Antigen-specific and non-specific CD4+ T cell recruitment and proliferation during influenza infection

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Abstract
To track epitope-specific CD4+ T cells at a single-cell level during influenza infection, the MHC class II-restricted OVA323–339 epitope was engineered into the neuraminidase stalk of influenza/A/WSN, creating a surrogate viral antigen. The recombinant virus, influenza A/WSN/OVAII, replicated well, was cleared normally, and stimulated both wild-type and DO11.10 or OT-II TCR transgenic OVA-specific CD4+ T cells. OVA-specific CD4+ T cells proliferated during infection only when the OVA epitope was present. However, previously primed (but not naive) transgenic CD4+ T cells were recruited to the infected lung both in the presence and absence of the OVA323–339 epitope. These data show that, when primed, CD4+ T cells may traffic to the lung in the absence of antigen, but do not proliferate. These results also document a useful tool for the study of CD4 T cells in influenza infection.

Keywords: Antigen; CD4+ T cell; Influenza

Introduction
The importance of CD4+ T cells in protection against viral pathogens is well documented. In murine models of viral infection, CD4+ T cells are necessary for the production of protective antibody, as well as formation of fully functional, long-lived effector and memory CD8+ T cells (Cardin et al., 1996; Matloubian et al., 1994; Riberry et al., 2000). The mechanisms used by CD4+ T cells to help in both long-lived antiviral antibody production and augmentation of effector CD8+ CTLs for the formation of memory are not completely understood. These studies have been confounded, in part, by the lack of readily available tools to effectively identify and track populations of antigen-specific CD4+ T cells during infection. The advent of MHC Class I tetramer technology has provided tremendous insight into the nature of antigen-specific CD8+ T cells at the single-cell level. However, similar methods for CD4+ T cells have been slower to develop.

Influenza infection has a number of features that make it a useful pathogen for study of T cell immune responses. Upon intranasal inoculation, the virus infects murine lung epithelium and is readily cleared at sublethal doses by CD8+ CTL and antibody (Doherty et al., 1997; Walker et al., 1992). The productive infection is localized to the lung tissue, and no persisting virus can be detected (Eichelberger et al., 1991). These features make influenza infection a good model for the study of both the effector and memory phases of a T cell response.

The ability to genetically engineer influenza virus has become a useful tool for research. The reverse genetics method for engineering recombinant negative-strand viruses...
was developed over a decade ago (Enami et al., 1990; Luytjes et al., 1989), and recent technological advances have developed more efficient means of engineering viruses in this way (Neumann and Kawaoka, 2001; Neumann et al., 1999). These viruses have proven useful in a wide array of research areas, including studies on viral pathogenicity, vaccine design, and the introduction and expression of foreign antigen (Maassab and Bryant, 1999; Neumann and Kawaoka, 2001). Several recombinant influenza viruses have been shown to productively infect cells and present engineered epitopes in the context of both MHC Class I and Class II (Castrucci et al., 1994; Garcia-Sastre et al., 1994; Miyahira et al., 1998; Nimmerjahn et al., 2003; Strobel et al., 2000). The utility of this system to study antiviral CD8 T cell responses was demonstrated with influenza A/WSN/OVA1 (WSN-OVA1), a recombinant influenza containing OVA 257 – 264 (Topham et al., 2001). For T helper responses, Walker et al. (1997) found that a recombinant influenza containing the HEL 46 – 63 epitope engineered into the neuraminidase (NA) stalk activated 3A9-TCR transgenic (Tg) CD4+ T cells (specific for HEL 46 – 63) in an MHC-restricted manner. This demonstrated that engineering MHC Class II-restricted immunogenic epitopes into the NA stalk of influenza allowed for processing and presentation of that epitope in the context of infection. We have used this technology to create influenza A/WSN/OVAII (WSN-OVAII), a novel recombinant influenza virus with the peptide OVA 323 – 339 inserted into the viral NA stalk.

The addition of an ovalbumin epitope to influenza has several advantages over other approaches to prime influenza- or OVA-specific CD4 T cells. Priming of OVA-specific CD4+ T cells during an infectious inflammatory response is less artificial than injection of high concentrations of purified or synthetic antigen. The infection is localized compared to several other OVA-transfected pathogens such as vaccinia and listeria (Pope et al., 2001; Restifo et al., 1995). Compared to other transgenic models for influenza, this system has the advantage of being able to track the antigen-specific CD4 T cells with defined antibodies. In the HNT CD4 TCR transgenic system, for example, there is neither a clonotypic antibody nor an allotype marker that can be used to track the cells in vivo. Thus, we have a system to track antigen-specific CD4 T cells at the single cell level during a respiratory infection.

