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Altered T-bet Dominance in IFN-γ–Decoupled CD4⁺ T Cells with Attenuated Cytokine Storm and Preserved Memory in Influenza

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Cytokine storm has been postulated as one of the major causes of mortality in patients with severe respiratory viral infections such as influenza. With the help of an influenza Ag–specific mouse experimental system, we report that CD4⁺ T cells contribute effector cytokines leading to lung inflammation in acute influenza. Although virus can no longer be detected from tissues 14 d post-infection, virus-derived Ag continues to drive a CD4⁺ T cell response after viral clearance. Ag-specific CD4⁺ T cells proliferate and evolve into memory CD4⁺ T cells efficiently, but the production of effector cytokines is seriously hampered during this phase. This decoupling of proliferation and effector cytokine production doesn’t appear in conjunction with increased suppression by regulatory T cells or decreased induction of transcription factors. Rather, GATA-3 and ROR-γt levels are elevated when compared with cells that have effector cytokine production. T-bet dominance over GATA-3 and ROR-γt decreases with the disarmament of effector cytokine production. Importantly, upon reinfection, these decoupled cells produce elevated levels of IFN-γ and were effective in virus eradication. These results provide a mechanism through altered T-bet dominance to dampen the cytokine storm without impeding the generation of memory T cells in influenza virus infection. *The Journal of Immunology, 2013, 190: 4205–4214.

Influenza strikes millions of people worldwide and causes significant death every year. The number of deaths increases in pandemic years (World Health Organization fact sheets, http://www.who.int/mediacentre/factsheets/fs211/en/). The symptoms of influenza are exaggerated by the patient’s immune system responding to the virus, rather than by the virus itself. The immune system’s prolonged and unbalanced response generates a cytokine storm and causes the lungs to become inflamed and significantly damaged (1–8). The fight continues in the damaged lung even after the virus is cleared from the body.

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Abbreviations used in this article: CTL, cytotoxic T cell; qRT-PCR, quantitative real-time PCR; Treg, regulatory T cell;

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tracted less compared with cells responding to acute infection. The induction of IFN-γ receptor 1 was less in residual Ag-driven CD4+ T cells compared with the cells responding to acute infection, whereas the Foxp3+ regulatory T cell (Treg) levels remained comparable between the two groups. Induction of transcription factors such as T-bet, GATA-3, and ROR-γt by residual Ag was not impaired either. However, the relative expressions of T-bet/GATA-3 and T-bet/ROR-γt became altered in the cells exhibiting the phenomenon of decoupled proliferation and effector cytokine production. Compared with the cells activated by acute infection, the decoupled cells were also very efficient for viral eradication upon reinfection with influenza virus. Interestingly, the decoupled cells provided a better memory response during a secondary influenza infection. Altered T-bet dominance may be a key mechanism for generating a more balanced immune response, and thereby restraining the devastating cytokine storm that is so detrimental for patients infected with influenza virus.

Materials and Methods

Mice and infection

The TCR-transgenic mouse line 6.5 expresses a TCR recognizing an I-Ek-restricted HA epitope (105SFERFEIPKE)105, provided by H. Von Bohmer, Harvard University, Boston, MA. The 6.5 and wild-type mice are of two kinds, Thy.1+ and Thy.1−. All mice are of C57Bl/10. D2 genetic background and are maintained in specific pathogen-free condition. All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of Chang Gung Memorial Hospital, Taiwan. The A/PR/8/34 (PR8) and WSN (A/WSN/33) strains of H1N1 influenza virus were produced in the allantoic fluid of 10-d-old embryonated chicken eggs and characterized by a core facility at the Chang Gung University, Taiwan. Although both the PR8 and WSN strains belong to H1N1 influenza virus, the WSN strain does not have the PR8 strain-specific HA epitope 105SFERFEIPKE105. The PR8-H3N2 (A/ Taiwan/447/2007) is a recombinant strain with heterologous HA and neuraminidase from H3N2 on a PR8 backbone and was provided by S.-R. Shih (Research Center for Emerging Viral Infections, Chang Gung University, Taiwan). Mice were inoculated via intranasal route during light diethyl ether anesthesia (J.T. Baker, Phillipsburg, NJ) with indi-
cated units of virus in 50 μl PBS.

