and P25 T cells, we proceeded to develop model systems that would allow us to answer the questions delineated above (Fig. 1C, 1D). For pulmonary BCG infections, we chose a dose of 5 × 103 CFUs, which is a hundred times lower than the dose we previously used for i.v. infections, given that 1% of i.v. administered mycobacteria arrive in the lung (32–34). Pulmonary infection with mycobacteria led to formation of mature granulomas defined as macrophage agglomerates in the lung by 8–12 wk and at this time point most of the BCG organisms were confined to these structures (Supplemental Fig. 2A). It has also been reported that the number of effector CD4+ T cells in the lung peaks at 8–12 wk after aerosol infection using a 5-fold lower dose of BCG than we employed in the present study (33). Additionally, we found that transferring
effector CD4+ T cells at earlier time points when granulomas had not yet formed resulted in poor effector cell recruitment to the lung (data not shown). For these reasons, we chose to study the 8- to 12-wk period after infection. This infection ran an indolent clinical course, with no weight loss and with minimal pulmonary pathology upon gross examination (Supplemental Fig. 2B). In a time frame ranging from 8 to 12 wk postinfection, mice received in vitro–generated effector CD4+ T cells derived from P25 (BCG-specific) and OTII (Ag-unspecific) TCR transgenic animals. For influenza infections we chose a sublethal dose, which led to significant weight loss and morbidity (Fig. 1C, 1D) and pulmonary pathology (Supplemental Fig. 2B). Days 6–7 postinfection represent the peak of the CD4+ and CD8+ T cell adaptive response to influenza (35), and therefore day 7 was chosen as the experimental end point. One day prior to analysis, mice received OTII T cells as well as P25 T cells as the non-Ag–specific control population.

**Effector T cells display distinct effector behavior in lungs infected with different agents**

To examine CD4+ T cell effector responses to mycobacterial Ags in the lung, mice were infected with BCG intranasally and 8–12 wk later received in vitro–generated Th1-polarized effector CD4+ T cells derived from P25 (BCG-specific) and OTII (Ag-unspecific) TCR transgenic animals. For influenza infections we chose a sublethal dose, which led to significant weight loss and morbidity (Fig. 1C, 1D) and pulmonary pathology (Supplemental Fig. 2B). Days 6–7 postinfection represent the peak of the CD4+ and CD8+ T cell adaptive response to influenza (35), and therefore day 7 was chosen as the experimental end point. One day prior to analysis, mice received OTII T cells as well as P25 T cells as the non-Ag–specific control population.

**Fig. 1.** Establishment of a robust in vivo system designed to study CD4+ T cell effector responses in lung tissue. (A) Day 7 in vitro–generated effector T cells (P25s or OTIIs) were incubated with an equal number of splenocytes pulsed with the cognate Ag at the indicated concentrations for 5 h. Percentage of IFN-γ–producing T cells is represented. Bar graph to the right indicates the percentage of IFN-γ+ P25 and OTII T cells when stimulated in vitro with PMA plus ionomycin. (B) Naive mice received ~1 million day 6 in vitro–generated effector T cells (P25s and OTIIs). After 18 h, the mice received equimolar amounts of both OVA323 and Ag85b. Two hours later, the mice were sacrificed and the percentage of IFN-γ–producing transferred cells in the spleen was determined. Data are representative of at least two independent experiments done in triplicates. Symbols and bars indicate means with indicated SEM. (C) Schematic representation of the experimental setup. (D) Percentage weight change over time. Symbols indicate mean of at least five animals, and error bars indicate the SEM. FC, Flow cytometry; IHC, immunohistochemistry; 2PM, two-photon microscopy.