Using this approach, we can further investigate which components of an immune response are required for proliferation versus recruitment of virus-specific CD4+ T cells to the site of infection. For example, it has been suggested that the proliferation and endothelial cell transmigration of human CD4+ T cells in response to antigen are mutually exclusive responses (Marelli-Berg et al., 1999). In addition, previous reports have demonstrated that OVA-primed Tg2 cells can be recruited to the lung in the presence of viral infection, LPS, or IgE cross-linking without specific antigen (Stephens and Chaplin, 2002; Stephens et al., 2002). For CD8+ T cells, it has been shown that activation requires specific antigen, but recently activated or memory cells could be recruited to the lung in an antigen-nonspecific manner during influenza infection (Topham et al., 2001).

This work focuses on the use of a novel viral system to study the recruitment and proliferation of naive and/or primed antigen-specific CD4+ T cells during infection. These studies extend previous work by measuring the kinetics of antigen-specific CD4+ T cell recruitment to lung tissue, as well as expansion and cytokine secretion during the acute response. We show that the requirement for antigen by CD4+ T cells differs with regard to recruitment and proliferation. The experiments establish the utility of the WSN-OVAII system for study of antigen-specific CD4+ T cell responses during respiratory virus infection.

Fig. 1. Modification of influenza A/WSN neuraminidase to include OVA 323 – 339 sequence and kinetics of viral replication and clearance. A) Map of the neuraminidase gene of parental A/WSN/33, and the modified A/WSN-OVAII. Reverse genetics (Enami et al., 1990; Luytjes et al., 1989) were employed to exchange the amino acid sequence for NA 43 – 58 with the sequence for OVA 323 – 339. Solid line indicates parental sequence; OVA 323 – 339 peptide is bracketed. B) Kinetics of viral replication and clearance of primary WSN-OVAII infection in C57BL/6 mice. 500 plaque-forming units (PFU) WSN-OVAII was administered intranasally (i.n.) to 8 week-old female mice. Individual lungs were homogenized and titrated for virus via MDCK plaque assay. Data points represent individual mice, with 3 – 5 animals per time point, and values expressed as log10 PFU per ml of lung homogenate.
Results

WSN-OVAII virus

Engineering of the WSN-OVAII virus involved replacement of a segment of the neuraminidase (NA) stalk by OVA323–339 (Fig. 1A). The resulting virus, with NA43–58 (Harley et al., 1989) replaced by OVA323–339, contained a slightly longer NA stalk. Since changes to the NA stalk of influenza can affect the efficiency of viral escape from host cells (Castrucci and Kawaoka, 1993), it was important to confirm that the modified WSN-OVAII virus could replicate in vivo. The lungs of wild-type C57BL/6 (B6) mice were sampled at varying time points during primary infection with WSN-OVAII. Virus peaked at day 6 post-infection, was rapidly reduced to minimal titers by day 10, and became undetectable in all mice tested by day 14 (Fig. 1B). The virus titers and clearance were not different than that obtained after infection with other recombinant WSN influenza viruses (data not shown).

Cellular immune response to WSN-OVAII infection

To test whether the engineered OVA epitope would be presented in the context of infection, mice were inoculated with 500 pfu WSN-OVAII intranasally, and ELISpot assays were performed over the infection course. Indeed, OVA323–339-specific CD4+ T cells secreting IFN-γ were detectable throughout the course of infection (Fig. 2A). This antigen-specific response was characterized by a defined peak on day 9 and was accompanied by increased cellularity in the lung and airway (BAL) (Fig. 2D), and correlated well with the decrease in viral load observed between days 8 and 10 post-infection (Fig. 1B).

Since our virus stocks were generated in embryonated chicken eggs, the possibility existed that the OVA323–339
response could have been due to contaminating ovalbumin in the inoculum. Three pieces of evidence made this unlikely. One, the ovalbumin content of the allantoic fluid is negligible by days 9–10 of gestation, and the allantoic virus suspensions are diluted 1000-fold before inoculation. Secondly, there was no detectable OVA<sub>323–339</sub> response in mice inoculated with WSN-recombinant influenzas missing the OVA<sub>323–339</sub> epitope. Thirdly, mice inoculated with allantoic fluid from uninfected day 9 embryonated chicken eggs did not have a detectable ovalbumin-specific immune response in the lung (data not shown), even when OVA-specific CD4 T cells had been transferred (Table 1). Together, these data support the conclusion that the OVA<sub>323–339</sub> response detected in the WSN-OVAII-infected mice was against the inserted epitope and not to contaminating ovalbumin from the eggs used to grow the virus.

In the WSN-OVAII-infected mice, the highest frequencies of antigen-specific T cells were found in the infected lung and airways (Fig. 2A), and low in the MLN and spleen. When compared to the CD8<sup>+</sup> T cell response against the influenza nucleoprotein epitope (NP<sub>264–272</sub>), the kinetics of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were nearly identical (Figs. 2A, B), but the magnitude of the OVA<sub>323–339</sub> response was approximately 10-fold lower in frequency (Fig. 2A) than that of the CD8<sup>+</sup> NP<sub>264–272</sub> response (Fig. 2B). However, the OVA<sub>323–339</sub> response was intermediate in frequency compared to CD4<sup>+</sup> T cell responses against two recently defined endogenous influenza epitopes, NA<sub>161–175</sub> and HA<sub>91–105</sub> (Crowe et al., in press) within the neuraminidase and hemagglutinin proteins, respectively (Fig. 2C). This suggests that the magnitude and kinetics of cytokine response to OVA<sub>323–339</sub> is comparable to that of endogenous influenza peptide responses.