Adoptive transfer

Clonotypic 6.5 CD4+ TCR transgenic T cells were prepared from pooled spleen and lymph nodes of 6.5 transgenic mice. Clonotypic percentage was determined by flow cytometry analysis. The naive phenotype was confirmed by activation marker CD44 and CD62L. The 6.5 CD4+ T cells (2.5 × 10⁶) were injected into the tail veins of the recipient mice in 0.2 ml HBSS.

For adoptive transfer of CFSE (5, 6-carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Eugene, OR)-stained cells, clonotypic T cells (1 × 10⁷ cells/ml) were stained with 5 μM CFSE in HBSS for 10 min. Cells were washed three times with complete RPMI 1640 medium, and CFSE-stained 6.5 CD4+ T cells (2.5 × 10⁶) were similarly transferred through the tail veins of the recipient mice.

Flow cytometry

Cell suspensions were incubated on ice with saturated concentrations of fluorochrome-labeled mAbs in FACS buffer (PBS plus 0.5% BSA and 0.02% NaN3). Donor T cells were identified using mAbs, as follows: biotin-conjugated anti-clonotypic TCR 6.5 (provided by H. Von Bohmer); avidin-PE (Caltag, Burlingame, CA) or avidin-alkaline phospho- cya tin (Caltag) and PerCP- or FITC-conjugated anti-CD4. All the fluorochrome-conjugated Abs were from BD Biosciences, except for T-bet and IgG1k (eBiosciences). Cells were acquired on FACSCalibur, and analysis was performed using CellQuest Pro (BD Biosciences) or FlowJo (Tree Star) software.

The anti-clonotypic TCR 6.5 Ab was replaced by anti-Thy1.1 Ab in the case of transferring Thy1.1+ donor cells into Thy1.2 recipient mice or anti-Thy1.2 Ab in case of transferring Thy1.2+ donor cells into Thy1.1 recipient mice.

Intracellular cytokine staining

Organs were harvested from the experimental mice on indicated days. Single-cell suspensions (5–10 million cells/well of a 24-well plate) were restimulated for 5 h with 100 μg/ml MHC class II-restricted HA peptide (105SFERFEIPKE105) for clonotypic CD4+ T cells and PMA (25 ng/ml) plus ionomycin (500 ng/ml) for endogenous T cells in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich). Concentration of brefeldin A was maintained throughout the intracellular cytokine staining. Re-
stimulated cells were surface stained with anti-6.5 and anti-CD4, as described above, fixed in intracellular fixation buffer (eBiosciences), washed, and stained in permeabilization buffer (eBiosciences) containing fluorochrome-conjugated Abs against target cytokines.

FOXp3 and Foxp3 were stained ex vivo without restimulation. After surface staining, cells were fixed and permeabilized in fixation/permeabilization buffer (eBiosciences), washed, and stained in permeabilization buffer (eBiosciences) containing fluorochrome-conjugated Abs.

Virus titer

We measured live virus in organs of influenza virus-infected mice using a modified Madin–Darby canine kidney (MDCK; American Type Culture Collection) cell plaque assay (14). Organs were collected at the indicated times in 1 ml DMEM, snap frozen in liquid nitrogen, and stored at −80°C until they are ready for use. MDCK monolayers were grown in DMEM supplemented with 10% FCS and antibiotic-antimycotic (100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Life Technologies BRL). The 10-fold dilutions of the tissue homogenates were prepared in DMEM supplemented with 0.00025% trypsin. A total of 500 μl of each dilution was added to confluent monolayers of MDCK cells in 6-well plates in duplicates for 1 h at 35°C. 5% CO2. Each well received 2 ml of an agar overlay (0.3%) in medium containing DMEM with 0.00025% trypsin. Cells were incubated for 3 d at 35°C and were fixed with 10% formalin for 20 min. The agar overlay was then removed, and fixed monolayers were stained by adding 1:10 dilution of 2% crystal violet prepared in 20% ethanol. The results are presented as PFU/ml = (mean number of plaques × 2) × (1/dilution factor).

