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Influenza A Virus Infection Results in a Robust, Antigen-Responsive, and Widely Disseminated Foxp3$^+$ Regulatory T Cell Response

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Foxp3$^+$ CD4$^+$ regulatory T cells (Tregs) represent a highly suppressive T cell subset with well-characterized immunosuppressive effects during immune homeostasis and chronic infections, although the role of these cells in acute viral infections is poorly understood. The present study sought to examine the induction of Foxp3$^+$ CD4$^+$ Tregs in a nonlethal murine model of pulmonary viral infection by the use of the prototypical respiratory virus influenza A. We establish that influenza A virus infection results in a robust Foxp3$^+$ CD4$^+$ T cell response and that regulatory T cell induction at the site of inflammation precedes the effector T cell response. Induced Foxp3$^+$ CD4$^+$ T cells are highly suppressive *ex vivo*, demonstrating that influenza virus-induced Foxp3$^+$ CD4$^+$ T cells are phenotypically regulatory. Influenza A virus-induced regulatory T cells proliferate vigorously in response to influenza virus antigen, are disseminated throughout the site of infection and primary and secondary lymphoid organs, and retain Foxp3 expression *in vitro*, suggesting that acute viral infection is capable of inducing a foreign-antigen-specific Treg response. The ability of influenza virus-induced regulatory T cells to suppress antigen-specific CD4$^+$ and CD8$^+$ T cell proliferation and cytokine production correlates closely to their ability to respond to influenza virus antigens, suggesting that virus-induced Tregs are capable of attenuating effector responses in an antigen-dependent manner. Collectively, these data demonstrate that primary acute viral infection is capable of inducing a robust, antigen-responsive, and suppressive regulatory T cell response.

Immune responses to foreign antigens must be carefully tempered in order to prevent inadvertent activation of self-reactive lymphocytes and to reduce collateral damage to normal tissue. A principal mechanism of immune moderation is mediated by regulatory T cells (Tregs), a distinct subset of T lymphocytes that contribute to immune system homeostasis and tolerance to self-antigens. A diverse assortment of Tregs have been described in both CD4$^+$ and CD8$^+$ T cell subsets, although Tregs expressing the CD4 coreceptor have been studied in greater detail and thus far have been ascribed greater physiological importance. In the CD4$^+$ Treg family, the transcription factor Foxp3 is a characteristic marker in mice and (to a lesser degree) humans, although Tregs not bearing this marker, such as Tr1 and Th3 Tregs, have been previously described (9, 17). CD3$^+$ CD4$^+$ Foxp3$^+$ Tregs can be broadly divided into 2 subsets, with natural, or constitutive, Foxp3$^+$ Tregs (natural Tregs) being generated in the thymus through major histocompatibility class II (MHC-II)-dependent T cell receptor (TCR) interactions, resulting in high-avidity selection (19, 21), while induced Foxp3$^+$ Tregs are generated in the periphery during an immune response and thus are likely to be potent regulators of ongoing inflammation (12, 13).

The ability of Tregs to recognize foreign antigens during influenza virus infection, and indeed during most encounters with microbial pathogens, is unknown. Utilizing Tregs collected from *Leishmania major*-infected animals and cultured with bone marrow-derived dendritic cells (BMDCs) in the presence of *L. major* metacyclics, Suffia and coworkers (35) noted that the majority of CD25$^+$ Foxp3$^+$ CD4$^+$ Tregs in the draining lymph node during chronic *L. major* infection were antigen specific, providing early evidence that antigen specificity may be a crucial factor in the Treg-mediated suppression of foreign-antigen-specific immunity. Ertelt and colleagues, however, were unable to identify antigen-specific Treg priming with a murine model of acute *Listeria monocytogenes* infection, suggesting that antigen-specific Treg responses are not engendered by every pathogen (14). The role of Tregs in acute infection is hypothesized to be driven by enhanced presentation of self-antigens, in which self-reactive natural Tregs limit effector responses in a bystander manner (3). Consistent with this, Liu and colleagues were able to identify only small numbers (<1%) of MHC class II epitope-specific Tregs in respiratory syncytial virus (RSV) infection (23). Similarly, very low numbers of influenza virus matrix 1-specific CD4$^+$ T cells demonstrating regulatory properties have also been identified in the peripheral blood mononuclear cells (PBMCs) of human donors (29).

Influenza A virus infection is characterized by robust activation of both the innate and adaptive arms of immunity and is associated with strong antigen-specific CD4$^+$ and cytotoxic CD8$^+$ T cell responses. Such lymphocyte activation, while important for the control of viral load, is also associated with considerable immunopathology leading to significant morbidity and death; thus, the induction of regulatory mechanisms may be vital (20). Recently studies identified a role for Foxp3$^+$ CD25$^+$ CD4$^+$ Tregs in murine models of RSV infection, further suggesting that Foxp3$^+$ Tregs may be influential in acute infection (16, 22, 32). The present study sought to examine the induction of Tregs in a murine model of nonlethal, primary influenza A virus infection and address the wider question of antigen specificity of Tregs in acute

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viral infection. Our findings demonstrate that influenza A virus induces a robust Foxp3$^+$ CD4$^+$ regulatory T cell response, particularly at early stages of infection, and that these cells are highly suppressive *ex vivo*. Influenza virus-induced Tregs proliferate in response to antigen, are widely disseminated, and stably express Foxp3. The ability of influenza virus-induced Tregs to suppress antigen-specific CD4$^+$ and CD8$^+$ T cell proliferation and cytokine production correlates with their antigen specificity, suggesting that such cells are capable of suppressing effector responses in an antigen-dependent manner.

MATERIALS AND METHODS

Mice and virus. C57BL/6 mice, C.Cg-*Foxp3$^{tm2(eGFP)Tch}$*/J mice in which enhanced green fluorescent protein (eGFP) was inserted downstream of the Foxp3 translational stop codon, resulting in all Foxp3$^+$ T cells coexpressing eGFP, and DO11.10 mice carrying the MHC class II-restricted rearranged transgene specific for $OVA_{323-339}$ were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in-house at the National University of Singapore animal facility. Mice were age and sex matched for each experiment. All experiments on mice were performed in accordance with institutional guidelines. Influenza virus A/PR/8/34 (H1N1) was purchased from the ATCC and propagated in the allantois of 10-day-old embryonated chicken eggs and stored at −80°C. Intranasal challenge of mice with influenza virus was performed by injecting mice with ketamine (Sigma) (100 mg/kg) and medotomidine (Orion Pharma) (15 mg/kg) intraperitoneally (i.p.) and administering 10 PFU of influenza virus in a 20-μl volume intranasally (i.n.), after which mice were administered atipamezole (Pfizer) (5 mg/kg) as the anesthesia reversal agent.