Despite the systemic nature of the BCG infection, very few P25 cells (1–2%) were producing inflammatory cytokines, such as IFN-γ (Fig. 2A, 2B) or TNF-α (Supplemental Fig. 3A), as assessed by intracellular staining. However, >50% of these cells had the potential to produce cytokine when they were stimulated in vitro with PMA plus ionomycin or in vivo with peptide (Fig. 2A, 2B, Supplemental Fig. 3A), indicating that cytokine production in the granuloma by effector T cells is limited by activation conditions in the infected tissue and not by T cell–intrinsic constraints on cytokine production capacity. In contrast to our previous analysis in liver (14), obtaining a peptide-dependent increase in cytokine production under these conditions required delivery of the Ag by both the i.v. and intranasal routes. Control OVA-specific T cells in these mice also behaved similarly, in that they produced virtually no cytokine when examined directly upon extraction from the tissue, but were able to do so when maximally stimulated (Fig. 2B, filled bars). In BCG-infected lungs, we did not detect any statistically significant differences in cytokine responses between Ag-specific and nonspecific effectors recovered from the tissue (Fig. 2B). Although we conducted these experiments when the number of CD4+ T cells in the granulomas is reported to reach a peak (33), another study suggested that cytokine production reaches a maximum earlier postinfection (36), and it was also possible that providing more Ag in the form of a higher inoculum of bacteria might change the outcome. We therefore infected mice systemically with a hundred times higher BCG dose ($5 \times 10^7$ CFUs) and studied earlier time points after intranasal infection. Even 2 wk after systemic BCG infection with this higher dose, very few (~5%) P25 cells in the lungs produced IFN-γ (Supplemental Fig. 3B). Additionally, we infected mice intranasally with...
FIGURE 2. Effector CD4+ T cells display distinct effector behavior in lungs infected with different agents. (A and C) Representative FACS plots showing the percentage of all transferred cells producing IFN-γ, as measured by intracellular cytokine stain, when taken from BCG- (A) or influenza-infected mice (C). Numbers indicate percentages. Gating strategy for lung single-cell suspensions involved gating on live cells with a generous forward and side scatter gate, then gating on CD45+ cells, followed by gating on the transferred T cell population of interest. Finally, CD4 by IFN-γ was plotted. Negative gate set was based on isotype control for each experimental condition. (B and D) Quantification of the percentage of IFN-γ+ P25 T cells or OTII T cells, when taken directly ex vivo from the lungs, after 4 h of in vitro PMA plus ionomycin stimulation, or 2 h after in vivo administration of cognate Ag. Responses of the control effector population not recognizing Ag are shown in the same graph depicted by different color bars. Data are represented as means ± SEM. Statistical analyses were performed using the unpaired Student t test. (E) Data represented as percentage of maximal IFN-γ production potential, obtained by comparing percentage IFN-γ+ ex vivo to percentage IFN-γ+ after PMA plus ionomycin stimulation. Data are representative of at least four independent experiments with three to five mice per group. Data are represented as means ± SEM. (F) Mice infected with BCG 11 wk prior were also infected with PR8OVA323. Seven days after PR8OVA323 infection (and thus 12 wk after BCG infection), effector T cells were transferred into the animals. Eighteen hours after transfer, transferred T cells were isolated and the percentage of IFN-γ-producing cells (gated on P25s or OTIs) was determined. Data are representative of two independent experiments with three mice per group. Data are represented as means ± SEM. IN, Intranasally; IV, intravenously; P/I, PMA plus ionomycin.

In contrast, cells isolated from influenza-infected mice behaved quite differently. A significant proportion of the isolated Ag-specific OTII T cells were actively producing cytokines (20–30%), as measured by IFN-γ (Fig. 2C, 2D) or TNF-α intracellular staining (Supplemental Fig. 3D). This fraction, however, was still lower than the maximal potential for the population, as stimulation with either PMA plus ionomycin in vitro or cognate peptide administration in vivo led to a greater frequency (40–70%) of cytokine-containing T cells (Fig. 2C, 2D, Supplemental Fig. 3D). Alternatively, control P25 T cells not specific for any influenza virus-encoded Ag barely produced cytokine when taken directly ex vivo, but did respond to pharmacologic reagents (Fig. 2D, open bars). The quantitative differences in ex vivo effector cytokine production by Ag-specific T cells seen in the BCG-infected versus the influenza-infected mice were even more apparent when we looked at effector cytokine production represented as a fraction of the maximal potential (Fig. 2E). Examined in this way, it was evident that a much smaller proportion of T cells in BCG-infected mice specific for a mycobacterial Ag are stimulated to produce cytokines in situ as compared with the T cells in the influenza-infected mice specific for an Ag encoded by this virus.