Influenza-specific Ab response

Influenza-specific Ab responses were determined by plaque reduction assay. Sera from influenza-infected mice were collected on the indicated days postinfection. They were heat inactivated for 30 min at 56°C to inactivate the complements. A total of 50 PFU live PR8 virus was allowed to infect a monolayer of MDME cells in the presence or absence of decomplemented sera with specified dilution for 1 h at 35°C. Infected cells were cultured for another 72 h in agarose-mixed DMEM at 35°C. Formation of plaques was counted manually after fixing with formalin and staining with crystal violet. Reduction of plaques due to inhibition by anti-influenza Ab in the sera was calculated.

Histopathology

Lungs from experimental mice were harvested at indicated days and fixed with 10% neutral buffered formalin solution. Following fixation, lungs were embedded in paraffin and 5-μm sections were cut. Sections were stained with H&E and scored blinded. The inflammatory cell infiltrations, including lymphocytes, neutrophils, and plasma cells, were separately scored as negative, 1+, 2+, 3+ according to the severity of each infiltration. Vascu-

Labitis and fibrosis were also scored as negative, 1+, 2+, 3+ on the basis of their intensity. An average of these scores was represented as overall inflammation in the lung.

In vitro proliferation

Single-cell suspension was prepared from the spleens. A total of 2 × 10⁶ cells was restimulated for 72 h with class II-restricted HA peptide (SFERFEIPKE) at 37°C in presence of 5% CO2. Tritiated thymidine was added 18 h prior to harvest. Cells were harvested, and the cpm was measured by a beta counter.

Quantitative real-time PCR analysis

The sorted HA-specific CD4+ T cells were immediately used for RNA extraction by TurboCapture 96 mRNA Kit (Qiagen). Reverse transcription was performed with Moloney murine leukemia virus high performance reverse transcriptase (EPICENTRE Biotechnologies). cDNA levels were analyzed by quantitative real-time PCR (qRT-PCR) with the iCycler Real-Time PCR Detection System (Bio-Rad, Hercu-
les, CA). Each sample was assayed in triplicate for the target gene together with β-actin as the internal reference in 25 μl final reaction volume containing 5 μl 10-fold diluted cDNA, 2.5 μl 10-fold PCR buffer, 2 μl dNTP (2.5 mM), 1.5 μl MgCl₂ (50 mM), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 0.1 μl platinum Taq DNA polymerase (5 U/μl) (Inviogene), 0.05 μl FluA (5 μM), 0.025 μl SYBR Green I (100 nM) (Invitrogen), and 12.825 μl dH₂O. The primers used for qRT-PCR are as follows: F-forward, 5'-CAA CAA CCC CCT TCC CAA AG-3' and reverse, 5'-TCC CCC AAG CAG TTG ACA GT-3'; GATA-3: forward, 5'-AGA ACC GCC CCC TTA TCA A-3' and reverse, 5'-AGT TCG CGC AGG ATG TCC-3'; ROBY: forward, 5'-CGC TTC ACC TGA CCT ACC-3' and reverse, 5'-TTG CCT CTG TCT GGA CTA TAC-3'; Ifng: forward, 5'-CGA GGT ATG AAT CAG GTG TGG AAG-3' and reverse, 5'-CAA AGA CCT AGG ATG GTG TGG TGT GCA CTT TTA TTG GTC TCA A-3' and reverse, 5'-TGG AAC GGT TGG TGT GCA CT TTA TTG GTC TCA A-3'. The relative mRNA frequencies were determined by normalization to the β-actin. Briefly, we normalized each set of samples using the difference in the power value of threshold cycles (Ct) between the target gene and the β-actin. The clonotypic percentage, CD44 up-regulation, CD62L downregulation, and production of effector cytokines were calculated as 2^ΔΔCt where ΔCt = (ΔCt sample/ΔCt naive sample).

Statistical analyses

Data represented as means ± SD. We used GraphPad Prism version 5 for Student t test analyses. We considered all p values >0.05 not to be significant.