T cell collection. Spleen, mediastinal lymph node (mLN), and mesenteric and axillary nondraining lymph node (NDLN) samples were collected from euthanized animals and pushed through a 61-μm-pore-size cell strainer (BD Falcon) to obtain a single-cell suspension. Bronchoalveolar lavage (BAL) samples were collected by four 0.5-ml instillations of phosphate-buffered saline (PBS) through the trachea of cannulated mice. Lung samples were digested for 45 min in Liberase (Roche) at 37°C prior to being passed through a cell strainer. Red blood cells were lysed using ammonium chloride. Cell samples were washed with MACS buffer (0.05 M PBS [pH 7.4], 2% fetal calf serum [FCS], 5 mM EDTA), filtered through a 61-μm-pore-size filter mesh, and stained for surface markers CD3 (clone 145-2C11; Biolegend) and CD4 (clone RM4-5; BD Pharmingen) for 30 min. Cells were washed twice, fixed using fixation/permeabilization buffer (BD Bioscience) for 1 h, washed twice in permeabilization buffer, and stained for Foxp3 (clone FKJ-16S; eBioscience) for 30 min. Cells were washed twice and examined on a CyAn flow cytometer (Beckman Coulter). Treg activation was determined on the basis of CD103 expression (clone 2E7; Ebioscience). Antigen-specific CD8$^+$ T cell numbers were determined using NP_{366} tetramer (Proimmune).

Treg suppression assay. CD4$^+$ eGFP$^+$ T cells collected from the lung, spleen, mLN, and NDLN of influenza virus-infected mice at day 8 postinoculation were sorted to >98% purity using a Moflo cell sorter (Dako) and labeled with 5 μM violet carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen)–PBS for 15 min at 37°C and washed twice in media containing 10% FCS. A total of 5×10^4 violet CFSE-labeled CD4$^+$ eGFP$^-$ or CD8$^+$ eGFP$^-$ responder cells were cultured per well in a U-bottomed 96-well plate with 0.75 μg/ml of α-CD3 (BD Pharmingen) in the presence of 5×10^4 irradiated T cell-depleted splenocytes and decreasing ratios of responder T cells collected from lung, mLN, NDLN, or spleen. After 3 days, cells were collected, stained, and examined for violet CFSE dilution via flow cytometery.

Ex vivo restimulation of CD4$^+$ eGFP$^+$ T cells. Bone marrow-derived dendritic cells were generated as described previously (26). A total of 5×10^4 violet CFSE-labeled CD4$^+$ eGFP$^+$ T cells sorted from the lung, mLN, NDLN, or spleen of day 8 postinoculation influenza virus-infected mice were cultured in the presence of 5×10^4 irradiated BMDCs in 200 μl of RPMI medium (Invitrogen) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μM 2-mercaptoethanol (2-ME) (all Sigma-Aldrich), and 10% FCS (HyClone) per well in a 96-well plate with or without influenza virus A/PR8/34 at a multiplicity of infection (MOI) of 0.1. CD4$^+$ eGFP$^-$ T cells collected from the lung were used a positive control. After 3.5 days of stimulation at 37°C, cells were collected, stained with CD4-allophycocyanin (CD4-APC) (clone RM4-5; eBioscience), and examined for violet CFSE dilution by flow cytometry. In subsequent experiments, lung CD4$^+$ eGFP$^+$ T cells collected from influenza virus-infected mice at day 11 postinoculation were cultured with BMDCs in the presence of anti-IA/IE MHC class II blocking antibody (eBioscience) (10 μg/ml) in addition to influenza A virus in order to determine MHC class II specificity. Similarly, lung CD4$^+$ eGFP$^+$ T cell/BDMC cultures were incubated with poly(I·C) (Sigma) (10 μg/ml) or CD40 agonist (eBioscience) (1 μg/ml) in place of influenza A virus to examine whether the proliferation was due to nonspecific DC activation. Lung CD4$^+$ eGFP$^+$ T cell/BDMC cultures were also incubated with MHC class II epitope HA (hemagglutinin)$_{126-139}$ (5 μg/ml) and, in a separate experiment, irrelevant peptide NP_{366} (5 μg/ml) (both peptides a kind gift from S. Balasubramanian) to determine whether Tregs proliferate in response to the direct presentation of influenza virus antigens.

BrdU treatment. Influenza virus-inoculated mice were administered 3 mg of bromodeoxyuridine (BrdU) (BD Bioscience) i.p. at day 8 postinoculation 6 h prior to euthanasia. Mice were culled, and cells from lung, mLN, NDLN, and spleen tissue were collected, processed as described above, and stained with anti-BrdU per the manufacturer's instructions (fluorescein isothiocyanate [FITC] BrdU flow kit; BD Biosciences) in addition to Foxp3, CD3, and CD4.

Antigen-specific CD4$^+$ T cell proliferation suppression assay. Lung, mLN, NDLN, and spleen samples were collected from C.Cg-*Foxp3$^{tm2(eGFP)Tch}$*/J mice at day 8 postinoculation or lymph node samples were collected from naïve DO11.10 mice, and samples were digested, strained, stained with CD4 antibody, and sorted on the basis of CD4 and eGFP expression (CD4 and CD25 for DO11.10 cells) as described above. A total of 5×10^4 violet CFSE-labeled CD4$^+$ eGFP$^-$ responder cells from the lung were cultured per well in a U-bottomed 96-well plate with influenza A virus at a MOI of 0.05 in the presence of 5×10^4 BMDCs and decreasing ratios of CD4$^+$ eGFP$^+$ T cells collected from lung, mLN, NDLN, or spleen tissue to examine the ability of Tregs from different tissues to suppress antigen-specific CD4$^+$ eGFP$^-$ responder cell proliferation. Tregs from naïve mice were used to confirm that naïve Tregs were unable to suppress influenza virus antigen-specific CD4$^+$ T cell proliferation. Violet CFSE-labeled DO11.10 CD4$^+$ CD25$^-$ T cells were cultured with lung Tregs from influenza virus-infected animals or CD4$^+$ GFP$^+$ Tregs from naïve animals in the presence of $OVA_{323-339}$ peptide (Mimotopes) (1 μg/ml) to determine whether influenza virus antigen-responsive Tregs are capable of suppressing proliferation to unrelated antigens. After 4 days in culture, the cells were collected and stained for CD4 expression, and the dilution of the violet CFSE intensity of CD4$^+$ eGFP$^-$ responder cells was examined by flow cytometry. CD4$^+$ and CD8$^+$ T cells were isolated from the lungs of influenza virus-infected mice at day 8 postinoculation and cultured in the presence of BMDCs and influenza virus as previously described with 5×10^4 Tregs from the lungs of influenza virus-infected mice, 5×10^4 Tregs from the spleens of naïve animals, or no Tregs as a baseline control. After 4 days, the effect of Tregs on CD8$^+$ T cell proliferation and CD4$^+$/CD8$^+$ T cell effector function was examined via CFSE dilution using flow cytometry and by measuring levels of gamma interferon (IFN-γ) in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA).

Statistical analysis. Statistic analysis was performed using GraphPad Prism (GraphPad software). Comparisons across groups were made using 1-way or 2-way analysis of variance (ANOVA), and dual comparisons were performed using Student's *t* test.