Recall potential of OVA<sub>323–339</sub>-specific cells primed by WSN-OVAII

An interesting feature of the primary CD4<sup>+</sup> T cell response elicited by the WSN-OVAII infection was that, although there was a substantial population of OVA<sub>323–339</sub>-specific cells in the lung and BAL during the acute phase, there were relatively few cells in the MLN and spleen (Fig. 2A). Furthermore, in recovered animals, the OVA<sub>323–339</sub>-specific CD4<sup>+</sup> response had fallen to levels at or below the limit of detection (<20/10<sup>6</sup>) of the assays in the MLN and spleen, while the response in the lung tissue was just above the limit of detection (Fig. 3B). This was in contrast to the response to whole virus, to which robust virus-specific

<table>
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<th>Table 1</th>
<th>Frequency and number of OT-II cells recovered day 9 post-treatment</th>
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<tr>
<td>Treatment</td>
<td>BAL Frequency&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WSN-OVAII</td>
<td>2.120</td>
</tr>
<tr>
<td>Control WSN</td>
<td>0.078</td>
</tr>
<tr>
<td>Allantoic fluid</td>
<td>0.467</td>
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<tr>
<td>Uninfected</td>
<td>0.765</td>
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<sup>a</sup> Frequency calculated from percentage of CD4<sup>+</sup> Thy1.2<sup>+</sup> cells in the lymphocyte gate.

<sup>b</sup> Numbers were calculated from the frequencies multiplied by the total cell counts for each organ.

Fig. 3. Presence of OVA<sub>323–339</sub>-specific CD4<sup>+</sup> T cell memory and recall responses following WSN-OVAII infection. Following infection with 500 PFU WSN-OVAII, animals were allowed to clear the virus and then assayed at day 30 post-infection by cytokine ELISpot. Briefly, single cell suspensions of enriched CD4<sup>+</sup> T cells isolated from lung, MLN, or spleen of 5–10 mice per experiment were cultured on cytokine antibody coated plates for 20 h with either whole, live WSN-OVAII virus (MOI = 2) (A) or 3 µg/ml OVA<sub>323–339</sub> (B). Bars represent the mean spot forming cells (SFC) per million CD4<sup>+</sup> lymphocytes, and error bars indicate the standard deviation of triplicate wells. C) The CD4<sup>+</sup> recall response in the lung against OVA<sub>323–339</sub> was tested 4 weeks after infection with WSN-OVAII by intranasal LPS/OVA instillation. Control animals had not been infected with WSN-OVAII. At day 5 after LPS/OVA administration, cells from BAL and lung were measured for IFN-γ and IL-2 secretion by ELISpot. Control animals were also assayed at day 7 for IL-2 production to be sure that a response had developed. Error bars indicate the standard deviation of triplicate wells. b.d. = below detection.
CD4+ T cell IL-2 or IFN-γ spot forming responses were detectable in recovered animals (Fig. 3A, note scale). The data show that a relatively small population of flu-primed CD4+ T cells might persist in the airways, spread over a greater number of single epitopes, especially compared to the CD8 response elicited by the same virus.

These observations raised questions regarding the functional qualities of a recall CD4+ response in the lung should antigen be re-encountered. To study the recall response of OVA-specific CD4+ T cells primed by the virus infection, mice were inoculated with WSN-OVAII and then allowed to clear the virus. Thirty days later, WSN-OVAII immune or uninfected cohorts were administered OVA323–339 with LPS by intranasal route. The WSN-OVAII immune cohort exhibited a strong OVA323–339-specific response by day 5 of LPS/OVA challenge, an early response characteristic of immune animals that was not detectable at day 5 in the unprimed mice (Fig. 3C). In the unprimed cohort, a predominantly IL-2 secreting (Fig. 3C) response to OVA323–339 was detectable at day 7, with few IFN-γ-secreting cells (data not shown). Both the rapid response and differentiated cytokine pattern exhibited in the WSN-OVAII immune mice suggest that a functional resident memory population of OVA323–339-specific CD4+ T cells exists.

Single cell analysis of CD4+ T cells in WSN-OVAII infection

Having demonstrated that WSN-OVAII could elicit an OVA323–339-specific response from wild-type CD4+ T cells, we sought to determine whether the CD4+ T cell response to influenza infection could be followed at the single cell level. Another advantage of the OVA323–339 epitope is that it is restricted by two distinct class II MHC alleles expressed by H-2d BALB/c and H-2b C57/Bl6 mice (Robertson et al., 2000). This allows investigation of an influenza primed CD4 response in two distinct genetic backgrounds. We used either IAn restricted DO11.10 (on BALB/c background) or IAb-restricted OT-II (on C57BL/6 background) OVA323–339-specific transgenic T cells. Another advantage over the wild-type or HNT systems is that the specific cells could be followed both in uninfected mice, or those infected with an otherwise identical virus missing the OVA epitope.