To test whether the inflammatory environment could be the basis for the difference in cytokine production in response to BCG and influenza infection, the ex vivo cytokine production by OTII and P25 T cells was compared in animals coinjected with BCG and PR8OVA323. Both OTII and P25 T cells isolated from coinjected animals maintained the cytokine production profile typical of their counterparts isolated from either BCG- or PR8OVA323-infected animals (Fig. 2F), arguing against the inflammatory environment as the basis for the low effector cytokine production in response to BCG infection. In fact, the maximal potential reached by the control (Ag-unspecific) T cell populations in the two infection models...
after transfer suggest that the milieu was more anti-inflammatory in influenza than in BCG-infected lungs. The OTII T cells resident in the BCG-infected lungs maintained the ability to produce cytokine to an extent similar to what the cells were able to achieve prior to transfer (70–80% in both cases) (Fig. 2B, filled bars, Supplemental Fig. 3E). Alternatively, the P25 T cells in the influenza-infected mice, when maximally stimulated, did not reach the same level of cytokine production as they could prior to transfer into the infected animals (40–50 versus 70–80%, respectively) (Fig. 2D, open bars, Supplemental Fig. 3F). These findings are consistent with earlier reports of anti-inflammatory activities in the lungs of influenza-infected animals (37–39), making even more striking the disparity between high rates of specific responses in influenza-infected tissue as compared with BCG-infected lungs. This disparity was not simply the result of employing TCR-transgenic effector cells in these studies, because similar differences were seen among endogenously responding T cells (Supplemental Fig. 3G).

**Differences in CD4+ T cell effector function are mirrored by differences in dynamic behavior**

Cytokine-producing cell frequency and arrested cell frequency were closely matched in liver granulomas (14), consistent with a model in which only those T cells engaging APCs with enough TCR ligand to cause signaling arrest migration and initiate an effector cytokine response. An alternative model has been proposed for tissue effector responses in which T cells sum subthreshold signals acquired by sequential interaction with many presenting cells until activation is achieved, leading to cytokine production without migration arrest (40, 41). Such a scenario would predict a marked difference between the fraction of cytokine-producing cells and the fraction of arrested Ag-specific T cells in each of these infection models. We therefore examined effector CD4+ T cell dynamic behavior in the lung.

For imaging, we developed a lung explant model (Supplemental Fig. 1). An intravital microscopy approach (where the lung is imaged in a live animal either by surgical exposure via a thoracotomy or through an implanted window), although theoretically preferable, has substantial limitations with regards to our question. Both BCG- and influenza-infected lung tissues are quite heterogeneous with respect to the distribution of cells and infected areas. Because of this heterogeneity, the ability to scan large areas of lung tissue is a critical requirement for avoiding sampling errors, and this capacity is provided with explants and not the presently available intravital techniques.

Similar to our previously published data examining liver granulomas in systemically infected mice (14), both Ag-specific and nonspecific effector T cells were found to accumulate in the lung granulomas (Fig. 3A). The mean velocities of the P25 and the OTII effector populations in the lung tissue were not significantly different (Fig. 3B, Supplemental Video 1), and, in line with this, total T cell displacement during the duration of imaging was also not significantly different between the two lymphocyte populations in the same tissue sample (Fig. 3C, Supplemental Video 1).

To determine whether these findings applied to other infectious settings, we conducted similar experiments using influenza A as the pulmonary pathogen, an infectious situation in which we anticipated that Ag abundance would not be limiting given the cytokine response data reported above. Six days after infection with PR8OVA123, mice received OTII T cells as the Ag-specific population as well as P25 T cells as the control population. Sixteen to 18 h later, lungs of influenza-infected mice were imaged using the explant technique described above. Both CD4+ T cell populations accumulated in the PR8-infected lungs 18–20 h after transfer (Fig. 3D). In contrast to what was seen in mycobacteria-infected pulmonary tissue, two-photon imaging of explanted lungs showed a significant difference in migration dynamics between the OTII T cells specific for a virally encoded Ag and the control P25 T cells. OTII T cells moved at a significantly slower mean velocity when compared with the P25 T cells in the same lung analyzed at the same time (Fig. 3E, Supplemental Video 2). Total displacement was also significantly less when comparing the Ag-specific T cell population to the control T cells (Fig. 3F, Supplemental Video 2). We also analyzed the difference in dynamic behavior between Ag-specific and nonspecific effector T cells in the BCG- and influenza-infected lungs by determining the frequency of cells moving at different velocities and representing these data as a frequency distribution (Fig. 3G, 3H). Both the Ag-specific population and the control population displayed a similar velocity distribution in the BCG-infected lung (Fig. 3G); in contrast, the OVA-specific OTII T cells showed a distribution skewed toward slower velocities in infected lungs expressing their cognate Ag OVA as compared with the Ag-unspecific P25 T cells in this same setting (Fig. 3H). This alternative way of displaying the data shows more clearly that most OVA-specific effector T cells in the influenza/OVA-infected lungs are moving at velocities considered to represent arrest in migration (at ~2 μm/min). Overall, these findings, which were in sharp contrast to the BCG infection model, suggested that the influenza-infected lung provided more opportunity for TCR engagement and signaling by the Ag-specific T cells. Furthermore, the significantly greater fraction of Ag-specific T cells displaying migration arrest in conjunction with a greater proportion of these T cells producing inflammatory cytokines in influenza-infected lungs compared with BCG-infected lungs was suggestive of an inverse relationship between cell migration and productive Ag sensing.