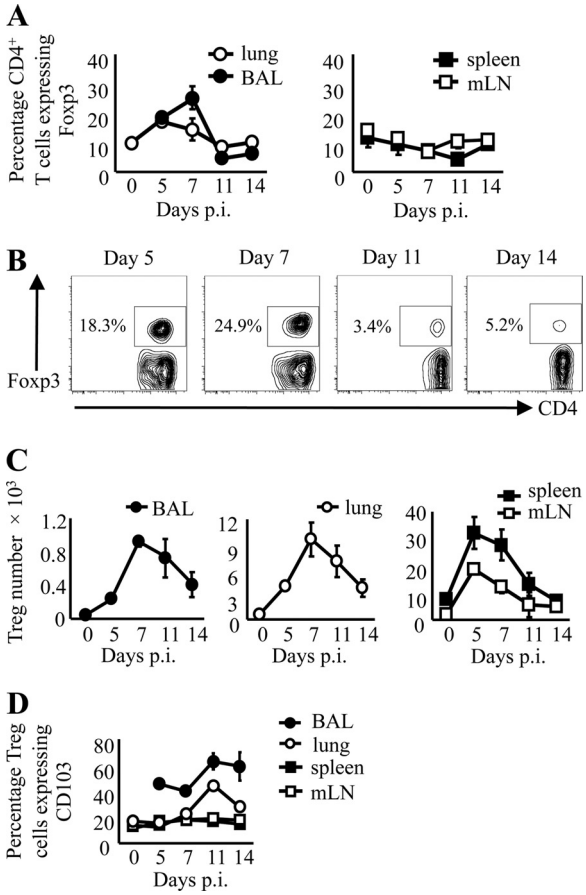

FIG 1 Influenza A virus infection results in a robust Foxp3$^+$ CD4$^+$ T cell response. Mice were infected with a sublethal dose of influenza virus A/PR/8/34, and Foxp3$^+$ CD4$^+$ T cell proportions and numbers in the BAL fluid, lung parenchyma, mediastinal lymph node, and spleen were examined. (A) Percentage of CD3$^+$ CD4$^+$ cells expressing Foxp3$^+$ in BAL fluid, lung, spleen, and mLN across a time course following influenza virus infection. (B) Representative fluorescence-activated cell sorter (FACS) plots of the proportion of CD4$^+$ cells expressing Foxp3 in the BAL compartment, gated on lymphocyte population and CD3$^+$ cells. Numbers represent percentages of CD4$^+$ T cells coexpressing Foxp3. (C) Total Foxp3$^+$ CD3$^+$ CD4$^+$ T cell numbers across a time course following influenza A virus infection. (D) Percentage of Foxp3$^+$ CD3$^+$ CD4$^+$ T cells coexpressing Treg activation marker CD103 in BAL fluid, lung parenchyma, mLN, and spleen across a time course following influenza A virus infection. $n = 3$ to 9 for all; data represent means ± standard errors of the means (SEM).

RESULTS

Influenza A virus infection resulted in a robust Foxp3$^+$ CD4$^+$ T cell response. Wild-type mice were administered a sublethal dose of influenza virus A/PR8 virus i.n. and sacrificed at days 5, 7, 11, and 14 postinoculation. BAL, lung, mLN, and spleen samples were collected and analyzed by flow cytometry. Samples were also taken from naïve animals (day 0).

Foxp3$^+$ cells constitute ~12% of CD4$^+$ T cells in the lung at the resting state, while all T cells were undetectable in the BAL fluid of naïve mice (Fig. 1A). The proportion of CD4$^+$ T cells expressing Foxp3 rapidly increased following the induction of the adaptive response to the virus, peaking at 22% in the lung at day 5 and 25.6% in the BAL fluid at day 7 postinoculation before declining. The fall was especially precipitous in the BAL compartment, with only 3.4% of CD4$^+$ T cells expressing Foxp3 by day 11 (Fig. 1B). The spleen and draining lymph node followed an inverse pattern, with the proportion of Foxp3$^+$ CD4$^+$ T cells decreasing during infection before increasing at later time points (Fig. 1A).

Total Foxp3$^+$ CD4$^+$ T cell numbers peaked at day 7 in both lung and BAL compartments, steadily decreasing thereafter (Fig. 1C). The slow decline in Treg numbers between days 7 and 11 is in contrast with the dramatic decrease in proportions of CD4$^+$ T cells expressing Foxp3 between days 7 and 11 as seen in Fig. 1B and suggests that the rapid decline in proportions was due to elevated CD4$^+$ Foxp3$^-$ T cell migration. Spleen and LN Foxp3$^+$ CD4$^+$ T cell numbers peaked earlier, at day 5, possibly reflecting the earlier priming of the T cell subset prior to migration to the site of inflammation (Fig. 1C). We examined the Foxp3$^+$ CD4$^+$ T cell expression of CD103, an $\alpha E\beta 7$ integrin that mediates T-cell retention in the epithelial compartment and is an excellent marker for identifying *in vivo*-activated Tregs (34, 39). Treg activation status remained static throughout all time points examined for Tregs isolated from the spleen and mLN (Fig. 1D). BAL fluid Foxp3$^+$ CD4$^+$ cells exhibited a higher baseline level of CD103 expression across all time points, together with Lung Foxp3$^+$ CD4$^+$ T cells, reaching a peak at day 11 postinoculation, possibly as a result of increased antigenic stimulation at later time points (31) (Fig. 1D). Collectively, these data indicate that influenza A virus infection results in a robust induction of a regulatory T cell response.

Foxp3$^+$ CD4$^+$ T cells induced during influenza virus infection are suppressive. To ensure that Foxp3$^+$ CD4 T cells induced by influenza virus infection possess a regulatory phenotype, eGFP Foxp3$^+$ CD4$^+$ cells were sorted from the lung, mLN, NDLN, and spleen of influenza A virus-infected C.Cg-*Foxp3*$^{tm2(eGFP)Tch}$/J mice at day 8 postinoculation. The sorted cells were cocultured with violet CFSE-labeled naïve CD4$^+$ or CD8$^+$ T cells, irradiated splenocytes, and anti-CD3. Increasing proportions of CD4$^+$ Foxp3$^+$ T cells dose dependently suppressed responder CD4$^+$ and CD8$^+$ T cell proliferation, as determined by violet CFSE dilution, with CD4$^+$ Foxp3$^+$ cells from the lung, mLN, spleen, and NDLN exhibiting equivalent abilities with respect to suppression of polyclonal CD4$^+$ (Fig. 2A) and CD8$^+$ (Fig. 2B) T cell proliferation, although CD8$^+$ T cells were less susceptible to Treg-mediated suppression of proliferation. These findings demonstrate that CD4$^+$ Foxp3$^+$ cells induced during influenza virus infection are phenotypically Tregs that exhibit highly suppressive activity.