Unprimed DO11.10 or OT-II spleen cells were adoptively transferred to the appropriate BALB or B6.PL naive recipients prior to infection with WSN-OVAII or a control WSN virus that contained an irrelevant epitope. On day 9, the animals were sampled for the presence of either KJ126+ (DO11.10 Y BALB) or Thy1.2+ (OT-II Y B6.PL) CD4+ T cells. WSN-OVAII infection resulted in recruitment of substantial numbers of both types of transgenic T cells to the lung and BAL during the peak of infection (Figs. 4A, B). As expected, these cells were uniformly CD44high (Figs. 4A, B), reflecting efficient activation (DeGrendele et al., 1997a, 1997b; Eichelberger et al., 1991; Ewing et al., 1995) in vivo by this virus. Though no transgenic CD4+ T cells appeared in the lung or airways of uninfected mice, a small number of CD44high transgenic CD4+ cells were detected in mice infected with a control influenza virus lacking the OVA sequence (data not shown). This opened the question as to whether the few cells observed in the control animals were a contaminating, primed population from the transgenics, or cells activated and recruited non-specifically by the virus infection.

Role of antigen in recruitment of naive OT-II CD4+ cells

To determine whether naive CD4+ T cells could be non-specifically activated and recruited to the lung by an antigenically unrelated virus infection, we sorted CD44low CD4+ T cells from unprimed Thy1.2+ OT-II donors and transferred 5 x 10^5 per mouse into Thy1.1+ congenic
The animals were left alone (uninfected) or infected with either WSN-OVA II or a control WSN influenza virus without the OVA sequence. Mock infection with allantoic fluid from uninfected eggs was used as a second negative control for non-specific activation of transgenic cells. As in the previous experiments, WSN-OVA II infection resulted in substantial activation and recruitment of the OT-II cells to the infected lung (Table 1 and data not shown). However, though OT-II cells could be detected in the lymph nodes and spleen, the frequency and number of OT-II cells in the lung tissue or airways of mice infected with control WSN were not different from either uninfected or allantoic fluid inoculated animals (Table 1). The total number of OT-II cells in the MLN of the control flu infected mice was higher than in the uninfected and mock-infected nodes, but the frequencies in these three groups were essentially the same, so the difference is due to the overall increase in cellularity of this node in the infection and does not reflect any selective recruitment or expansion of the OT-II cells. In fact, an increase in the overall number of OT-II cells recovered was apparent only in the WSN-OVA II-infected mice (Table 1). This clearly demonstrates that T cell activation via specific antigen is required for expansion and recruitment of naive CD4+ T cells during influenza infection.

Role of antigen in the recruitment of primed CD4+ T cells to the lung

The previous experiments looked at the ability of WSN-OVA to activate and recruit unprimed or naive CD4+ T cells. The first series of experiments with unsorted cells suggested that some previously activated CD4+ T cells might be non-specifically recruited to the lung during infection. To determine whether primed CD4+ T cells could be non-specifically recruited to the lung, in vitro primed, TH1 polarized OT-II cells were adoptively transferred prior to infection. OT-IIxB6.PL (Thy1.1+) CD4+ T cells were stimulated with OVA323–339 peptide in vitro for 4 days along with IL-12 and anti-IL-4 mAb, then washed and transferred into Thy1.2+ B6 recipients. Cohorts were left uninfected, inoculated with control virus or WSN-OVA II. Thy1.1+ OT-II CD4+ T cells could be detected in the lungs of infected mice regardless of which virus was present. However, infection with WSN-OVA II resulted in increased cell recovery from the airways, lung, and MLN over control virus infection (Fig. 5), suggesting that the presence of specific antigen in these sites promoted the recruitment or retention of specific CD4+ T cells. Few cells were found in lymph nodes that did not drain the infected lung. Though not all possible organs were examined, the net number of OT-II cells recoverable from the animals was the same in both WSN-OVA II (4.9 × 10^6) and control (5.5 × 10^6) influenza-infected mice, both higher than in the uninfected controls (2.9 × 10^6). Together the data suggest that infection is not a requisite for bystander recruitment of previously activated T cells to the lung, since cell recovery from BAL, lung, and MLN were similar in the control virus and no infection groups (Fig. 5). However, it suggests infection may promote the non-specific expansion or survival of previously activated CD4+ T cells.

