Costimulation Modulation Uncouples Protection from Immunopathology in Memory T Cell Responses to Influenza Virus[1]

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The rapid effector functions and tissue heterogeneity of memory T cells facilitate protective immunity, but they can also promote immunopathology in antiviral immunity, autoimmunity, and transplantation. Modulation of memory T cells is a promising but not yet achieved strategy for inhibiting these deleterious effects. Using an influenza infection model, we demonstrate that memory CD4 T cell-driven secondary responses to influenza challenge result in improved viral clearance yet do not prevent the morbidity associated with viral infection, and they exacerbate cellular recruitment into the lung, compared with primary responses. Inhibiting CD28 costimulation with the approved immunomodulator CTLA4Ig suppressed primary responses in naive mice infected with influenza, but was remarkably curative for memory CD4 T cell-mediated secondary responses to influenza, with reduced immunopathology and enhanced recovery. We demonstrate that CTLA4Ig differentially affects lymphoid and nonlymphoid responses to influenza challenge, inhibiting proliferation and egress of lymphoid naive and memory T cells, while leaving lung-resident memory CD4 T cell responses intact. Our findings reveal the dual nature of memory T cell-mediated secondary responses and suggest costimulation modulation as a novel strategy to optimize antiviral immunity by limiting the memory T cell response to its protective capacities. *The Journal of Immunology,* **2009, 182: 6834–6843.**

The ability of memory T cells to mediate rapid effector function and reside in diverse tissue sites results in recall responses that are kinetically, functionally, and spatially distinct from primary responses initiated by naive T cells. These unique properties of memory T cells enable them to mediate protective immunity, yet they can also predispose them to promote immunopathology in antiviral immunity (1, 2), autoimmunity (3), and transplantation (4). Regulation of memory T cell-mediated responses is therefore a critical consideration for T cell-directed immunotherapies to optimize their protective abilities and inhibit deleterious consequences. However, inhibiting pathways that control or suppress naive T cell responses have been shown to be either ineffective or differentially effective with memory T cells (5, 6), and clinical immunomodulation of memory T cells in disease has not yet been achieved.

The CD28 costimulatory pathway is required for activation of naive T cells and has emerged as a key target for immunotherapy. CTLA4Ig (abatacept) is the first approved costimulation modulator that inhibits the CD28 pathway by binding its ligands CD80 and CD86 with high affinity (7). CD28 costimulation was previously thought to be dispensable for memory T cell activation, based on memory T cell activation in the absence of B7 ligands (8, 9). However, we and others recently showed that inhibiting CD28 costimulation in vivo reduced memory CD4 and CD8 T cell proliferation and effector function (10–12). Moreover, abatacept has shown efficacy in adults with chronic rheumatoid arthritis and psoriasis (13, 14), diseases associated with infiltration of memory T cells into inflamed sites. Taken together, these results suggest that immunotherapies targeting CD28 costimulation may affect memory T cell responses, although the impact of CD28 inhibition on physiological secondary responses and protective immunity by memory T cells is not known.

The prevalence of memory T cells in adult immune responses is well documented in viral infections due to previous exposures and cross-reactivity to heterologous viruses (15, 16). For ubiquitous viruses such as influenza, flu-specific memory T cells have been detected in the peripheral blood and lungs of healthy individuals (17, 18). In particular, influenza-specific memory CD4 T cells generated from exposure to seasonal strains were found to cross-react with avian influenza (H5N1) epitopes (19, 20). These results suggest that memory CD4 T cells could form a "first-line" defense in responses to new or variant influenza strains that evade neutralizing Ab responses; however, the ability of memory CD4 T cells to direct secondary responses to influenza has not been defined. Moreover, the immune response to influenza, particularly against pandemic strains, is associated with disease severity and heightened mortality (21, 22), although the cellular mechanisms and effect of preexisting memory CD4 T cells on this immunopathology are not known. There are currently no effective means for modulating the immune response to reduce morbidity and mortality to influenza while still maintaining its protective features.

We demonstrate here that influenza-specific memory CD4 T cells can direct a secondary response to influenza challenge with enhanced viral clearance compared with primary responses in the

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context of extensive lung immunopathology and morbidity. Strikingly, protection and immunopathology of this memory CD4 T cell-driven secondary response can be uncoupled by inhibiting the CD28 pathway with CTLA4Ig. We show that in primary responses to influenza infection, CTLA4Ig suppresses the CD4 T cell response, resulting in reduced viral clearance and recovery. In contrast, CTLA4Ig treatment of mice with influenza-specific memory CD4 T cells resulted in improved clinical outcome and reduced morbidity to sublethal influenza infection, as well as increased survival to lethal influenza challenge. We demonstrate that CTLA4Ig treatment maintains enhanced and rapid lung viral clearance mediated by memory CD4 T cells, yet reduces lung immunopathology. In vivo, CTLA4Ig inhibits naive and memory CD4 T cell lymphoid responses and T cell recruitment to the lung, while not affecting in situ lung-specific memory T cell responses, accounting for differential effects on primary vs secondary responses. These results suggest a new strategy to optimize antiviral immunity to influenza and other ubiquitous pathogens where memory T cells readily develop and persist, and they further emphasize the importance of the host immune status in determining the outcome of immunotherapies.

Materials and Methods

Mice

BALB/c mice (8–16 wk of age) were obtained from the National Cancer Institute Biological Testing Branch, and congenic BALB/c (Thy1.1) mice were bred as homozygotes. Influenza hemagglutinin (HA)[3]-TCR transgenic mice expressing a transgene-encoded TCR (clonotype 6.5) specific for HA peptide (110–119) and I-E^d (23) were bred as heterozygotes onto BALB/c (Thy1.2) or BALB/c (Thy1.1) hosts. RAG2$^{-/-}$ mice on BALB/c genetic backgrounds were obtained from Taconic and maintained under specific pathogen-free conditions. Mice were maintained in the Animal Facility at the University of Maryland School of Medicine (Baltimore, MD), and animal protocols were approved by the Institutional Animal Care and Use Committee.

Reagents

The following purified Abs were purchased from Bio X Cell: anti-CD8 (TIB 105), anti-CD4 (GK1.5), anti-I-A^d (212.A1), and anti-Thy-1 (TIB 238). The 6.5 anti-clonotype Ab directed against the HA-TCR (23) was purified and conjugated to biotin (Pierce). Allophycocyanin- or PE-conjugated CD62L, PE-conjugated CD90.1 and CD90.2, FITC-conjugated CD90.1 and CD90.2, and PerCP-conjugated anti-CD4 were purchased from BD Pharmingen. PE-conjugated FoxP3 Ab was purchased from eBioscience. Murine CTLA4Ig was obtained from Bristol Myers-Squibb, and murine IgG2a isotype control was obtained from Bio X Cell. Influenza HA peptide (110–120, SFERFEIFPKE) was synthesized by the Biopolymer Laboratory at the University of Maryland School of Medicine.

Influenza virus infection

Influenza virus (A/PR/8/34) was generously provided by Dr. Walter Gerhard (Wistar Institute) and grown in the allantoic fluid of 10-day-old embryonated chicken eggs as described (24). Determination of influenza viral titers in viral stocks, lung homogenates, or bronchoalveolar lavage (BAL) fluid was accomplished by the tissue culture infectious dose 50% assay ($TCID_{50}$) as described (25), with titers expressed as the reciprocal of the dilution of lung extract that corresponds to 50% virus growth in Madin-Darby canine kidney (MDCK) cells, calculated by the Reed-Muench method.