Peak regulatory T cell induction occurs prior to maximal CD4$^+$ and CD8$^+$ T cell responses. We next correlated the regulatory T cell response with the dynamics of the effector CD8$^+$ and CD4$^+$ Foxp3$^-$ T cell response across the course of infection. As noted in Fig. 1A, Foxp3$^+$ CD4$^+$ T cell numbers peaked at day 7 postinoculation in both BAL fluid and lung (Fig. 3A), before the peak in CD8$^+$ T cell numbers (Fig. 3B). To determine the relationship between antigen-specific CD8$^+$ T cell and Foxp3$^+$ CD4$^+$ T cell infiltration to the site of infection, we examined the distribution of CD8$^+$ T cells specific for primary influenza virus A PR/8/34 infection with immunodominant MHC class I epitope NP_{366} (5) by the use of tetramer staining, noting that the migration of CD8$^+$ $NP_{366}{}^+$ T cells to the lung and BAL fluid followed kinetics similar to that of total CD8$^+$ T cell numbers (Fig. 3C). We also examined activated CD8$^+$ T cell numbers, as determined by expression of the α-chain of the interleukin-2 (IL-2) receptor CD25. Negligible numbers of activated CD8$^+$ T cells were observed at the peak of the Foxp3$^+$ CD4$^+$ T cell response, with the maximal activated

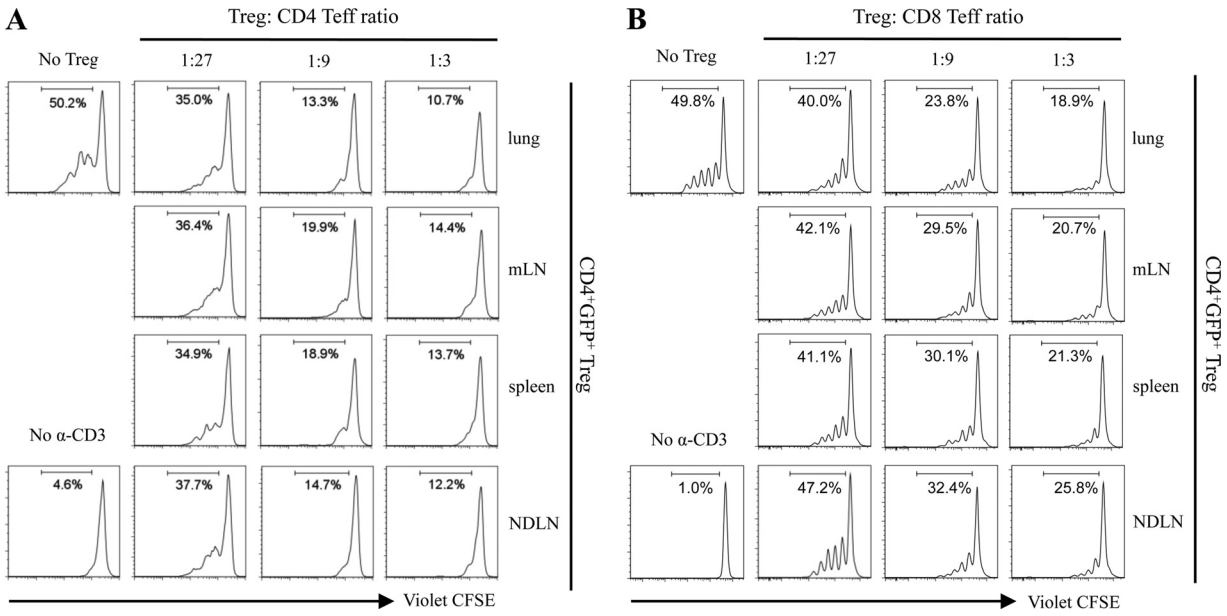

FIG 2 Foxp3$^+$ CD4$^+$ T cells induced during influenza A virus infection are highly suppressive. (A) Foxp3$^+$ CD4$^+$ T cells were sorted from whole lung, mLN, spleen, and NDLN on the basis of eGFP expression from the lungs of influenza virus-infected mice at day 8 postinoculation and cultured in increasing proportions with violet CFSE-labeled Foxp3$^-$ CD4$^+$ responder cells collected from naïve mice and irradiated splenocytes for 3 days, after which responder cell proliferation was examined. (B) Naïve CD8$^+$ T cells were used as responder T cells. The number in each plot represents the percentage of cells that had gone through at least one cell cycle division. Results are representative of 3 independent experiments for panel A and 2 independent experiments for panel B.

CD8$^+$ T cell response occurring at day 11 postinoculation and declining rapidly thereafter (Fig. 3D). Similarly, there was little correlation between the Foxp3$^+$ CD4$^+$ and Foxp3$^-$ CD4$^+$ T cell responses (Fig. 3E). Our findings demonstrate that the regulatory T cell response to influenza A virus precedes that of the effector T cell response, which is consistent with previous studies demonstrating that Tregs are capable of cocoordinating early responses to viral infection (25, 32).

Foxp3$^+$ regulatory T cells induced during influenza virus infection are antigen responsive and widely disseminated and stably express Foxp3. In order to examine the antigen specificity of Foxp3$^+$ Tregs induced during influenza A virus infection, Foxp3$^+$ CD4$^+$ cells were sorted from the lungs, mLN, NDLN, and spleen of infected mice at day 8 postinoculation, labeled with violet CFSE, and cocultured with BMDCs in the presence or absence of influenza A virus. Day 8 was chosen as it was close to the time of peak Treg response at the site of infection, while there were also significant numbers of Tregs in the draining LN. Tregs isolated from the lung proliferated extensively in response to stimulation with influenza A virus-pulsed BMDCs, with more than 57% of these cells demonstrating reduced violet CFSE intensity and that they had gone through up to 5 cell cycle divisions (Fig. 4A and Table 1). Surprisingly, Tregs isolated from the mLN exhibited significantly lesser reduction of violet CFSE intensity, possibly because the majority of antigen-responsive Tregs generated had already migrated toward the site of inflammation (Fig. 4A). There was a significant degree of proliferation of Tregs isolated from the spleen, although there was only minor proliferation in cells collected from the NDLN (Fig. 4A).

Tregs that exhibited CFSE dilution in response to stimulation *ex vivo* with influenza virus-infected BMDCs were also evaluated for Foxp3 expression following 4 days in culture. Proliferating cells from all tissues examined retained high levels of Foxp3 expression in the presence of influenza virus-infected BMDCs (Fig. 4B). These data demonstrate that proliferation in our cultures was predominately by Foxp3$^+$ T cells and that Foxp3 expression in proliferating Tregs was stable and represented a stable Treg lineage.

To investigate the high level of basal proliferation in our *ex vivo* cultures and to confirm that the Tregs had enhanced proliferation *in vivo* at the site of infection, we treated mice with BrdU 6 h prior to sacrifice on day 8 postinoculation and examined BrdU incorporation into Tregs from various compartments by flow cytometry. Of the Tregs in the lung, 8.9% exhibited BrdU incorporation compared to 3.2% in the mLN, 0.3% in the NDLN, and 1.5% in the spleen (Fig. 4C). As with our observations of a proportionally greater Treg presence at the early stages of influenza virus infection, Foxp3$^+$ CD4$^+$ T cells exhibited a higher degree of BrdU incorporation than their Foxp3$^-$ counterparts (Fig. 4C). These data suggest that the high degree of proliferation in the absence of influenza A virus in our *ex vivo* lung Foxp3$^+$ CD4$^+$ cultures was likely a consequence of these cells having already entered the cell cycle *in vivo* and thus rapidly replicating when removed from the lung and placed into culture.