Antigen-specific proliferation of CD4+ T cells in response to influenza infection

To distinguish whether the increased cell recovery in the lung and BAL was due to proliferation, unprimed CD4+ TCR transgenic T cells were CFSE-labeled prior to adoptive transfer and infection the next day with WSN-OVA II or control virus. In mice receiving unprimed (but not sorted) CD4+ T cells, identical numbers of transgenic cells were detected by flow cytometry in both groups 48 h after transfer (1 day after infection), indicating that the adoptive transfer efficiency was satisfactory in both groups. At day 5 post-infection, WSN-OVA II-infected mice had large numbers of transgenic T cells actively proliferating in the MLN, with few proliferating cells found in the lung tissue and spleen (Fig. 6A). This correlates well with the observations in wild-type mice without adoptively transferred transgenic

![Fig. 5. Recruitment of in vitro activated T cells by WSN-OVA II infection. OT-II cells were isolated and polarized in vitro to a TH1 phenotype by culture with 10 μg/ml OVA323–339 peptide, 10 μg/ml α-CD28, 10 ng/ml rIL-2, 5 ng/ml rIL-12 and 10 μg/ml α-IL4 antibody for 4 days along with IL-12 and anti-IL-4 mAb, then washed and transferred into Thy1.2+ B6 recipients. Cohorts were left uninfected, inoculated with control virus or WSN-OVA II. Thy1.1+ OT-II CD4+ T cells could be detected in the lungs of infected mice regardless of which virus was present. However, infection with WSN-OVA II resulted in increased cell recovery from the airways, lung, and MLN over control virus infection (Fig. 5), suggesting that the presence of specific antigen in these sites promoted the recruitment or retention of specific CD4+ T cells. Few cells were found in lymph nodes that did not drain the infected lung. Though not all possible organs were examined, the net number of OT-II cells recoverable from the animals was the same in both WSN-OVA II (4.9 × 10^6) and control (5.5 × 10^6) influenza-infected mice, both higher than in the uninfected controls (2.9 × 10^6). Together the data suggest that infection is not a requisite for bystander recruitment of previously activated T cells to the lung, since cell recovery from BAL, lung, and MLN were similar in the control virus and no infection groups (Fig. 5). However, it suggests infection may promote the non-specific expansion or survival of previously activated CD4+ T cells.](image-url)
cells in which the OVA\textsubscript{323–339}–specific IFN-\textgamma response was not detectable until around day 6 (Fig. 2A). Interestingly, in control influenza-infected mice, though little to no dilution of CFSE was apparent (Fig. 6A), a population of donor CFSE\textsuperscript{+} cells could still be recovered from the lung and MLN of the control virus group, lending further support of bystander recruitment of CD4\textsuperscript{+} T cells during influenza infection, and suggesting that non-specific recruitment and proliferation of CD4\textsuperscript{+} T cells can be uncoupled.

In addition to studies with unprimed cells, T\textsubscript{H}1-primed transgenic cells were also tested for their ability to proliferate in the absence of specific antigen. In vitro polarized T\textsubscript{H}1 cells were rested via injection into naive hosts for 1 week. These cells were then re-isolated and injected i.v. into hosts that subsequently received no infection, control virus, or WSN-OVA\textsubscript{I\textgamma}. Transgenic cell proliferation was monitored by BrdU incorporation. Fig. 6B shows substantial BrdU incorporation by CD4\textsuperscript{+} T cells in the airways of WSN-OVA\textsubscript{I\textgamma}-infected mice at 6 days post-infection. BrdU levels in control virus-infected mice, on the other hand, were not different from uninfected controls. Together with the data from Fig. 5 above, the findings suggest little non-specific expansion of primed CD4\textsuperscript{+} T cells occurs in the absence of antigen, even though there is some evidence of antigen-independent trafficking to the lung. The fact that we observed proliferation, but no increase in cell recovery of donor T\textsubscript{H}1 OT-II cells in WSN-OVA\textsubscript{I\textgamma}-infected mice over control WSN infection could be explained by either increased apoptosis of the antigen-activated cells, decreased apoptosis of transgenic cells in the control-infected mice, or distribution to other tissues that were not sampled. Investigations are underway to resolve this dilemma.

**Discussion**

We have shown WSN-OVA\textsubscript{I\textgamma} is an extremely useful tool for the study of CD4\textsuperscript{+} T cell biology during viral infection. The insertion of the class II MHC-restricted OVA\textsubscript{323–339} epitope into the virus did not alter the infection significantly from that of similar influenza viruses containing an antigenically neutral engineered epitope. In addition, the generation of a specific CD4\textsuperscript{+} T cell response against OVA\textsubscript{323–339} suggests the engineered OVA\textsubscript{323–339} epitope is efficiently presented during the infection. Using defined OVA\textsubscript{323–339}\textsuperscript{+} specific TCR transgenic cells showed the responding cells were activated and recruited to the lung tissue, where they contributed to the overall flu response by secreting IFN-\textgamma.

Of note is the low frequency, and number, of the OVA-specific response in both wild-type B6 and adoptive transfer systems. The frequencies of CD4 T cell responses to two other, recently identified, immunodominant class II restricted, endogenous epitopes in influenza virus were, respectively, higher or lower than the response to the inserted OVA epitope. Thus, the OVA-specific response in the context of influenza is in the same range as the responses to naturally occurring epitopes. The data suggest that class II restricted CD4\textsuperscript{+} T cell responses to individual influenza virus epitopes, on the whole, are programmed to be low in frequency and abundance. This is compared to the substantial response to whole virus, suggesting the overall response must be spread among many epitopes. Similar results were observed after infection with a natural murine respiratory virus, Sendai virus (Ewing et al., 1995). These observations raise doubts as to the utility of class II MHC
tetramers and even intracellular cytokine staining methods for accurately enumerating the CD4⁺ T cell response to infection, especially in the memory phase. The low frequencies of single-epitope-specific CD4⁺ T cells will make it a challenge to reliably see the antigen-specific cells above background unless the techniques and reagents are highly developed.