**Local Ag abundance dictates T cell effector function as well as dynamic behavior**

Additional experiments were conducted to further examine the hypothesis that differences in Ag abundance play a major role in determining the distinct fraction of effector T cells responding in the two infection models. Because providing more peptide in vivo enabled larger fractions of the OTII and especially the P25 T cells to produce cytokines (Fig. 2B, 2D), it did not seem that the number of APCs or MHCII expression by APCs in the tissue was a limiting factor. To look more directly at the role of Ag abundance, we stained paraformaldehyde-fixed lung frozen sections for the presence of Ag85b and PR8 (owing to the lack of an Ab that reliably identifies OVA123 peptide, we employed a polyclonal serum against PR8, using polyclonal anti-RSV serum as a specificity control; Supplemental Fig. 4). Ag85b was restricted to the center of granulomas and was present in small amounts, as previously reported (14) (Fig. 4A). Alternatively, influenza protein staining was much stronger and more widespread in the lung (Fig. 4B). Although this staining represents the protein source of antigenic peptides and not the processed, MHC-bound fraction, these data do suggest that there is greater availability of the influenza-encoded Ag for generation of pMHC ligands, in agreement with the above findings.

To determine whether in this virally infected tissue with widespread inflammation and Ag presence cytokine production was focal and targeted, we examined whether a finer grained relationship existed between Ag (virus) location within the lung tissue and effector T cell cytokine production by determining the location of IFN-γ–producing cells in relation to virally infected cells (Fig. 4C). Through histocytometric image analysis (30), we found a nonrandom distribution of the IFN-γ–producing Ag-specific CD4+ T cells, nearly all of which were located within ~100 μm of the influenza-infected cells (Fig. 4D, 4E). Thus, whereas both Ag-specific and
nonspecific effector CD4+ T cells are located in the general area of infection, nearly all IFN-γ–producing Ag-specific cells are within 100 μm of the infected cells, and most imaged Ag-specific cells in these virally infected areas show an arrest of migration. We conclude that influenza-specific and nonspecific effector CD4+ T cells are efficiently recruited to infected areas where the level of Ag presentation is sufficient to induce influenza-specific T cell arrest and IFN-γ production. Although not all the T cells extracted from the entire influenza-infected lung show IFN-γ production without restimulation, ~70% of the Ag-specific cells are in the regions of high Ag density (Fig. 4D), and of the cells that are in these regions, ~50% are arrested (Fig. 3H). This 35% of the total OTII T cells corresponds closely to the fraction of T cells making IFN-γ directly ex vivo. Hence, in the case of influenza infection, there is a strong relationship between Ag-induced stopping and cytokine response, as we previously observed in the liver granuloma model (14).

Discussion
In this study, we present a careful analysis of effector CD4+ T cell dynamic behavior and cytokine production in Mycobacterium versus influenza-infected lungs in an effort to determine whether our previous report of severe Ag restriction leading to very limited CD4+ T cells responses in mycobacterial liver granulomas was generalizable to other organs (the lung) and whether the same limitation in T cell responses was seen with an acute severe infection.