While our data in Fig. 4A demonstrate that regulatory T cells proliferated in the presence of influenza virus and BMDCs, this in itself does not prove that those Tregs were responding to influenza virus antigens. As influenza A virus is a potent activator of dendritic cells (27), it is possible that influenza virus-induced activation of DCs resulted in nonspecific proliferation of Tregs in our cultures. To exclude this possibility and ensure that our Tregs were responsive to influenza virus antigens, we cultured Tregs collected from the lungs of influenza virus-infected mice at day 11 postinoculation, a time point past the peak proliferation of Tregs and therefore representing a period of lower basal proliferation

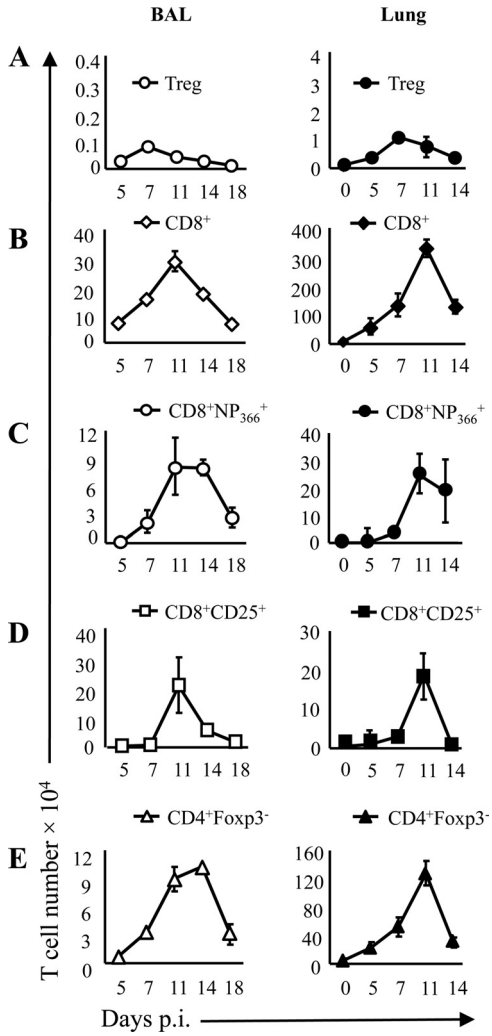

FIG 3 The peak Foxp3$^+$ CD4$^+$ T cell response occurs prior to maximal adaptive immune response within the lung. Foxp3$^+$ CD4$^+$ T cell numbers in BAL fluid and lung parenchyma (A) correlated with total CD8$^+$ T cell (B), $NP_{366}{}^+$ CD8$^+$ T cell (C), CD25$^+$ CD8$^+$ T cell (D), and Foxp3$^-$ CD4$^+$ T cell (E) numbers across a time course following influenza A virus infection. $n = 3$ to 6 for all; data represent means ± SEM.

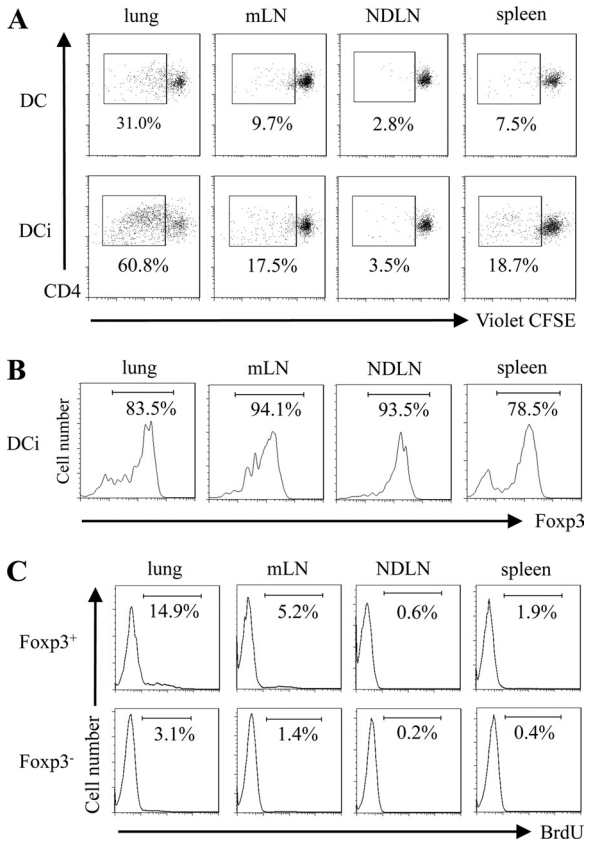

FIG 4 Influenza A virus infection-induced Foxp3$^+$ CD4$^+$ T cells are antigen responsive and widely disseminated and stably express Foxp3. (A) Foxp3$^+$ CD4$^+$ T cells were collected from whole lung, mLN, spleen, and NDLN of influenza A virus-infected mice at day 8 postinoculation, stained with violet CFSE, and cultured with BMDCs in the presence (DCi) or absence (DC) of influenza A virus for 4 days, after which proliferation was examined by FACS analysis. (B) Stability of Foxp3 expression in proliferating Treg collected from whole lung, mLN, NDLN, and spleen of influenza virus-infected mice following *ex vivo* stimulation with influenza virus-infected BMDCs. (C) Influenza A virus-infected mice at day 8 postinoculation were administered 3 mg of BrdU 6 h prior to euthanasia, after which the proportion of Foxp3$^+$ CD4$^+$ and Foxp3$^-$ CD4$^+$ T cells incorporating BrdU from whole lung, mLN, spleen, and NDLN was determined. Data are representative of the results of 2 to 4 independent studies.

and resulting in a cleaner assay. While the Tregs did not proliferate as strongly as at day 8 in our system, basal proliferation was considerably lower and we observed that the Tregs proliferated to a degree comparable to that seen with the CD4$^+$ GFP$^-$ T cells that were included as a positive control (Fig. 5). The addition of $HA_{126\text{-}138}$, one of approximately 30 known influenza virus A PR8/34 MHC class II epitopes (11), to our cultures in place of influenza virus resulted in modest but significant proliferation, demonstrating that at least a proportion of Tregs are specific for a known influenza virus epitope.