For in vivo infection using sublethal doses of influenza, mice were anesthetized with isoflurane, and 20 μl of PR8 influenza virus containing 500 $TCID_{50}$ was administered intranasally. For lethal influenza infection, mice were infected as above with 5000 $TCID_{50}$ PR8 influenza ($2LD_{50}$), and weight loss and mortality were monitored daily. All infected mice were housed in the biocontainment suite, the University of Maryland at Baltimore animal facility, where tissue harvest from infected mice was also performed. Isolation of BAL fluid was obtained from anesthetized mice by flushing the alveolar space with PBS followed by withdrawal of lavage liquid. BAL fluid samples were centrifuged to pellet cells, and the supernatant was analyzed for viral content by the $TCID_{50}$ assay described above.

Hemagglutination inhibition assay

The concentration of neutralizing anti-influenza Abs was measured in serum from 10-day-infected animals using the HA inhibition assay as described (26). Briefly, serum was heat inactivated for 30 min at 56°C, diluted 1/5 in PBS, and preadsorbed with 1% chicken RBC for 30 min. Serial 2-fold diltutions of serum were subsequently incubated in duplicate wells with 4 agglutinating units of virus for 1 h at room temperature, then 50 μl of a 1% chicken RBC solution was then added to each well and incubated for 45 min at room temperature. The HA inhibition titer was expressed as the reciprocal of the serum dilution where agglutination was inhibited in duplicate wells.

Generation of influenza-specific memory CD4 T cells

Generation of HA-specific memory CD4 T cells in congenic BALB/c (Thy1.1) hosts was accomplished as previously described (27, 28). Briefly, naive CD4 T cells were purified from spleens of HA-TCR mice and primed in vitro by culture with 5.0 μg/ml HA peptide and mitomycin C-treated, T-depleted BALB/c splenocytes as APCs in complete Click's media (Irvine Scientific) for 3 days at 37°C. The resultant activated HA-specific effector cells were transferred into congenic BALB/c (Thy1.1) hosts (5×10^6 cells/mouse) to yield "HA-memory" mice with a stable population of HA-specific memory CD4 T cells (27–29). HA-specific memory CD4 T cells were also generated by transfer of 5×10^6 primed, HA-specific effector cells into RAG2$^{-/-}$ recipient mice and harvested 2–3 mo posttransfer as previously described (12, 27, 30, 31). HA-specific memory CD4 T cells isolated from these RAG2$^{-/-}$ recipients were labeled with 5 μM CFSE (Invitrogen) and adoptively transferred into secondary BALB/c (Thy1.1) hosts, which were subsequently infected with influenza.

Polyclonal memory CD4 T cells specific for influenza were generated by infecting BALB/c mice intranasally with 500 $TCID_{50}$ PR8 influenza. Total splenic CD4 T cells containing influenza virus-specific memory CD4 T cells were harvested 12–16 wk postinfection. The relative frequencies of influenza-specific IFN-γ- and IL-2-secreting memory CD4 T cells in response to stimulation with HA peptide or whole influenza virus particles were determined using ELISPOT as previously described (27, 31), and spots were enumerated using the ImmunoSpot ELISPOT reader (CTL; BD Biosciences).

Flow cytometry and intracellular cytokine staining

Cells were stained with fluorochrome-conjugated Abs as described (12), fixed and acquired using an LSR II flow cytometer (BD Biosciences) with a minimum acquisition of 100,000 events and analyzed using FACSDiva software (BD Biosciences). Intracellular cytokine staining was performed as described previously (27). Briefly, lymphocytes from the spleen and lungs of influenza infected mice treated with CTLA4Ig or IgG2a were isolated 6 days postinfection, cultured in vitro for 4 h in the presence of PMA (25 ng/ml), ionomycin (1 μg/ml), and monensin (1 μl/ml) (GolgiStop; BD Pharmingen), surface stained, fixed in Cytoperm/Cytofix solution (BD Pharmingen), and stained intracellularly with IFN-γ or isotype control IgG1 Ab in Perm/Wash solution (BD Pharmingen). Stained cells were analyzed using an LSR II flow cytometer and FACSDiva software (BD Biosciences).

Histopathology of lung samples

For preparation and isolation of lung tissue for histological examination, mice were euthanized by isofluorane inhalation, trachea were exposed, and lungs were inflated with 4% paraformaldehyde at constant pressure. Lungs were then removed from the chest cavity, fixed in paraformaldehyde, embedded in paraffin wax, sectioned and stained with H&E by the Pathology Core Facility (University of Maryland at Baltimore), and analyzed by light microscopy.

In vivo BrdU labeling

Influenza virus-infected mice treated with CTLA4Ig or IgG2a were administered BrdU (1 mg, i.p.) for 3 consecutive days starting at day 3 postinfection. Spleen and lung lymphocytes were harvested at day 6 postinfection and resuspended in stain buffer. Cells were surface stained, fixed and permeabilized (Cytofix/Cytoperm, Perm/Wash; BD Biosciences), incubated with DNase (Sigma-Aldrich), and stained intracellularly with fluorescently labeled anti-BrdU Abs at 4°C. Cells were subsequently analyzed on the LSR II (BD Biosciences).

[3] Abbreviations used in this paper: HA, hemagglutinin; BAL, bronchoalveolar lavage; $TCID_{50}$, tissue culture infectious dose 50%.

Statistics

Results are expressed as the mean value from individual groups ± SD indicated by error bars. Significance between experimental groups was determined by the two-tailed Student's *t* test, assuming a normal distribution for all groups.

Results

Model for analyzing memory CD4 T cell-mediated secondary responses to influenza virus challenge

To analyze secondary responses to influenza virus infection directed exclusively by memory CD4 T cells, we established complementary models using TCR-transgenic and polyclonal influenza-specific T cells. In the TCR-transgenic model, naive TCR-transgenic CD4 T cells specific for influenza HA were obtained from HA-TCR transgenic mice (23), primed in vitro with HA peptide and APCs, and the resultant HA-specific effector cells were transferred into unmanipulated, congenic BALB/c hosts where they develop into long-lived, resting memory T cells (27, 28). The resultant "HA-memory" mice contain a stable population of HA-specific memory CD4 T cells, which comprise 0.5–5% of total endogenous CD4 T cells (Ref. 27 and data not shown) and exhibit the phenotype, function, and heterogeneous tissue distribution of in vivo-primed polyclonal memory CD4 T cells, as we previously showed (12, 27, 29–31). For generating polyclonal influenza-specific memory CD4 T cells, we infected BALB/c mice intranasally with a sublethal dose of PR8 influenza, isolated CD4 T cells 2–4 mo postinfection, and determined the frequency of influenza-specific memory CD4 T cells by ELISPOT (12). Equal numbers of CD4 T cells from previously primed mice were transferred into BALB/c hosts to generate "polyclonal flu-memory" recipients with a full complement of endogenous T cells. The total numbers of flu-specific memory CD4 T cells in these flu-memory hosts were back-calculated based on the ELISPOT results.

We assessed whether influenza-specific memory CD4 T cells could coordinate a protective immune response to influenza challenge, initially by comparing responses in BALB/c naive and HA-memory hosts infected with a sublethal dose of PR8 influenza (500 $TCID_{50}$) with mock-infected mice as controls. We assessed the progression of disease by monitoring daily weight loss, and analyzed viral clearance by determining lung viral titers at day 6 when naive mice have not yet cleared virus (32–34). HA-memory mice challenged with influenza exhibited similar daily weight loss as did flu-infected naive mice (Fig. 1*A*, *left*), yet they had a highly significant (>2 log) decrease in lung viral titers compared with infected naive mice (Fig. 1*A*, *right*). The rapid viral clearance in HA-memory mice was apparent as early as day 3 postinfection, with nearly complete clearance by day 7, contrasting naive infected mice with significant viral loads at day 7, and complete viral clearance only by day 10 (Fig. 1*B*). We obtained similar results following influenza challenge of polyclonal flu-memory compared with naive mice, which exhibited reduced lung viral titers at day 6 postinfection (Fig. 1*C*), yet comparable weight loss through the course of infection (data not shown). The extent of enhanced viral clearance seen with polyclonal memory CD4 T cells was typically lower than for HA-specific memory CD4 T cells due to their lower frequency in a polyclonal T cell population. Taken together, these results indicate that influenza-specific memory CD4 T cells can direct a classic secondary immune response to influenza challenge with enhanced kinetics of viral clearance; however, they do not appear to protect against the morbidity of viral infection as measured by weight loss.