Compared to CD8⁺ responses to flu, there is an approximate ten-fold difference in frequency between individual CD4⁺ and CD8⁺ antigen reactive pools, similar to other pathogens (Seder and Ahmed, 2003). This difference may be due to the capacity of CD8⁺ T cells to proliferate more extensively than CD4⁺ T cells (Foulds et al., 2002), thus resulting in smaller clonal populations of CD4⁺ T cells against any given viral epitope. However, viral epitope density and clonal T cell survival also contribute to the observable T cell pool, and therefore it cannot be assumed that the magnitude of an antiviral CD4⁺ T cell response is reduced simply because of reduced proliferative capacity. In any case, it seems clear that intrinsic differences exist in the programming of CD4⁺ and CD8⁺ T cell activation, proliferation, and acquisition of effector function.

Nevertheless, in spite of the low frequencies and abundance of OVA 323–339-specific cells, a subset of these cells survived the contraction phase following viral clearance, and recovered animals have an enhanced OVA 323–339-specific recall response characterized by rapid onset and greater magnitude over primary challenge. These results suggest that a viable memory population of OVA 323–339-specific CD4⁺ T cells is generated in response to WSN-OVAHI infection.

An advantage of our engineered system is that it is now possible to use readily available TCR transgenics to overcome some of the problems associated with low-frequency T cells. This has given us the capability to study virus-specific CD4⁺ cells at the single cell level, and has in turn revealed some interesting aspects of CD4⁺ T cell biology. Not surprisingly, it is clear that OVA 323–339-specific T cells proliferate and acquire differentiated effector function when their activating antigen is present in the virus. However, after infection with a virus lacking the activating antigen, small numbers of primed OVA 323–339-specific TCR transgenic T cells are recruited to the lung tissue during the acute inflammatory response. This does not occur if the cells have been sorted for a naive (CD44⁺ CD8⁻) phenotype. Even when deliberately activated prior to transfer, though they traffic to the lung, the majority of these non-specifically recruited cells do not appear to proliferate significantly. This suggests in vivo proliferation is not a prerequisite for extravasation into tissue and that antigen regulates CD4⁺ T cell proliferation, even during active inflammation. This implies that T cells which are localized to inflamed tissue and subsequently under the influence of inflammatory mediators may not be responsive to these stimuli unless a recent encounter with specific antigen has occurred. This fits with the idea that CD4⁺ T cells acquire full differentiation as effectors in the draining lymph node subsequent to encounter with specific antigen (Jenkins et al., 2001).

By tracking a population of surrogate virus-specific CD4⁺ cells, we found evidence for the trafficking of primed CD4⁺ T cells to the lung with specificities unrelated to the viral infection. In particular, small numbers of in vitro primed CD4⁺ T cells localized to the lung regardless of whether infection or antigen was present. This trafficking is therefore largely independent from the presence of inflammation in the lung, perhaps reflecting a steady state recruitment of previously activated cells into this site. The localization of memory CD4⁺ T cells to non-lymphoid tissues has been previously shown (Jenkins et al., 2001), but it is not clear whether the presence of these cells reflects tissue residence or constant replacement of tissue memory from the blood. Our data suggest the latter, but do not rule out the possibility that a small resident population exists. In addition, since the tissue memory cells appear to undergo minimal proliferation, it is likely that this population is constantly replaced by the lymphoid tissues.

Recently, it has been suggested that memory CD8⁺ T cells appear in the infected lung in three distinct phases (Woodland and Randall, 2004). There is a resident tissue memory population at the outset, and a second population that is recruited from the blood in an antigen-independent manner. Neither of these first two populations has recently proliferated. The third population is derived from the lymphoid compartment, and has proliferated in response to antigen. It is not yet known whether primed and memory CD4⁺ T cells behave in a similar fashion during virus infections. However, our data suggest that, like the CD8 memory cells, primed CD4⁺ T cells can either reside or be recruited to the infected lung early in the infection without regard to antigen-specificity or requiring recent cell division. It is also clear that the later accumulation of specific cells is an antigen-driven process for both CD4⁺ and CD8⁺ T cells.