In order to determine the MHC class II specificity of the proliferation of Tregs, we examined the ability of MHC class II blocking antibody to attenuate influenza A virus-induced Treg proliferation. Tregs isolated from the lungs of influenza virus-infected mice at day 8 postinoculation and incubated in the presence of BMDCs and MHC class II blocking antibody exhibited attenuated proliferation in the absence of virus, which is consistent with previous findings of Suffia and coworkers (35) determined using MHC class II$^{-/-}$ BMDCs, suggesting that the small degree of baseline proliferation in our Treg- and BMDC-only cultures may have been due to self-antigen presentation (Fig. 5). The addition of MHC class II blocking antibody greatly attenuated Treg proliferation in response to influenza A virus, demonstrating virus-induced Treg proliferation in our system to be MHC class II specific (Fig. 5). Lung Tregs incubated with agonist α-CD40 antibody failed to proliferate above baseline levels, demonstrating that BMDC activation alone is not sufficient to induce Treg proliferation. The addition of poly(I·C) to our Treg/BMDC cultures similarly failed to induce proliferation, demonstrating that the influenza virus-induced Treg proliferation was not the result of virus-mediated TLR3 activation (Fig. 4C). These findings demonstrate that our Tregs responded to the presentation of an influenza virus MHC class II epitope, the virus-induced proliferation we observed was MHC class II dependent, and influenza virus-induced Tregs do not proliferate in response to nonspecific activators of DCs.

TABLE 1 Antigen-specific stimulation of CD4$^+$ Foxp3$^+$ T cells by influenza virus-infected BMDCs[a]

No. of cell divisions	% of Foxp3$^+$ CD4$^+$ T cells isolated from:							
	Lung		mLN		NDLN		Spleen	
	DC	DCi	DC	DCi	DC	DCi	DC	DCi
0	69.0	39.2	90.3	82.5	97.2	96.5	92.5	81.3
1	15.7	14.7	6.2	8.2	2.8	2.0	4.0	8.2
2	9.2	17.7	2.0	2.5	0.6	0.7	1.2	2.1
3	2.1	13.1	0.9	2.2	0	0.6	0.6	3.1
4	2.9	8.9	0.3	2.2	0	0.1	0.8	3.0
5	1.1	6.4	0.3	2.4	0	0.1	0.9	2.3

[a] GFP$^+$ CD4$^+$ T cells were sorted from whole lung, mLN, NDLN, and spleen tissue of influenza virus-infected mice at day 8 postinoculation. A total of 5×10^4 violet CFSE-labeled T cells and 5×10^4 BMDCs were cultured for 3.5 days in the presence (DCi) or absence (DC) of influenza virus A/PR8/34 at an MOI of 0.1. Cells were analyzed using flow cytometry, and numbers of cell divisions were determined on gated CD4$^+$ violet CFSE$^+$ cells by the use of Summit software. Data are representative of the results of 2 independent experiments.

Collectively, these data demonstrate that Foxp3$^+$ CD4$^+$ T cells induced during influenza A virus infection are antigen responsive and widely disseminated throughout the site of infection, the draining lymph node, and the spleen and have enhanced *in vivo* proliferation at early time points in infection compared with their CD4$^+$ Foxp3$^-$ counterparts.

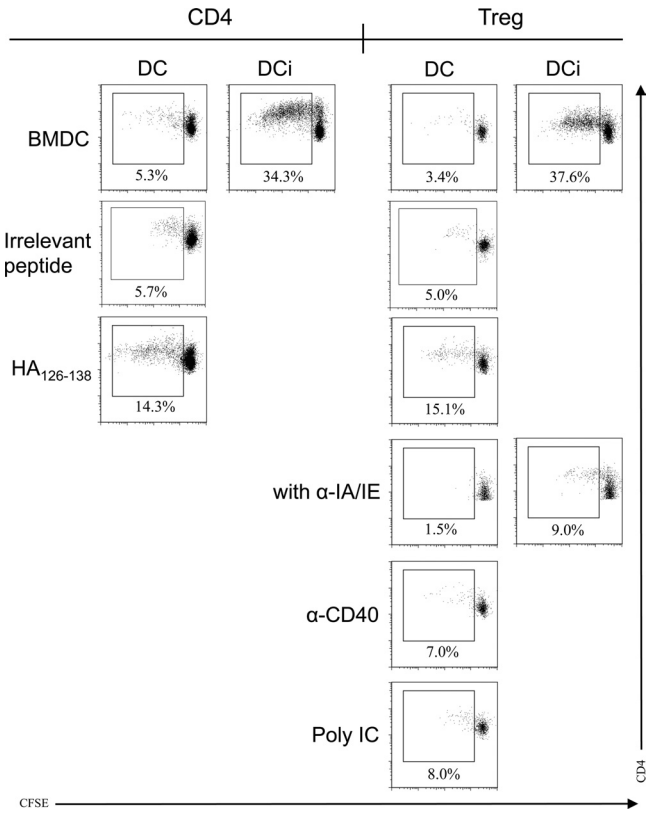

FIG 5 Influenza virus-induced lung Tregs proliferate in response to an influenza virus MHC class II epitope. Foxp3$^+$ CD4$^+$ T cells and positive-control Foxp3$^-$ CD4$^+$ T cells were collected from whole lung of influenza A virus-infected mice at day 11 postinoculation, stained with violet CFSE, and cultured with BMDCs for 4 days with (DCi) or without (DC) influenza A virus, negative-control influenza virus MHC class I influenza A epitope NP_{366}, or MHC class II epitope $HA_{126-138}$ in the presence or absence of IA/IE MHC class II blocking antibody, agonistic α-CD40 antibody, or poly(I·C). Proliferation was examined by FACS analysis after 4 days in culture. Data are representative of the results of 3 to 4 separate experiments.

Antigen-responsive Tregs suppress antigen-specific CD4$^+$ T cell proliferation. After establishing that Tregs induced by influenza A virus are responsive to antigens to various degrees, we examined whether these Tregs were capable of constraining effector responses by examining their ability to suppress antigen-specific CD4$^+$ T cell proliferation. Tregs isolated from the lung potently suppressed antigen-specific CD4$^+$ Foxp3$^-$ proliferation up to approximately 60% (Fig. 6A). Tregs from spleen and mLN tissue suppressed CD4$^+$ Foxp3$^-$ proliferation by 40%, while Tregs isolated from NDLN were able to suppress proliferation by only 20% (Fig. 6A), demonstrating that the ability of influenza virus-induced Tregs to suppress proliferation correlated with their ability to proliferate in response to influenza virus antigens. To ensure that our Tregs suppressed antigen-specific CD4$^+$ T cell proliferation in an antigen-dependent manner and that suppression was not merely a result of higher activation status, we cultured Tregs collected from the lungs of influenza virus-infected animals or the LN of naïve animals as a control with CD4$^+$ T cells specific for the irrelevant antigen $OVA_{323-339}$ in the presence of peptide-pulsed BMDCs. Tregs from the lung of influenza virus-infected animals or from the LN of naïve animals were unable to suppress $OVA_{323-339}$-specific CD4$^+$ T cell proliferation (Fig. 6B). After determining that influenza virus-induced Tregs can suppress antigen-specific CD4$^+$ T cell proliferation in an antigen-dependent manner, we examined the ability of Tregs to constrain effector T cell cytokine production. Tregs collected from the lungs of influenza A virus-infected mice, but not Tregs from the spleens of naïve donors, modestly suppressed antigen-specific CD4$^+$ T cell IFN-γ production *ex vivo* (Fig. 6C). Similarly, influenza virus-induced lung Tregs but not naïve Tregs suppressed influenza virus antigen-specific CD8$^+$ T cell proliferation and IFN-γ production (Fig. 6D and E). Collectively, these data show that Tregs from influenza virus-infected mice suppress influenza-specific effector CD4$^+$ and CD8$^+$ T cell proliferation and IFN-γ production, demonstrating influenza A virus-induced Tregs are able to constrain effector responses.