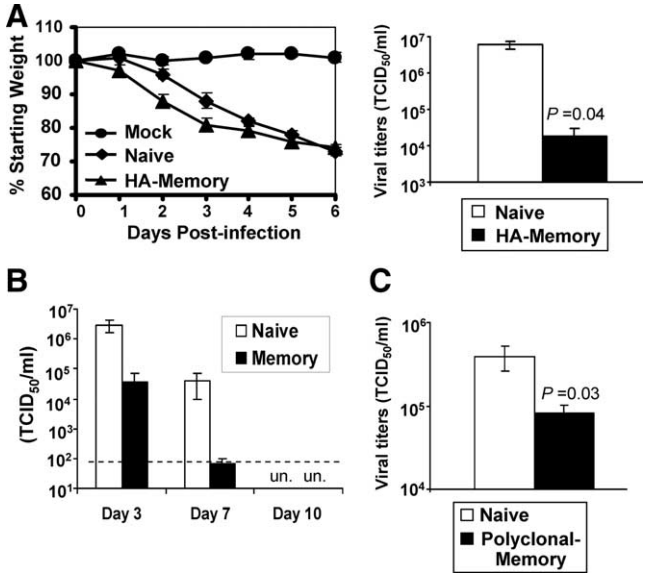

FIGURE 1. *A*, Influenza-specific memory CD4 T cells mediate secondary responses to influenza challenge. Naive or HA-memory mice were infected intranasally with 500 $TCID_{50}$ PR8 influenza virus and monitored 1–6 days postinfection. *Left*, Daily weight loss expressed as percentage of starting weight (100%) in naive and HA-memory mice following influenza challenge. *Right*, Lung viral titers determined by $TCID_{50}$ assay (see *Materials and Methods*) from lung homogenates harvested 6 days postinfection. Value is $p = 0.04$ for difference in titers between naive and memory mice; $n = 4$ for each group; representative of five independent experiments. *B*, Kinetic analysis of influenza viral titers in naive and HA-memory mice. Titers from BAL supernatants isolated at days 3, 7, and 10 postinfection are expressed as $TCID_{50}$/ml (see *Materials and Methods*), with "un." (undetectable) indicating viral titers below the detection limit of the assay ($n = 3$ mice/group). *C*, Viral titers from lung homogenates harvested 6 days postinfection from naive or BALB/c recipients of 50,000 polyclonal influenza-specific memory CD4 T cells. Value is $p = 0.03$ for difference in titers between naive and polyclonal memory mice; titers were compiled from three independent experiments with three to five mice per group.

CTLA4Ig treatment improves the clinical outcome of memory CD4 T cell responses to influenza challenge while maintaining viral clearance

We compared the effects of inhibiting CD28 costimulation using CTLA4Ig, on the physiological outcomes of primary and memory T cell responses to influenza challenge. For costimulation modulation in vivo, we treated naive, HA-memory, or polyclonal-memory mice with murine IgG2a or CTLA4Ig at the 10 mg/kg clinical dose (12) before and following influenza challenge (Fig. 2*A*) and measured weight loss and viral titers as in Fig. 1*A*. In naive mice, both control- and CTLA4Ig-treated animals lost extensive weight following influenza challenge (Fig. 2*B*, *left*), with CTLAIg-treated naive infected mice having higher lung viral loads and mortality at 6 days postinfection compared with infected IgG2a control-treated naive mice (Fig. 2*B* and data not shown). This suppression of antiviral primary responses is consistent with a previous report (35) and the known CD28 requirement for naive T cell activation.

In contrast to the undesirable effects of CTLA4Ig on primary immune responses to influenza, CTLA4Ig treatment of mice with influenza-specific memory CD4 T cells improved the clinical outcome to influenza challenge. Whereas IgG2a-treated HA-memory mice exhibited progressive weight loss from 1 to 6 days postinfection comparable to infected naive mice, CTLA4Ig-treated HA-memory mice lost weight initially and then began to recover weight by day 4, with a steady weight gain until necropsy at day

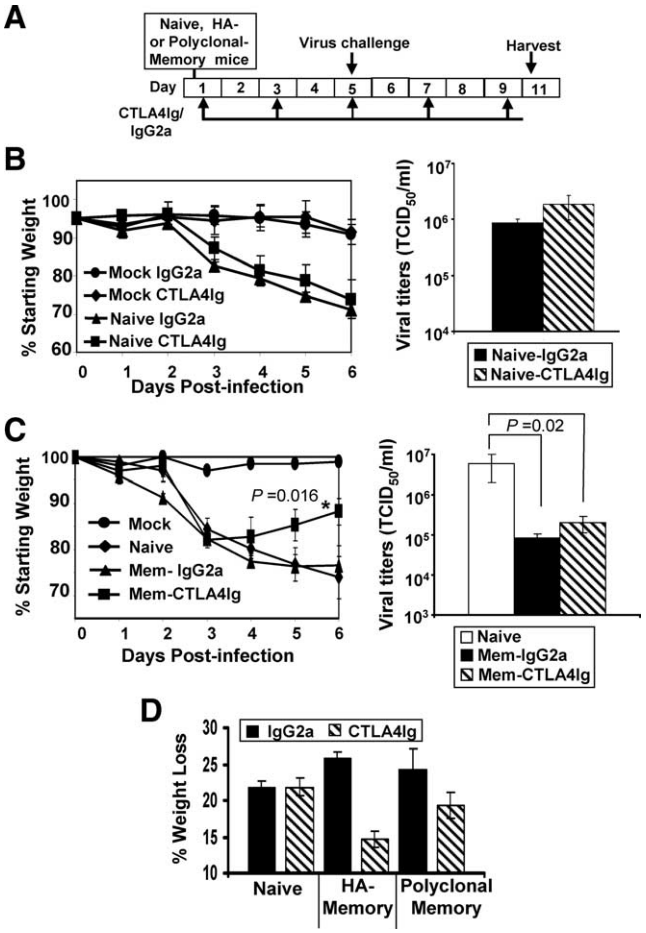

FIGURE 2. CTLA4Ig optimizes secondary responses to influenza, while suppressing primary responses. *A*, Protocol for CTLA4Ig treatment of naive and HA-memory mice. Lower arrows denote time points for administration of CTLA4Ig or IgG2a, and upper arrows indicate time points for infection and mouse harvest. *B*, CTLA4Ig effects on the primary response to influenza in naive BALB/c mice treated and infected as in Fig. 2*A*. *Left*, Daily weight loss. *Right*, Lung viral titers 6 days postinfluenza virus challenge determined as in Fig. 1. Results are from four to five mice per group and are representative of three independent experiments. *C*, CTLA4Ig effects on the memory CD4 T cell-mediated secondary response in HA-memory mice treated and infected as in *A*. *Left*, Daily weight loss following influenza infection of control IgG2a- or CTLA4Ig-treated HA-memory mice compared with naive or mock-infected mice (*, $p = 0.016$ for weights of CTLA4Ig- vs IgG2a-treated mice at day 6; $n = 4$ mice/group). *Right*, Viral titers from lung homogenates harvested 6 days postinfection as in Fig. 1. ($p = 0.02$ between naive and IgG2a- or CTLA4Ig-treated HA-memory mice; $n = 4$ mice/group). Results are representative of six independent experiments. *D*, Cumulative weight loss at day 6 postinfection of naive, HA-memory, or polyclonal-memory mice treated and infected as in *A*. Weight loss data are compiled from three independent experiments for naive mice ($n = 9$), three experiments using polyclonal memory mice ($n = 8$), and four experiments with HA-memory mice ($n = 10$). Each experiment contained three to five mice per experimental group. Value is $p = 0.0001$ when comparing HA-memory mice treated with IgG2a vs CTLA4Ig and $p = 0.01$ comparing recipients of polyclonal memory CD4 T cells treated with IgG2a and CTLA4Ig.