The significance of tissue-resident memory CD4⁺ T cells and early non-antigen-specific recruitment to the pulmonary immune response is not clear. Prior vaccination for a CD4⁺ T cell response, or direct intratracheal transfer of virus-specific CD4⁺ T cells into the lung can produce an early reduction in viral load (Hogan et al., 2001; Zhong et al., 2000), but also appears to inhibit the subsequent CD8⁺ response (Hogan et al., 2001; Zhong et al., 2000). In a model of dermal contact sensitivity, early (but not late) CD4⁺ T cell recruitment was dependent on selectin-mediated interactions (Hwang et al., 2004). Late CD4⁺ T cells accumulation was more dependent on a-4-integrin (Hwang et al., 2004). Blockade of P- and E-selectins within the first 2 h of antigen exposure prevented early CD4⁺ T cell trafficking to the challenge site and abrogated the later antigen-specific inflammatory response (Hwang et al., 2004). Together, the data suggest that the small numbers of CD4⁺ T cells that appear early in the immune response can profoundly impact later events. It also suggests that...
early non-specific and late antigen-specific trafficking is regulated by distinct adhesion interactions.

In addition to their potential impact during the acute response, these observations raise questions as to the role of non-specifically recruited cells after the infection is resolved. For instance, it is unknown whether they persist following infection or whether these cells affect future pulmonary infection or antigen encounters. With regard to persisting cells in the lung tissue, even with cognate activation and recruitment in the context of specific antigen, within a month of infection, antigen-specific CD4+ T cells were below detection in the airways, and only small numbers of cells were detectable in the lung tissue. It is likely that the antigen-independent resident cells would be at even lower frequencies and numbers, thus limiting the impact of these local cells on subsequent encounters with antigen. However, as suggested by the contact sensitivity model mentioned above, resident cells and early non-specific recruits from the blood may combine to shape later events in the immune response. Future work will be aimed at gaining insight into these important issues.

The WSN-OVA2 viral system, together with currently available transgenic mice and antibodies, constitute an effective model to study antigen-specific CD4+ T cells on a single-cell level during infection. It has several advantages over previous approaches including the ability to track the antigen-specific cells with antibodies, and the ability to study the CD4+ T cell response to an identical antigen and infection in two distinct strains of mice. The lung-specific tropism of influenza also makes it a good model system with which to study tissue-specific trafficking and function of CD4+ effector and memory subsets (Sallusto et al., 1999). Results reported in this paper confirm the importance of specific antigen in activation and accumulation of effector T cells in the lung during influenza infection. Further work aimed at characterizing the nature of bystander T cells may contribute to our understanding of the requirements for CD4+ T cell activation, recruitment, effector function, and survival during and after viral infection.

Materials and methods

Cell culture

Madin Darby Canine Kidney (MDCK) cells were maintained in C-MEM supplemented with non-essential amino acids and sodium pyruvate, and were grown at 37 °C with 5% CO2. At 1 day prior to determination of viral titers via plaque assay, MDCK cells were plated in 6-well plates at a density of 3 × 10^5 cells/ml in 2 ml maintenance medium.

Reverse genetics

Engineering of influenza/A/WSN was carried out as previously described (Enami et al., 1990) using the pT3WSN-OVAII plasmid, with the NA gene flanked by an upstream T3 RNA polymerase promoter and a downstream Ksp6321 site. Transcripts were complexed with purified nucleoprotein and polymerase proteins. The resultant complexes were then transfected into MDCK (bovine) cells with helper virus. The progeny virus of the 1st infection was recombinant, as tested by sequencing PCR-amplified cDNA. Recombinant virus was subsequently purified, titered on MDCK cells, and stored at −80 °C.

Mice

C57BL/6 (H-2b) and BALB/c (H-2b) mice were purchased from Taconic laboratory (Germantown, NY). Thy1.1+ B6.PL (H-2b) and H-2d (IAd-restricted) BALB/c TCR Tg DO11.10 and H-2b (IA^b-restricted) C57BL/6 OT-II Thy 1.2+ mice were bred and maintained at the University of Rochester Animal Housing facility (Rochester, N.Y.) in specific pathogen-free conditions. OT-II × B6.PL Thy 1.1+ mice were bred and maintained at the Sidney Kimmel Cancer Center (San Diego, C.A.) in specific pathogen-free conditions.

Cell polarization and adoptive transfer

Spleen and lymph nodes of DO11.10 or OT-II TCR Tg mice were removed and placed in HBSS. Tissues were ground in homogenizers, and the resultant homogenate was passed through nylon mesh into a 15 ml conical-bottom tube. For unpolarized DO11.10 or OT-II cells: cells were either left untreated or stained with CFSE. After treatment, 1–5 × 10^7 cells were injected i.v. into either BALB/c (for DO11.10) or B6.PL (for OT-II) recipients. For OT-II Th1 cells: CD4+ T cells were magnetically separated using negative selection with a CD4 enrichment cocktail from BD (Imag) according to the manufacturer’s protocol. To generate Th1 cells, the cells were cultured for 4 days at 10^6/ml with an equal number of C57BL/6 spleen cells that were pretreated with 25 mg/ml mitomycin C. Media (RPMI 1640) containing 10% FBS, 2% PSG, 1 mM HEPES, and 5 × 10^{-5} M 2-ME was supplemented with 10 μg/ml OVA323–339 peptide, 10 μg/ml anti-CD28 (37N51.1.), 10 ng/ml rIL-2, 5 ng/ml rIL-12, and 10 μg/ml anti-IL-4 (11B11). On day 4, the cells were harvested, washed, and 5 × 10^6 Tg+ cells were injected i.v. into C57BL/6 recipients.