DISCUSSION

There is an increasing body of evidence to suggest that Foxp3$^+$ CD4$^+$ Tregs act not only to maintain immune homeostasis through self-antigen-specific pathways but also to shape adaptive

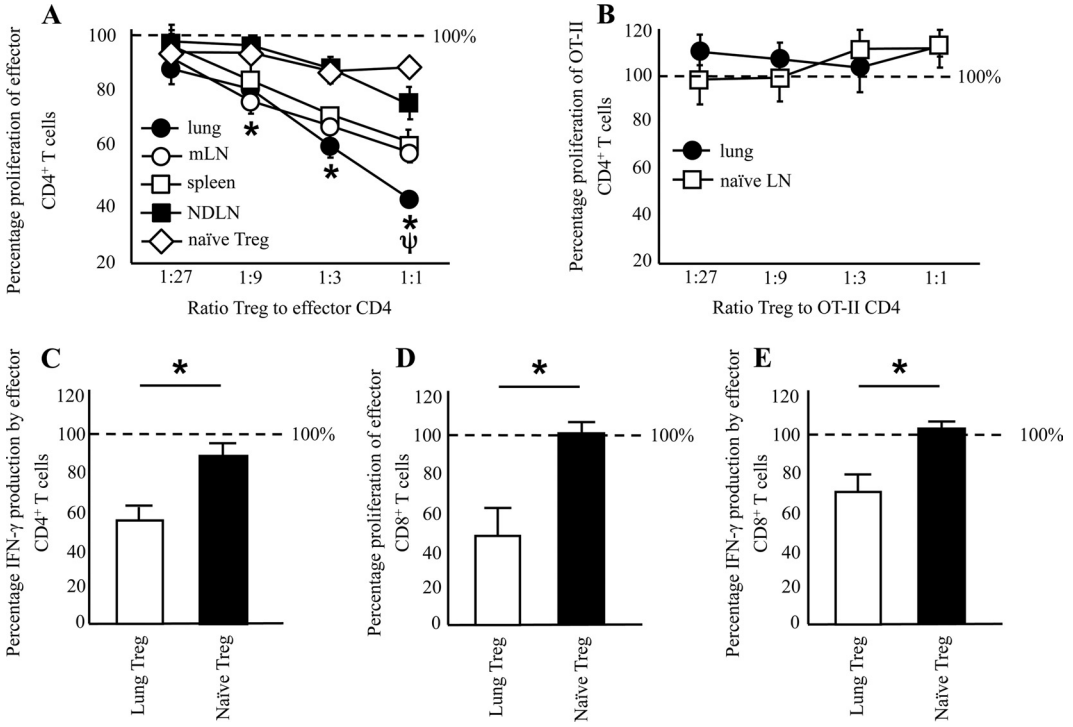

FIG 6 Foxp3$^+$ CD4$^+$ T cells induced during influenza A virus infection suppress antigen-specific CD4$^+$ and CD8$^+$ T cell proliferation. Foxp3$^+$ CD4$^+$ T cells were sorted from whole lung, mLN, and NDLN of influenza virus-infected mice at day 8 postinoculation and cultured in increasing proportions with violet CFSE-labeled Foxp3$^-$ CD4$^+$ responder cells collected from the lungs of influenza virus-infected animals in the presence of BMDCs and influenza A virus. The number in each plot represents the percentage of cells that had gone through at least one cell cycle division. (A) Representative FACS dot plots. Plots were gated on a live T cell FS/SS population. *, $P < 0.05$ for each lung/mLN/spleen data point compared to the corresponding NDLN data point; ψ, $P < 0.05$ for each lung data point compared to the corresponding mLN/spleen data point (2-way ANOVA). (B) Cumulative data are representative of the results determined with 5 samples over 2 independent experiments with multiple mice per sample. Data represent means ± SEM. (C) Foxp3$^+$ CD4$^+$ T cells collected from the lungs of influenza virus-infected animals or naïve Foxp3$^+$ CD4$^+$ T cells were cultured in increasing proportions with violet CFSE-labeled CD4$^+$ CD25$^-$ responder cells collected from the lymph nodes of DO11.10 mice in the presence of $OVA_{323-339}$ peptide-pulsed BMDCs. Cumulative data are representative of the results determined with 5 samples over 2 independent experiments with multiple mice per sample. (D) Virus-induced CD4$^+$ T cells from influenza virus-infected mice at day 8 postinoculation were cultured with Tregs from the lung of influenza virus-infected mice or the spleens of naïve mice for 4 days and IFN-γ levels determined by ELISA. (E) Virus-induced CD8$^+$ T cells from influenza virus-infected mice at day 8 postinoculation were cultured with Tregs from the lung of influenza virus-infected mice or the spleens of naïve mice for 4 days, and the degree of proliferation compared to Treg-free cultures was determined by CFSE dilution. (D) IFN-γ production by virus-induced CD8$^+$ T cells from influenza virus-infected mice at day 8 postinoculation cultured with influenza virus-induced lung Tregs or naïve compared to Treg-free cultures. (D and E) Data represent means ± SEM ($n = 3$ to 4). * denotes $P < 0.01$ (Student's t test).

immune responses to foreign antigens. Chronic microbial infection across an array of animal models results in robust antigen-specific regulatory T cell responses (1, 4, 38), although the role of Tregs in acute infections is poorly understood. Previous investigators have noted an inability of acute microbial infection to mediate induction of Foxp3 by conventional CD4$^+$ T cells, possibly as a result of the highly inflammatory environment induced by an acute infection not favoring the emergence of Foxp3$^+$ Tregs (15). Acute herpes simplex virus 2 (HSV-2) infection, however, results in the accumulation of Tregs at the site of infection and the draining lymph node, demonstrating that viral infection is capable to inducing a local Treg response (25). The findings of the present study show that acute pulmonary infection with a prototypical respiratory virus results in a robust systemic Treg response, with the activation status of Tregs at the site of infection increasing across the course of infection, peaking at day 11 postinoculation. In contrast to the findings of Lund and coworkers, however, we find that influenza virus infection results in the preferential accumulation of Foxp3$^+$ CD4$^+$ T cells at the site of inflammation at the early stages of the T cell response prior to the maximal adaptive immune response, consistent with previous findings in studies employing murine models of RSV infection (16, 22, 32), while the proportion of Tregs rapidly declines below the normal steady-state level at later time points. This dramatic decrease in the Treg:Teff ratio is likely due to an infiltration of Foxp3$^-$ CD4$^+$ T cells as opposed to extensive Foxp3$^+$ CD4$^+$ T cell deletion such as has been observed in other systems (30). We sought to confirm the phenotype of these Foxp3$^+$ CD4$^+$ T cells identified during infection to ensure that they possessed regulatory capacity. Our data indicate that influenza virus-induced Foxp3$^+$ CD4$^+$ T cells were highly suppressive in an *ex vivo* proliferation assay, demonstrating that these cells are phenotypically Tregs and thus may represent a constraining factor with respect to effector responses.