6 (Fig. 2*C*, *left*). Importantly, CTLA4Ig treatment did not appreciably affect the ability of HA-specific memory T cells to clear virus as seen by the comparable low viral titers in the lungs of IgG2a- and CTLA4Ig-treated HA-memory mice 6 days after influenza challenge (Fig. 2*C*, *right*). In polyclonal flu-memory mice, CTLA4Ig treatment also resulted in reduced weight-loss morbidity

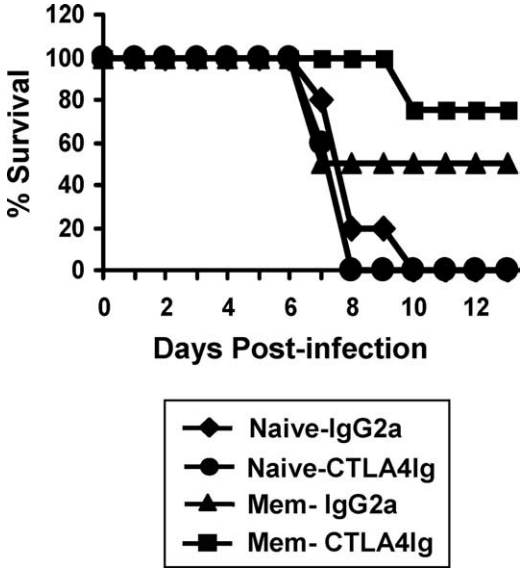

FIGURE 3. Enhanced survival of memory but not naive mice from lethal influenza virus challenge in the presence of CTLA4Ig. Naive BALB/c or HA-memory mice were treated with CTLA4Ig or IgG2a as in Fig. 2 and infected with $2LD_{50}$ of PR8 influenza virus. Survival of differentially treated naive and memory mouse groups up to day 14 postinfection is shown, with surviving mice (only in memory groups) recovering weight loss by days 12–14. Data were compiled from four to five mice per group.

(Fig. 2*D*) and maintenance of lung viral clearance (data not shown). Comparing morbidity data from multiple experiments (Fig. 2*D*) reveals that CTLA4Ig treatment did not affect morbidity of naive mice infected with influenza, while it significantly reduced morbidity of HA- and polyclonal-memory mice, with CTLA4Ig-treated mice losing only 10–15% of their body weight compared with 25–30% weight loss of IgG2a-treated memory mice. HA- and polyclonal-memory mice treated with CTLA4Ig also exhibited fewer clinical signs of influenza-induced morbidity, including ruffled fur and hunched posture, compared with IgG2a-treated mice (data not shown). These results indicate that CTLA4Ig administration appears to optimize memory CD4 T cell-mediated antiviral responses by reducing morbidity while maintaining viral clearance, contrasting its suppressive effect on primary anti-influenza responses.

The reduced morbidity in response to influenza challenge observed in CTLA4Ig-treated HA-memory mice prompted us to ask whether CTLA4Ig treatment would provide protection from a lethal influenza virus challenge. We challenged CTLA4Ig or IgG2a-treated naive or HA-memory mice with a lethal dose ($2LD_{50}$) of influenza virus and monitored morbidity and mortality daily. Mortality from this lethal dose began at days 7–8 postinfection, with all mice within IgG2a- and CTLA4Ig-treated naive groups succumbing to lethal challenge at 8–10 days postinfection (Fig. 3). The presence of memory CD4 T cells in HA-memory mice results in partial protection from lethal influenza infection, with 50% of IgG2a-treated mice succumbing to infection (Fig. 3). CTLA4Ig treatment of HA-memory mice resulted in improved survival from lethal challenge, with surviving mice experiencing less weight loss overall (Fig. 3 and data not shown). These results show that CTLA4Ig treatment can also improve protective immunity to lethal challenge in the presence of influenza-specific memory CD4 T cells.

Because CTLA4Ig inhibits primary T cell and Ab responses (7) and Abs are considered essential for complete viral clearance in naive mice (36), we asked whether the improved clinical outcome

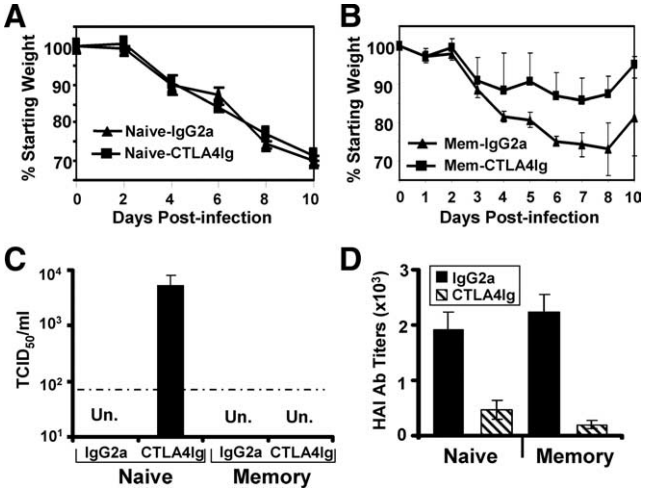

FIGURE 4. CTLA4Ig promotes enhanced recovery during a secondary influenza response while suppressing anti-influenza serum Ab responses. Naive BALB/c mice (*A*) or HA-memory mice (*B*) were treated and infected as in Fig. 2*A* and monitored and weighed until day 10 postinfection, when viral titers and serum anti-influenza Ab titers were determined. Daily weight loss was recorded as percentage of starting weight. *C*, Lung viral titers 10 days postinfluenza virus challenge from naive and HA-memory mice treated with CTLA4Ig or IgG2a were determined as in Fig. 1. The designation "un." (undetectable) indicates viral titers below the detection limit of the assay. *D*, Anti-influenza virus Ab titers in serum determined by HA inhibition assay (see *Materials and Methods*) 10 days postinfection in naive and HA-memory mice. Titers are expressed as the reciprocal dilution equaled to 1 HAI. Value is $p = 0.02$ when comparing IgG2a- and CTLAIg-treated naive mouse groups; $p = 0.006$ when comparing IgG2a- vs CTLA4Ig-treated HA-memory groups. Results are representative of two independent experiments with three to five mice per experimental group.