Cell proliferation

CFSE: unpolarized DO11.10 cells were prepared as above. Cells were resuspended at a concentration of 50 × 10^6/ml, and CFSE was added at a final concentration of 5 μM for 5 min prior to adoptive transfer. BrdU: OT-II Tg+ cells were prepared as above, and then rested for 5 days by transfer of 7 × 10^6 into normal C57BL/6 recipients. The cells were isolated from the spleens and pooled peripheral LN by magnetic separation using...
negative selection with a CD4 enrichment cocktail and Thy 1.2 conjugated beads (Imag). 3–5 × 10^6 Tg^+ cells were injected i.v. into a second set of recipients that were uninfected or were infected i.n. with 1000 PFU of either WSN-FLAG or WSN-OVA<sub>H</sub>. On the day of cell transfer, all groups of mice were injected i.p. with 1 mg BrdU and were treated with BrdU water (800 μg/ml) for 6 days.

**Viral infection**

Stocks of WSN-OVA<sub>H</sub> or control WSN-HEL (Walker et al., 1997), WSN-FLAG (Castrucci et al., 1992), or WSN-OVA<sub>I</sub> (Topham et al., 2001) recombinant flu viruses in allantoic fluid were thawed from −80 °C to room temperature. Virus was diluted in 10 ml cold DPBS so that a 30-μl volume would contain 500–1000 PFU of virus. Recipient mice were first sedated with avertin (2,2,2-tribromoethanol) i.p., then given a 30-μl intranasal inoculation of virus.

**Organ harvest**

Mice were Avertin anesthetized and exsanguinated via brachial artery. Bronchoalveolar lavage (BAL) samples were collected by three intratracheal lung washes (with C-mem) using a Teflon cannula attached to a 1-ml syringe. Lung tissue, mediastinal lymph node (MLN), and spleen were homogenized and filtered through nylon mesh. All organ cells were maintained in C-mem prior to use.

**Plaque assay**

Lungs were removed from infected mice, frozen at −80 °C, thawed and then homogenized in 1 ml HBSS. Virus was then serial diluted 10-fold in HBSS/BSA. 0.5 ml of each dilution of virus was added to individual wells of 6-well plates containing a monolayer of fresh MDCK cells (see Cell Culture). Plates were incubated 60 min for viral uptake. Virus was then washed off with 2 ml HBSS/BSA. Equal volumes of 1.2% SeaKem agarose (BioWhittaker, Wakefield, MD) and 2× MEM supplemented with 100 μg/ml gentamycin (BioWhittaker, Wakefield, MD), 0.4% 1× phenol red and 0.5 μg/ml TPCK trypsin (Worthington, Lakewood, NJ) were mixed and added to cell monolayers at 2 ml/well. Plates were incubated 48 h at 37 °C with 5% CO<sub>2</sub>. Plaques were visualized by staining agar overlay with 2 ml/well 1% crystal violet in 10% normal buffered formalin for 2 h. After rinsing and drying plates, plaques were enumerated and average PFU of triplicate wells was reported.

**Flow cytometry**

All Abs used for flow cytometric analysis were purchased from BD PharMingen (San Diego, CA). Flow cytometry was performed on a FACS Calibur (Becton Dickinson) and analyzed with Cell Quest Pro (BD) analysis software. Cell sorting was performed on a FACS Aria (BD) and analyzed with FACS DiVa (BD) software.

**ELISpot assay**

IFN-γ (R4-6A2) or IL-2 (JES6-1A12) capture Ab and biotinylated α- IFN-γ (XMG1.2) or α-IL-2 (JES6-5H4) secondary Abs were purchased from BD PharMingen. C57BL/6 or BALB/c naïve splenocytes, 500,000/well, were peptide-pulsed (5μg/ml for 2 h) with OVA<sub>323–339</sub> (sequence SQAVHAAHAINEAGR), NA<sub>161–175</sub> (A/WSN/33 sequence SVAWSASACHDGVGW) or HA<sub>91–105</sub> (A/WSN/33 sequence LPARSWYSIVETPNS), infected with influenza (10 PFU/cell), or left untreated and then used as APCs in the 16–20 h overnight culture. Detection of secondary Ab involved incubation with streptavidin-conjugated alkaline phosphatase (Jackson Immunoresearch Labs, West Grove, PA) and the Vector alkaline phosphatase substrate kit (Burlingame, CA). ELISpot plates were analyzed using the CTL Immunospot plate reader and counting software (Cellular Technology).

**LPS/OVA**

LPS (Sigma serotype 0111:B4) and OVA<sub>323–339</sub> peptide were mixed at 1 mg/ml and 2 mg/ml, respectively, in DPBS. LPS/OVA was administered intranasally in 30 μl to Avertin-anesthetized mice. Mice were monitored for recovery from anesthesia.

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