It is unclear whether antigen specificity of Tregs is required to suppress immunity during infection. As Tregs have a high affinity for self-antigens, recognition of self-antigens may be sufficient to drive their expansion and activation in the inflammatory milieu (3), particularly during acute infection with a high degree of pathology as found in influenza virus infection, which may result in potentiated self-antigen presentation. To address this possibility,

we isolated Tregs from influenza virus-infected animals and stimulated them with influenza virus-pulsed BMDCs to determine whether they would proliferate in response to the presentation of live influenza virions. Tregs isolated from the lungs, mLN, and spleen proliferated in response to influenza A virus, suggesting antigen specificity in the Treg population at the site of infection and, to a lesser extent, in the primary and secondary lymphoid organs. However, a significant number of non-influenza virus antigen-responsive Tregs also appear to be recruited to the lung, as is the case with effector $CD4^+$ T cells in influenza virus infection (8). This is, to our knowledge, the first demonstration of a broad Treg response to foreign antigens in an acute viral infection. The degree to which Tregs respond to foreign antigens is still poorly understood, but as Tregs possess a TCR repertoire at least as diverse as that of conventional $CD4^+$ T cells, it appears that Tregs are theoretically capable of recognizing most if not all microbes (18, 28). MHC class II tetramers containing epitopes recognized by effector $CD4^+$ T cell populations commonly do not bind to $Foxp3^+$ Tregs, possibly as a result of Tregs recognizing epitopes that do not overlap or that only partially overlap with those of effector $CD4^+$ T cells (2, 7, 33), and as such may at least partially account for the negative results noted by Ertelt and coworkers (14) in a study of *Listeria monocytogenes* infection. Such disparities may also account for the low number of MHC class II tetramer-specific Tregs identified in RSV infection by Liu and coworkers (23). There have been approximately 30 MHC class II epitopes identified in studies of primary influenza virus infection (11), and, given that immunodominant epitopes for $CD4^+$ $Foxp3^-$ cells may differ from those of Tregs, there are practical difficulties with respect to quantifying the magnitude of the antigen-specific Treg response by the use of peptides or MHC class II tetramers, where available. The systemic nature of the Treg response examined in the present study contrasts with the infected site-restricted response previously observed in a study of *L. major* infection (35), although it is not known whether the distributions of responding Tregs differ due to different inflammatory response induced by the pathogens or due to various levels of TCR affinity for antigen. Initial studies of a pathogen-induced antigen-specific Treg lineage suggested antigen-specific Treg responses to be the result of expansion of a preexisting pool of Tregs as opposed to the conversion of antigen-specific effector T cells to a Foxp3 phenotype in the periphery (33). A more detailed examination of Treg lineage during influenza A virus infection awaits the wider development of MHC class II tetramers.

Our findings also indicate that Tregs proliferate more vigorously than their $Foxp3^-$ counterparts at the early stages of the adaptive immune response, consistent with our observations of increased proportions of $Foxp3^+$ $CD4^+$ T cells as a percentage of the total $CD4^+$ T cell population at the site of infection at early time points and the high degree of BrdU incorporation by $Foxp3^+$ T cells in the lung. There is some evidence that removal of $CD25^+$ Tregs delayed the migration of $CD8^+$ T cells to the lung in a murine model of RSV infection (32), and it is thus possible that the early appearance of an antigen-specific regulatory T cell subset acts to coordinate early immune responses in a way similar to that described for HSV-2 infection (25). Our data do confirm that influenza A virus-induced Tregs from the lung are capable of suppressing antigen-specific $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production, in contrast to previous reports suggesting that Treg-mediated suppression induced during influenza virus infection is attenuated via an IL-6-dependent mechanism (24). Our finding that Tregs from the lung and lymphoid organs of influenza virus-infected mice equally suppress proliferation of polyclonal $CD4^+$ and $CD8^+$ T cells but that suppression of antigen-specific $CD4^+$ T cell proliferation correlates closely with the antigen specificity of the Tregs isolated from the tissues of influenza virus-infected mice raises the possibility that foreign antigen recognition by Tregs during acute infection represents a major mechanism of Treg-mediated immune suppression. The influenza virus-induced Tregs in our system were less efficacious in suppressing effector T cell cytokine production than those observed in the *L. major* model of Belkaid et al., which we attribute to the high degree of nonspecific T cell recruitment to the site of inflammation during influenza virus infection (8) resulting in a lower proportion of antigen-responsive Tregs compared to the highly antigen-specific population identified by Belkaid et al. during chronic infection.

On the broader question of why an acute response requires regulatory T cell suppression, our data and that of groups recently examining $CD25^+$ Tregs and RSV indicate that the rapid emergence of $Foxp3^+$ Tregs acts to attenuate adaptive immunity (16, 22, 32). Depletion of Tregs in RSV infection also results in increased virus-induced immunopathology and potentiated innate immunity (16, 22, 32). Given that the majority of the adaptive immune response to influenza virus infection takes place after the virus has been cleared during a primary infection, our data suggest that antigen-specific Tregs in cases of influenza virus infection may be particularly influential in limiting $CD8^+$ T cell-mediated immunopathology. Recent studies by Sun et al. have shown that $CD8^+$ T cell-derived IL-10 is a major contributor to protection against immunopathology and that $CD4^+$ T cell help is required for $CD8^+$ T cell IL-10 production (36, 37). These studies highlighted the complex nature of regulatory networks, and we hypothesize that regulatory T cells constitute one aspect of a broad regulatory response. Studies in our laboratory utilizing PC61 CD25-depleting antibody demonstrated incomplete depletion and rapid regeneration of $Foxp3^+$ Tregs (6), consistent with previous findings in acute murine models of infection (10). In our hands, influenza virus A PR/8/34 is of dramatically higher pathogenicity than RSV in mice; thus, we suggest that, in such a highly inflammatory environment, PC61 antibody is insufficient to deplete Tregs to the extent that clinical effects are observed. Further clarification of the role of Tregs in influenza virus infection awaits the wider availability of Foxp3-DTR conditional-knockout mice.

In summary, influenza A virus infection results in a robust $Foxp3^+$ $CD4^+$ regulatory T cell response, which preferentially accumulates in the lung at early stages of infection before diminishing in the wake of $CD4^+$ and $CD8^+$ T cell responses. Induced $Foxp3^+$ $CD4^+$ regulatory T cells are highly suppressive, proliferate in response to influenza A virus antigen, are widely disseminated, and are capable of suppressing effector $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production.

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