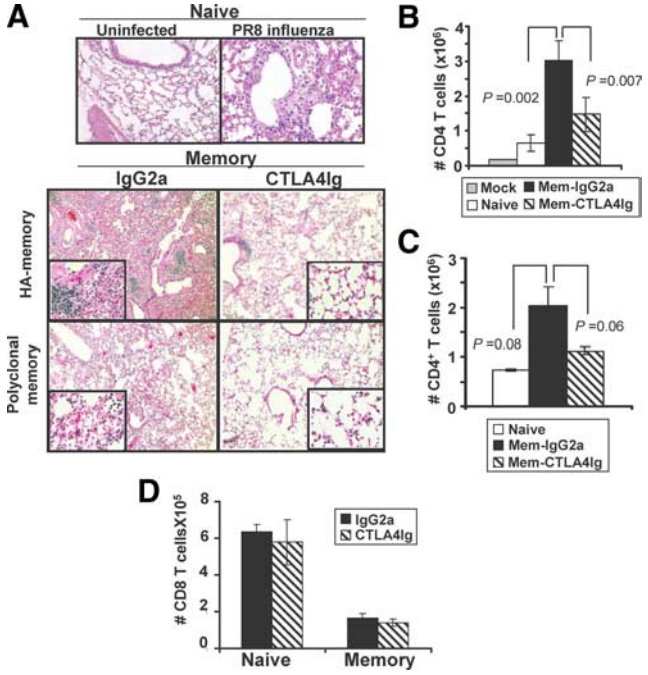

FIGURE 5. Lung immunopathology in the presence of HA- or polyclonal influenza-specific memory CD4 T cells is ameliorated by CTLA4Ig treatment. *A*, H&E-stained lung sections obtained from an uninfected mouse (*upper left*) or a naive mouse 6 days following infection with influenza PR8 (*upper right*) shown as ×20 magnification. *Lower*, H&E-stained sections of lungs derived from HA-memory mice (*top row*) and polyclonal memory mice (*bottom row*) treated with IgG2a or CTLA4Ig 6 days postinfection with influenza, shown as ×10 magnification and ×40 magnification (*insets*). Results are representative of three independent experiments. *B*, Total number of endogenous CD4 T cells in the lungs of uninfected or influenza-infected naive mice or IgG2a- or CTLA4Ig-treated HA-memory mice. Value is $p = 0.002$ between naive and IgG2a-treated HA-memory groups, and $p = 0.007$ between IgG2a- and CTLA4Ig-treated HA-memory groups. *C*, Number of endogenous CD4 T cells in the lungs of naive or IgG2a- and CTLA4Ig-treated polyclonal memory mice (*right*). Value is $p = 0.08$ when comparing untreated naive mice with mice receiving polyclonal memory CD4 T cells treated with IgG2a, and $p = 0.06$ when comparing polyclonal memory CD4 T cell recipients treated with IgG2a or CTLA4Ig. *D*, Total number of $CD8^+$ cells in the lungs of influenza-infected naive mice treated with IgG2a or CTLA4Ig (*left*) or IgG2a- and CTLA4Ig-treated recipients of HA-memory CD4 T cells. Results are representative of three independent experiments with four to five mice per group.

and viral clearance in CTLA4Ig-treated memory mice persisted at later times postinfection. We assessed influenza responses of differentially treated naive and memory mice up to day 10 postinfection, which corresponds to the peak Ab response and complete viral clearance in naive animals. For naive mice, CTLA4Ig- and IgG2a-treated mice exhibited comparable progressive weight loss until day 10 postinfection (Fig. 4*A*), although the efficiency of virus clearance and Ab production differed in these groups. Control-treated naive mice completely cleared virus at day 10 coincident with high levels of flu-specific serum Ab. In contrast, virus persisted in the lungs of CTLA4Ig-treated naive mice (Fig. 4*C*) and Ab production was inhibited (Fig. 4*D*), consistent with the known effect of CTLA4Ig in suppressing immune-mediated viral clearance (35).

In contrast to the inhibitory effect of CTLA4Ig on long-term viral clearance in naive infected mice, CTLA4Ig treatment of memory mice resulted in enhanced recovery. CTLA4Ig-treated HA-memory mice began to gain weight as early as day 4 postinfection, recovering 95–100% of their starting weight by day 10, whereas IgG2a-treated HA-memory mice only began to recover weight at day 10 postinfection (Fig. 4*B*). Viral clearance was complete in both memory groups (Fig. 4*C*), despite disparate levels of influenza-specific serum Ab, which was high in IgG2a-treated and suppressed in CTLA4Ig-treated memory mice (Fig. 4*D*). These results indicate that while the diminished Ab response in CTLA4Ig-treated naive mice correlated with morbidity and reduced viral clearance, CTLA4Ig-treated memory mice experienced an improved clinical outcome and complete protection despite a similarly suppressed Ab response.

CTLA4Ig treatment of memory mice reduces lung immunopathology

The comparable viral clearance, yet disparate clinical outcomes in CTLA4Ig vs IgG2a-treated, flu-infected memory mice, prompted examination of lung pathology in these differently treated groups following influenza challenge. We examined H&E-stained sections from influenza-infected naive, IgG2a- or CTLA4Ig-treated HA-memory and polyclonal flu-memory mice. As compared with uninfected mice, lungs from infected naive mice contained mononuclear infiltrates within the interstitial tissue and near the large airways along with moderate airway damage characterized by hypertrophy in the alveolar epithelium. Additionally, these mice had moderate epithelial hypertrophy with dispersed consolidation surrounding the bronchial airways (Fig. 5*A*). In contrast, lungs from influenza-challenged control mice with either HA-specific or polyclonal flu-specific memory CD4 T cells had extensive diffuse mononuclear infiltrates around the airways and throughout the interstitium, leading to disruption of normal alveolar architecture and

severe consolidation near most of the bronchial airways. In tandem, we observed acute damage to the airway epithelium as evidenced by desquamation throughout the alveoli and sloughing within the bronchial airways (Fig. 5*A*), connoting extensive lung immunopathology. Importantly, this lung immunopathology in flu-infected memory mice was dramatically reduced by CTLA4Ig treatment, as exemplified by reduced mononuclear cell infiltration and alveoli hypertrophy, and an increased number of alveoli with normal architecture in CTLA4Ig-compared with IgG2a-treated polyclonal- and HA-memory mice (Fig. 5*A*).

Consistent with the extensive infiltration in memory mice observed by histopathology, we also found increased numbers of endogenous CD4 T cells in the lungs of influenza-challenged HA-memory (Fig. 5*B*) and polyclonal flu-memory mice (Fig. 5*C*) compared with flu-infected naive mice. This enhanced accumulation of CD4 T cells in the lungs of memory mice was reduced by CTLA4Ig treatment in both HA and polyclonal memory groups (Fig. 5, *C* and *D*). We also investigated whether there were increased numbers of CD8 T cells in the lungs of flu-memory mice and whether CD8 T cell recruitment to the lungs was affected by CTLA4Ig. Interestingly, we found a decreased number of CD8 T cells in the lungs of flu-infected memory compared with naive mice (Fig. 5*D*), possibly due to reduced CD8 T cell priming due to early lung viral clearance in HA-memory mice (Fig. 1*B*). These results indicate that increased CD8 T cell recruitment to the lung does not occur in the presence of flu-specific memory CD4 T cells. Moreover, CTLA4Ig treatment did not significantly decrease or alter the number of CD8 T cells in the lungs of influenza-infected naive or memory hosts (Fig. 5*D*). These results show that CTLA4Ig has more profound inhibitory effects on the endogenous CD4 compared with the CD8 T cell compartment during influenza virus infection.

CTLA4Ig reduces the accumulation and expansion of memory CD4 T cells in spleen and lung following influenza challenge

To determine mechanisms for the improved antiviral response and clinical outcome mediated by memory CD4 T cells in the presence of CTLA4Ig, we used the HA-memory model to analyze the effects of costimulation inhibition on the responding memory CD4 T cell population. In control-treated HA-memory hosts, influenza infection resulted in extensive expansion and accumulation of HA-specific memory T cells in both the spleen and lungs, with HA-specific memory T cells comprising 25–50% of total lung CD4 T cells at 6 days postinfection (Fig. 6*A*, *left*). However, in flu-challenged CTLA4Ig-treated mice, there was a marked reduction in the frequency and absolute numbers of HA-specific memory T cells in the spleen and lungs (Fig. 6*A*). Comparing the absolute numbers of HA-specific memory cells in lung and spleen from IgG2a- and CTLA4Ig-treated infected mice (Fig. 6*A*, *right*) reveals that CTLA4Ig treatment inhibited the accumulation of memory T cells in the lung (5-fold inhibition) to a greater extent than in spleen (2-fold inhibition).