We found that as in BCG- or M. tuberculosis–infected liver, Ag85b-specific CD4+ T cells showed very limited activation in BCG-infected lungs, with only a few percent of the cells showing TCR-mediated migration arrest or containing IFN-γ or TNF-α at

FIGURE 3. Differences in CD4+ T cell effector function are mirrored by differences in dynamic behavior. (A-C) Lung BCG granuloma image acquired by two-photon microscopy and analysis thereof. (D-F) Image of inflammatory patch in an influenza-infected lung acquired by two-photon microscopy and analysis thereof. (A and D) Maximum projection rendering of imaging volume. Colors of the words correspond to colors in the image, here and throughout. In (A), the dotted line delineates the margins of the granuloma in the image. P25s are labeled with the intracellular dye CMTMR and OTIIs with the intracellular dye CMF2HC. In (D) OTIIs are labeled with the intracellular dye TAMRA and the P25s with the intracellular dye CMF2HC. (B and E) Mean velocity of OTIIs and P25s; red line indicates mean value. (C and F) Total displacement over the duration of the imaging session (red line indicates mean value) and representation of the tracks of OTIIs and P25s over the duration of the video, all translated to the same starting point. (G and H) Frequency distribution of P25 and OTII velocities in BCG (G) and influenza-infected tissue (H); bin width, 2 μm. Dashed lines indicate mode of the distributions. All data are representative of at least three similar experiments, with at least three mice per group. Statistical analysis was performed using the unpaired Student t test.
the time of extraction, although most of the T cells could make cytokine when stimulated ex vivo or in vivo using antigenic peptide. In striking contrast, OTII CD4+ T cells specific for a peptide encoded by recombinant influenza virus exhibited substantial migration arrest in infected lungs and a corresponding high frequency of cytokine-producing cells. Using quantitative image analysis by histocytometry, we could demonstrate that a large fraction of effector T cells in influenza-infected lungs reside within 100 μm of a rich source of viral protein Ag and that most IFN-γ-producing T cells are within this radius, with a very close correspondence between migration arrest and cytokine production. Given our evidence that the P25 T cells specific for Ag85b are more sensitive to Ag display than OTII T cells, these findings clearly indicate that Ag paucity restricts the effector T cell response in mycobacterial infection, but that such a limitation is specific to only a subset of pathogens.

We assessed T cell dynamic behavior in lung tissue using an explant approach to lung imaging. This technique does not require excessive ex vivo manipulation of the organ (such as inflation with agarose gel or sectioning of the tissue), allows for imaging of immune cell behavior in the lung for up to 2 h, and permits survey of a much larger volume of tissue than intravital approaches using windows. With this technique, we not only generated data showing the same correlation between migration arrest and in vivo cytokine production in influenza-infected lung tissue as seen in mycobacterial liver granulomas (14), but we also made additional observations related to the effects of infection state on T cell motility in general. For example, the P25 T cells in the influenza-infected

FIGURE 4. Ag abundance dictates T cell effector and dynamic behavior. (A and B) Immunofluorescent staining of BCG-infected mouse lung with anti-Ag85b Ab (arrow indicates Ag85b stain) (A) or of influenza-infected mouse lung with anti-PR8 Ab (B). P25 and OTII T cells were labeled with an intracellular dye prior to transfer. In (A), the dotted line delineates the margins of the granuloma in the image. P25s are labeled with the intracellular dye CMTMR and the OTIIs with the dye CMF2HC. In (B) and (C) P25s are labeled with the intracellular dye CMFDA and OTIIs with the dye CMTMR. (C) IFN-γ staining in an influenza-infected mouse lung. Insets shown are magnifications of the areas in the gray boxes for panels (A)–(C). (D) Two-dimensional contour plot depicting data from fluorescent immunohistochemical analysis of influenza-infected mouse lungs, after importing position and mean fluorescent intensity data obtained in Imaris into FlowJo, as described in Materials and Methods. Data are representative of two independent experiments. (E) Percentage of IFN-γ-producing OTIIs within the population of cells that reside either beyond or within 100 μm of influenza-infected cells. Each dot represents one experiment and the red line represents the mean.
tissue lacking their cognate Ag moved at a greater mean velocity than did OTII T cells in BCG-infected lungs lacking the relevant Ag. This reduced velocity of control T cells in BCG-infected lungs might partially be the result of the dense myeloid environment that T cells must migrate through in granulomas (14). These data emphasize the importance of ensuring the presence of a control population in all imaging experiments, as the comparison can then be made with a cell population exposed to the same physical and chemical environmental cues as the cellular population of interest. They also indicate that the factors influencing T cell motility are quite divergent in the two infectious states and suggest it will be of importance to characterize this distinct milieu and the impact that slower T cell effector motility has on the clearance of pathogen (42). Computational studies have indicated that the rate of pathogen detection, a parameter that is highly dependent in turn on effector cell migration velocity, plays a key role in determining the balance between pathogen eradication versus continued expansion (43, 44).