We asked whether the reduced numbers of memory T cells in the spleen of CTLA4Ig-treated mice resulted from reduced proliferation of memory T cells in vivo, by analysis of CFSE-labeled HA-specific memory CD4 T cells. Memory CD4 T cells isolated from RAG2$^{-/-}$ adoptive hosts were CFSE labeled and transferred to mice treated and infected as in Fig. 2*A*. We found extensive in vivo proliferation of HA-specific memory T cells in both IgG2a- and CTLA4Ig-treated groups; however, the proportion and absolute numbers of minimally divided (CFSE^{high}) CD4 T cells was higher in CTLA4Ig-compared with control-treated mice (Fig. 6*B*). These results show that CTLA4Ig reduces the proliferative expansion of splenic memory CD4 T cells in response to influenza infection.

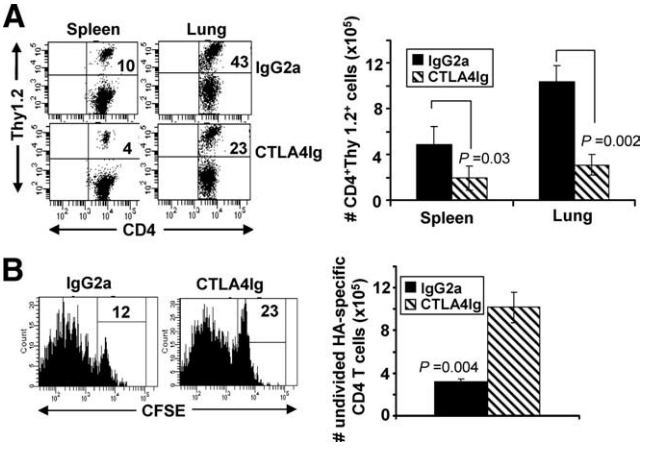

FIGURE 6. CTLA4Ig treatment inhibits proliferation and expansion of influenza-specific memory CD4 T cells to viral challenge. *A*, Reduced frequency and absolute numbers of HA-specific memory CD4 T cells in flu-infected HA-memory mice treated with CTLA4Ig. *Left*, Flow cytometry plots show the frequency of CD4$^+$Thy1.2$^+$ HA-specific memory CD4 T cells in the spleen and lung 6 days following influenza challenge of IgG2a- and CTLA4Ig-treated HA-memory mice, with the percentage of HA-specific memory CD4 T cells from total CD4 T cells indicated in each plot. The absolute number of HA-specific memory CD4 T cells in spleen and lung tissue was calculated from microscopic cell count by trypan blue exclusion of dead cells. Values are $p = 0.03$ and 0.002 when comparing the absolute numbers of Thy1.2$^+$ memory CD4 T cells in the spleen and lung tissues, respectively, from mice treated with IgG2a and CTLA4Ig. Results are representative of six independent experiments with four to five mice per group. *B*, CTLA4Ig treatment reduces in vivo proliferation of HA-specific memory CD4 T cells. CFSE-labeled HA-specific memory CD4 T cells were transferred (1×10^6/mouse) into congenic BALB/c hosts, which were subsequently infected with 500 $TCID_{50}$ PR8 influenza virus. *Left*, CFSE dilution of HA-specific memory CD4 T cells 5 days postinfection, with the marker indicating percentage of undivided memory cells. *Right*, Absolute number of undivided HA-specific memory CD4 T cells expressed as an average of four mice per group. Value is $p = 0.004$ when comparing the absolute numbers of undivided Thy 1.2$^+$ memory CD4 T cells in IgG2a- and CTLA4Ig-treated mice.

The reduced accumulation of flu-specific memory CD4 T cells in the lung could be due to diminished T cell expansion and/or altered homing and recruitment to the lung. To address potential differences in homing capacity, we examined expression of the lymph node homing receptor molecule CD62L on memory CD4 T cells in CTLA4Ig- vs IgG2a-treated, uninfected, or flu-infected memory mice. While CTLA4Ig treatment did not alter CD62L expression by resting HA-specific memory CD4 T cells in uninfected mice (data not shown), profound differences in CD62L expression were observed on splenic HA-specific memory CD4 T cells in IgG2a- compared with CTLA4Ig-treated mice following influenza infection. In IgG2a-treated memory mice, HA-specific memory CD4 T cells in spleen exhibited a predominant CD62L^{low} effector-memory phenotype following infection (Fig. 7*A*), consistent with the CD62L^{low} profile of activated effectors and tissue-homing memory T cells (27, 37). In contrast, spleen-derived HA-specific memory CD4 T cells exhibited a predominant CD62L^{high} or central-memory phenotype in CTLA4Ig-treated HA-memory mice following influenza challenge (Fig. 7*A*). Interestingly, HA-specific memory CD4 T cells in the lung of both IgG2a- and CTLA4Ig-treated infected mice were predominantly CD62L^{low}

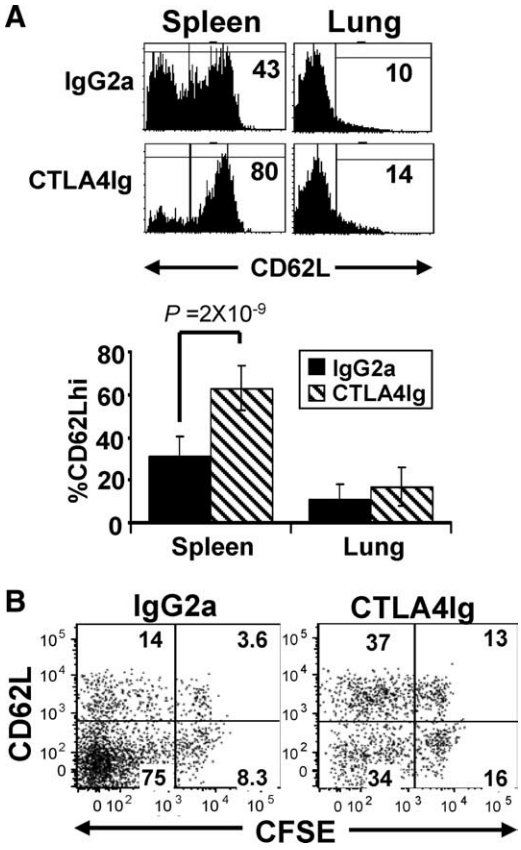

FIGURE 7. CTLA4Ig treatment alters homing receptor expression of activated HA-specific memory CD4 T cells. *A*, Increased CD62L expression on HA-specific memory CD4 T cells in CTLA4Ig- vs IgG2a-treated mice following influenza challenge. *Upper*, CD62L expression by $CD4^+Thy1.2^+$ spleen and lung HA-specific memory CD4 T cells isolated from IgG2a- and CTLA4Ig-treated, flu-challenged HA-memory mice, with percentage $CD62L^{high}$ indicated in each histogram. *Lower*, The frequency of $CD62L^{high}$ memory CD4 T cells in spleen and lung tissues compiled from five independent experiments ($n = 22$). Value is $p = 2 \times 10^{-9}$ for the frequency of CD62L expression HA-specific memory T cells between IgG2a- and CTLA4Ig-treated mice. *B*, Maintenance of $CD62L^{high}$ expression on proliferating memory CD4 T cells in the presence of CTLA4Ig. CFSE-labeled memory CD4 T cells were transferred into congenic hosts and analyzed after infection and treatment as in Fig. 6*B*. Plots show CD62L expression vs CFSE dilution on gated $CD4^+Thy\ 1.2^+$ memory T cells 5 days postinfection of IgG2a- and CTLA4Ig-treated mice.

(Fig. 7*A*), indicating that CTLA4Ig did not affect the CD62L profile of lung memory CD4 T cells and rather had biased effects on CD62L expression by spleen memory CD4 T cells.

The predominant $CD62L^{high}$ phenotype of splenic memory CD4 T cells in CTLA4Ig-treated flu-infected mice could result from impaired memory CD4 T cell activation or from reduced CD62L down-regulation by activated memory T cells. To address these possibilities, we analyzed the CD62L profile of CFSE-labeled HA-specific memory CD4 T cells transferred into differentially treated mice as in Fig. 6*B*. This analysis clearly shows that maximally divided memory CD4 T cells ($CFSE^{low}$) were predominantly $CD62L^{low}$ in control-treated mice and were equally divided between $CD62L^{high}$ and $CD62L^{low}$ phenotypes in CTLA4Ig-treated mice (Fig. 7*B*). These results indicate that CTLA4Ig partially inhibits CD62L down-regulation on memory CD4 T cells responding to influenza virus, suggesting that the capacity of lymphoid memory CD4 T cells to home to nonlymphoid sites, such as the lung, is curtailed.

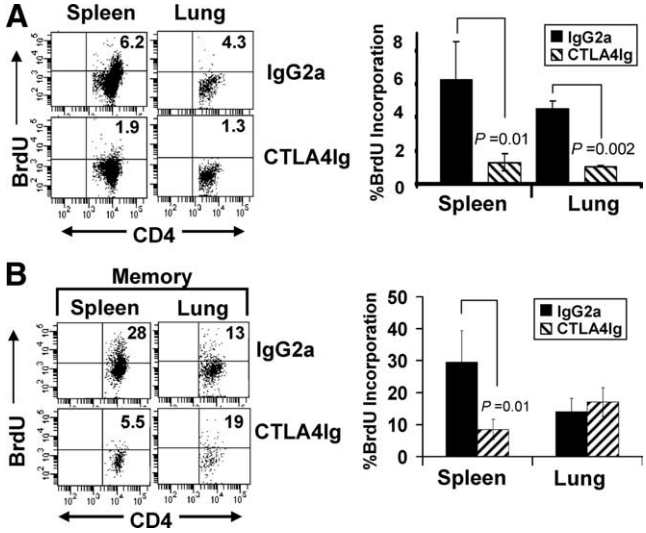

FIGURE 8. CTLA4Ig treatment differentially inhibits lung vs spleen memory CD4 T cell responses to influenza. Naive BALB/c mice (*A*) or recipients of HA-specific memory CD4 T cells (*B*) treated and infected as in Fig. 2*A* were administered BrdU (1 mg/dose) on days 3, 4, and 5 postinfection, and spleen and lung lymphocytes were harvested on day 6 postinfection. *A*, *Left*, BrdU incorporation of lung and spleen $CD4^+(FoxP3^-)$ T cells from flu-infected naive mice treated with CTLA4Ig or IgG2a. The percentage of $CD4^+BrdU^+$ cells is indicated in each plot. *Right*, Graph shows mean BrdU incorporation (±SD) of CD4 T cells harvested from spleen and lung tissues. Values are $p = 0.04$ and $p = 0.002$ when comparing mean BrdU incorporation of spleen and lung CD4 T cells from IgG2a- vs CTLA4Ig-treated mice, respectively. *B*, CTLA4Ig treatment differentially inhibits responses of influenza-specific memory CD4 T cells in spleen and lung tissue. *Left*, BrdU incorporation of Thy 1.2^+ HA-memory CD4 T cells expressed as percentage of total CD4 T cells (*upper right corner*) for both spleen and lung cells 6 days after influenza challenge. *Right*, Mean BrdU incorporation (±SD) compiled from four to five mice per group of $Thy1.2^+$ memory CD4 T cells. Value is $p = 0.01$ when comparing mean BrdU incorporation of Thy 1.2^+ HA-memory CD4 T cells from IgG2a- and CTLA4Ig-treated mice. Results are representative of two independent experiments with three to five mice per group.

CTLA4Ig treatment has biased effects on lymphoid memory CD4 T cells

To evaluate the cellular mechanism for the differential effects of CTLA4Ig treatment on primary and secondary immune responses to influenza infection, we analyzed in vivo responses of naive and memory CD4 T cells in both lymphoid and nonlymphoid tissues by BrdU incorporation. We administered BrdU to naive or HA-memory mice infected and treated as in Fig. 2*A*, harvested spleen and lung tissue 6 days postinfection, and measured the extent of BrdU incorporation in each tissue from the differentially treated groups. In naive mice infected with influenza, BrdU incorporation of endogenous CD4 T cells in both the spleen and lung of control-treated mice was substantially inhibited by CTLAIg treatment (Fig. 7*A*), with mock-infected controls having minimal BrdU incorporation in both tissues (0.5–1% and 1–3% in spleen and lung, respectively). In flu-infected HA-memory mice, both spleen and lung-resident memory CD4 T cells in control-treated mice exhibited extensive BrdU incorporation following influenza infection (Fig. 8*B*, *left*) that exceeded BrdU incorporation in the primary CD4 T cell response (Fig. 8*A*). In the presence of CTLA4Ig, BrdU incorporation by spleen-memory CD4 T cells was markedly reduced (5-fold reduction), whereas BrdU incorporation by lung-memory CD4 T cells was not affected (Fig. 8*B*, *top left* and *bottom left*). BrdU incorporation of endogenous CD4 and CD8 T cells in

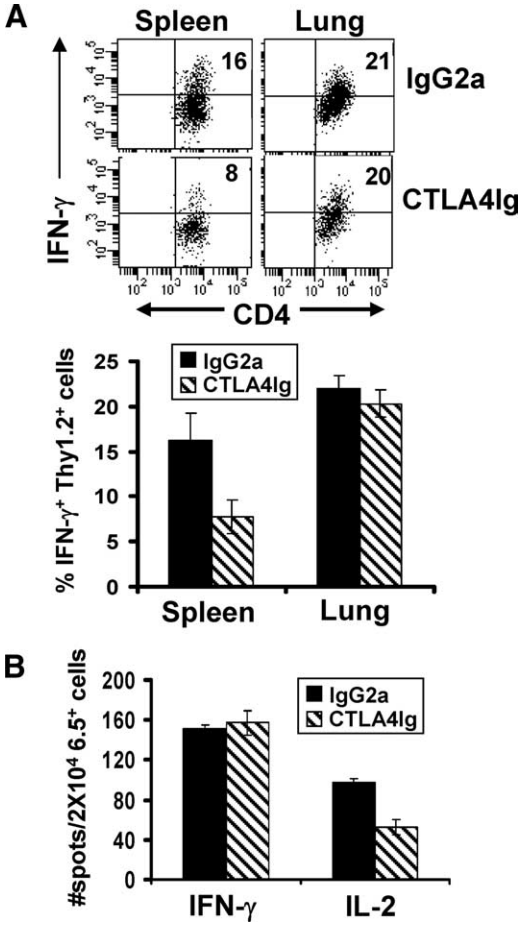

FIGURE 9. Differential effect of CTLA4Ig on cytokine production by lung and spleen memory CD4 T cells. *A*, CTLA4Ig treatment inhibits IFN-γ production from spleen but not lung memory CD4 T cells during influenza challenge. HA-specific memory CD4 T cells were isolated 6 days postinfection from the spleen and lungs of influenza-infected, HA-memory mice treated with IgG2a or CTLA4Ig and stimulated in vitro with PMA and ionomycin for 4 h. *Upper*, IFN-γ production from Thy 1.2^+ HA-specific memory CD4 T cells from both spleen and lung. Quadrants were drawn based on isotype control. *Lower*, IFN-γ production of Thy1.2^+ memory CD4 T cells compiled from three mice per group. *B*, IFN-γ and IL-2 production from HA-specific memory CD4 T cells stimulated in vitro with HA peptide in the presence of 50 μg/ml CTLA4Ig or IgG2a for 18 h. Results are shown as means ± SD of triplicates and are representative of two independent experiments.

spleen and lung of infected memory mice was inhibited by CTLA4Ig treatment, similar to that seen in naive mice (data not shown). These results strongly suggest that CTLA4Ig preferentially inhibits spleen or lymphoid-derived naive and memory CD4 T cells, while leaving intact in situ lung memory CD4 T cells responses; however, we cannot rule out that $BrdU^+$ cells in the lung may have migrated from lymphoid sites.