Our model involves the transfer of in vitro-activated effector T cells. Although we could explore P25 function and behavior starting with small numbers of transferred naive cells in the liver infection model in which mature granulomas appear within 2–3 wk, genetic incompatibilities between the presently available P25 TCR transgenic animals and the C57BL/6 recipients preclude such studies over the longer (8–12 wk) time frame required for granuloma development in the lung infection model. However, we have performed experiments in influenza-infected mice where the effector T cells have been generated in vivo, by transferring naive T cells prior to infection at numbers close to what would be expected to be the precursor frequency of several hundred cells per mouse (45). These experiments recapitulated the data obtained with the in vitro–generated effector T cells (data not shown), namely, that a substantial fraction of all effector T cells actively engaged in effector function. Additionally, to address the question of whether the use of transferred effector cells had an impact on the fraction of cytokine-producing cells recovered, we also analyzed the endogenous CD4+ T cell pool for the production of cytokine. We again found that cytokine production was significantly higher in the influenza-infected mice than in the case of the BCG-infected animals.

Taken together, these findings are in agreement with our hypothesis that Ag abundance is in fact responsible for regulating T cell dynamic behavior in peripheral tissue, with greater Ag presence associated with a significant reduction in motility and this in turn associated with greater cytokine production. Although some investigators have proposed that summation of shorter contacts can also lead to T cell activation under certain conditions (40), our data are in agreement with observations made by others (5, 6, 8, 14) that stopping and prolonged TCR stimulation most closely correlates with cytokine production. Additionally, we show that although Ag-unspecific effector CD4+ T cells are effectively recruited to the effector site, the inflammatory environment does not lead to substantial Ag-unspecific effector responses and that effector responses are strictly dependent on the presence of Ag at the effector site. This conclusion is supported by the lack of cytokine production by the Ag-unspecific effector control populations under infectious conditions, the cytokine production profile displayed by the Ag-specific cells under coinfection conditions, and the cytokine responses seen when using in vivo peptide to maximize effector responses by one of the two effector populations. We speculate that the lack of Ag-unspecific effector function may be an evolutionarily acquired defense mechanism designed to minimize the deleterious effects of excessive cytokine production in vital organs, such as the lung. Finally, using a new method for multicolor quantitative immunohistochemistry (30), we demonstrate that cytokine-producing effector CD4+ T cells are in close proximity to infected cells, further supporting the idea that cytokine production is focal, specific, and Ag-driven.

The ex vivo flow cytometric analysis of whole tissue homogenates we use in this study provides information about cellular behavior at a population level, but it does not provide information on single cell behavior in the tissue of interest that can be directly linked to the dynamic behavior of that cell. Unfortunately, existing gene expression reporter systems that encode long-lived cytoplasmic fluorescent proteins do not provide an accurate picture of cytokine expression because they persist for much longer than cytokine is made and secreted by activated T cells (46). However, by combining an ex vivo approach with in vivo imaging we have been able to gain insight into the origin of the heterogeneity of effector T cell migratory and functional behavior at the effector site, providing evidence that Ag density plays a major role in the extent of the response by tissue-homing effector T cells and that this parameter differs markedly in infections with diverse pathogens. More widespread and robust Ag presentation in the setting of an acute pulmonary influenza infection leads to engagement and activation of a larger fractional percentage of the effector T cell pool compared with a chronic nonresolving mycobacterial infection. Nevertheless, even during acute pulmonary influenza infection, effector function may not reach its maximal potential. This insight suggests that one way to improve local effector responses is to increase Ag display in the infected site. This approach may be constrained, however, by difficulty in targeting such Ag to focal sites of pathogen replication rather than broadly within a tissue, organ, or even the whole body, with the risk of inducing immunopathology due to overexuberant responses. Additional studies will be needed to determine whether it is possible to balance the host protective effects of such Ag administration with the possibility of disruption of tissue homeostasis.

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Disclosures

The authors have no financial conflicts of interest.

References

Ag ABUNDANCE CONSTRAINS CD4+ T CELL EFFECTOR FUNCTION