A hallmark of memory CD4 T cell recall is their rapid effector function. We therefore measured the capacity of HA-memory CD4 T cells recovered from the spleen and lung of CTLA4Ig- or IgG2a-treated mice to produce IFN-γ 6 days after influenza virus challenge. We observed a biased reduction in early IFN-γ production from spleen memory CD4 T cells (2-fold) of CTLA4Ig-treated mice, with no significant reduction in IFN-γ production from lung-resident memory CD4 T cells (Fig. 9*A*). These results show that inhibition of CD28 costimulation differentially affects rapid cytokine secretion from lymphoid and nonlymphoid memory CD4 T cells.

Our findings that lung memory CD4 T cells retain effector function in the presence of CTLA4Ig in vivo suggested either that the functional recall of lung memory CD4 T cells was independent of CD28, or that CTLA4Ig was not present in sufficient quantities in the lung in vivo. To distinguish between these possibilities, we examined the functional properties of Ag-specific lung memory CD4 T cells in vitro in the presence of ample quantities of CTLA4Ig. We found that lung memory CD4 T cells produce predominantly IFN-γ and to a lesser extent IL-2 following antigenic stimulation (Fig. 9*B*). Antigenic stimulation of lung-memory CD4 T cells in the presence of CTLA4Ig resulted in significant inhibition of IL-2, while IFN-γ production was unchanged from control-treated Ag-stimulated cells (Fig. 9*B*). When taken together, our results demonstrate that effector function from lung memory CD4 T cells is intrinsically independent of CD28 costimulation.

Discussion

We demonstrate herein that memory CD4 T cells mediate secondary responses to influenza infection characterized by efficient viral clearance in the context of extensive immunopathology and morbidity. Strikingly, the physiological outcome of a memory CD4 T cell-mediated secondary response to influenza can be significantly improved by targeting the CD28 pathway with the costimulation modulator CTLA4Ig. While CTLA4Ig is suppressive for primary immune responses to influenza, leading to increased viral loads, reduced lung function, and increased morbidity, CTLA4Ig treatment of memory CD4 T cell secondary responses to influenza is remarkably curative, resulting in less morbidity and immunopathology, and enhanced recovery. We demonstrate that CTLA4Ig specifically inhibits lymphoid memory CD4 T cell responses and reduces their capacity to migrate to nonlymphoid sites. Moreover, the ability of lung memory T cells to respond to influenza in situ and mediate rapid effector function is independent of CD28 costimulation and remains intact in CTLA4Ig-treated mice. Our results reveal a novel role for CD28-based immunotherapy for optimizing antiviral secondary responses by differential effects on lymphoid vs lung memory CD4 T cells.

Our findings that CTLA4Ig treatment resulted in disparate clinical outcomes for primary and secondary responses to influenza can be attributed to the disparate functional and spatial attributes of primary and memory responses. Naive T cells reside and become activated in lymphoid tissue and require CD28 costimulation for IL-2 production, as well as differentiation into effector cells (38, 39), which will ultimately migrate to the site of infection. CTLA4Ig treatment of naive mice infected with influenza suppressed the initiation of T cell and Ab responses in lymphoid tissues, impairing the antiviral response. In contrast, memory CD4 T cells are present in both lymphoid and lung tissue, and they require CD28 costimulation mainly for Ag-driven IL-2 production and proliferation (12). While CTLA4Ig inhibited lymphoid memory CD4 T cell expansion, it did not affect in situ lung memory CD4 T cell expansion and effector cytokine production, and therefore viral clearance was maintained. Our results further reveal a specific role for CD28 costimulation in homing to nonlymphoid sites during a viral infection, and they are consistent with earlier findings that CD28 controls T cell migration to peripheral sites in the absence of infection (40). These effects of CTLA4Ig treatment on T cell homing may be a mechanism for the clinical efficacy of abatacept in reducing immunopathology in rheumatoid arthritis, known to be perpetuated by memory CD4 T cells (14).

In addition to its differential effects on lymphoid and nonlymphoid responses, CTLA4Ig treatment had disparate effects on cytokine production by memory CD4 T cells. We show herein that CTLAIg preferentially inhibits IL-2 production from lung memory

CD4 T cells, while leaving intact IFN-γ production. We propose that the ability of CTLA4Ig to differentially inhibit IL-2 vs IFN-γ responses may be directly related to the uncoupling of immunopathology and protection in secondary influenza responses. IFN-γ production has been shown to be crucial for protection in secondary responses to influenza and other viral infections (41, 42), although it can be dispensible for clearance of influenza virus during primary responses (43, 44). The ability of lung memory CD4 T cells to rapidly produce IFN-γ in the presence of CTLA4Ig despite a suppressed Ab and endogenous CD4 and CD8 T cell responses suggests that IFN-γ production in situ may mediate rapid viral clearance by memory CD4 T cells, a possibility we are presently investigating. Conversely, IL-2 production by memory CD4 T cells, which is important for their expansion (12), can contribute to increased infiltration into lung tissue and the resultant immunopathology. Thus, highly expansive memory T cells may be detrimental when site-specific immunity is required in respiratory virus infections. We propose that for protective immunity to influenza, the quality and location of memory T cells is more important than their absolute frequency, also a key issue for vaccine design (45).

We demonstrate that targeting CD28 costimulation can optimize influenza-specific antiviral secondary responses, suggesting a new clinical strategy for ameliorating influenza morbidity. Morbidity and mortality from influenza infection have been attributed to pathological immune responses characterized by excessive cytokine secretion and inflammatory infiltration into the lung (21, 46); however, a cellular mechanism for influenza-induced immunopathology has not been identified. We show herein that memory CD4 T cells can exacerbate infiltration and inflammation in the lung in secondary responses to influenza, similar to findings of memory CD4 T cell-mediated immunopathology in other viral systems, including respiratory syncytial virus (47, 48), dengue virus (49), and hepatitis (50). Additionally, previous studies have identified a role for CD8 T cells in lung immunopathology during primary influenza infection (51, 52). As memory CD8 T cells have also been shown to require CD28 costimulation for optimal proliferation in vivo (10, 11), CTLA4Ig treatment may also show efficacy in preventing CD8 T cell-mediated immunopathology. Thus far, strategies for reducing immunopathology through inhibition of inflammatory cytokines (53) or global T cell immunosuppression (54) have been ineffective or have blocked protective immune responses, impairing viral clearance. Here, we show that CTLA4Ig may provide the appropriate type of immunosuppression to differentially curtail pathological immune reactions while maintaining site-specific antiviral responses mediated by memory T cells.

Memory T cell responses to influenza are clinically relevant given their presence in healthy individuals (17, 18), as well as recent identification of memory CD4 T cells that cross-react with avian influenza (H5N1) epitopes in the peripheral blood of healthy humans exposed to seasonal influenza variants (19, 20). These findings emphasize the clinical importance of understanding memory T cell responses to influenza and other viruses, and the clinical applicability of immunotherapies that enhance a memory T cell response. We propose that an illness resulting from influenza infection in an immune-experienced individual may mask the underlying memory T cell-mediated viral clearance, and that immunomodulation may be an effective way to manifest the protective features of T cell memory.

Our findings strongly suggest that considering both the mode of immunomodulation together with the host immune status are critical parameters for evaluating the efficacy of immunotherapies. Previous studies in transplantation have found that the presence of memory T cells interferes with or prevents the effectiveness of tolerance induction strategies or immunosuppression (55, 56), indicating that memory T cells may represent a barrier to effective treatment. We demonstrate herein that immunomodulation of a memory response can result in a positive clinical outcome to a respiratory virus infection. These studies, together with our results, suggest that considering memory T cells when designing and testing immunotherapies is important for evaluating their efficacy and potential utility in antiviral immunity, autoimmunity, and transplantation.

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