Results

Exaggerated immune response causes lung inflammation upon influenza virus infection

Naive influenza HA-specific naive CD4⁺ T cells were adoptively transferred into syngeneic mice. The recipients were infected with 2.5 × 10⁶ PFU influenza virus on the same day. On day 4, HA-specific CD4⁺ T cells expanded in number and accumulated in the lungs of infected mice. CD44 was upregulated and CD62L was downregulated on the membrane of these clonotypic cells. HA-specific CD4⁺ T cells produced effector cytokines such as IFN-γ, IL-2, and TNF-α. The clonotypic percentage, CD44 up-regulation, CD62L downregulation, and production of effector cytokines increased further on day 7. The activation status of HA-specific CD4⁺ T cells waned thereafter. However, in comparison with the donor cells of uninfected mice, the activation status of the donor cells on day 14 was significantly higher in infected mice (Fig. 1A). The gross appearance of the lung changed postinfection and the tissue was inflamed, with highest congestion and black patches by day 7 (data not shown). Histological analysis of lung sections revealed infiltration of immune cells such as lymphocytes, neutrophils, and plasma cells, in addition to the swelling of the bronchial wall on day 4 postinfection. The degree of inflammation increased further on day 7. Inflammation decreased thereafter, although the persistence of infiltration was evident in lung sections prepared from mice sacrificed on day 14 postinfection (Fig. 1B).

Postinfection with 2.5 × 10³, 5 × 10³, or 1 × 10² PFU influenza virus, the mice successfully cleared the virus from the...
body within 14 d of infection. Live virus could be detected in the lungs on day 4 and 7 postinfection with all doses; however, virus could no longer be detected by day 14 (Fig. 1C). Although virus was detected in the lung, plaque assays showed the lymph nodes and spleen to be virus free, even at all three doses (data not shown).

**Decoupling of proliferation and IFN-γ response hampers the production of effector cytokines**

On day 14 postinfection, naive HA-specific naive CD4+ T cells were adoptively transferred. From this point, these cells will be referred to as late transferred cells. In parallel, naive HA-specific CD4+ T cells were similarly transferred into mice infected on the same day as transfer. These cells are the early transferred cells.

The production of IFN-γ and IL-2 by the late and early transferred cells was comparable on day 4 after adoptive transfer (Fig. 2A, 2B). IFN-γ production by the late transferred cells was greatly reduced thereafter, and production level was almost equivalent to the level of naive cells on days 7 and 14. However, IFN-γ production by the early transferred cells increased further on day 7 and remained elevated on day 14 (Fig. 2A). For IL-2 production, both late and early transferred cells were equally potent on days 4 and 7. In fact, late transferred cells produced more IL-2 in the lungs on day 14 (Fig. 2B). Expansion of the late transferred cells was marginally lower than early transferred cells on day 7, as evidenced by the CFSE profile. However, there was a significant expansion of the late transferred cells compared with the control naive cells that were similarly transferred into uninfected mice (Fig. 2C). On day 7, the late transferred cells displayed an upregulation of activation markers such as CD44 and CD69; there was a downregulation of CD62L, although not to the level of early transferred cells (Fig. 3A). The expression of chemokine receptors CCR5 and CCR7 was comparable between late and early transferred cells (Fig. 3A). In addition to IFN-γ, reduced amounts of TNF-α, IL-10, and IL-17a were produced by the late transferred cells (Fig. 3B). IL-2 and IL-6 production did not differ between the two groups (Fig. 3B).

**Better proliferation and less contraction of HA-specific CD4+ T cells responding with decoupled proliferation and effector cytokine production**

On day 4 postransfer, clonotypic percentages did not differ between late and early transferred HA-specific CD4+ T cells in the lung and spleen. Upon restimulation with cognate HA peptide, the in vitro proliferation of the late and early transferred cells was similar as well (Fig. 4A). On day 7 postransfer, clonotypic percentages were lower for late transferred cells than early transferred cells. However, when stimulated in vitro, the late transferred cells proliferated better than early transferred cells (Fig. 4A). On day 14 postransfer, clonotypic percentages for late transferred cells exceeded the early transferred cells. The late transferred cells also showed greater proliferation in vitro than early transferred cells (Fig. 4A).

The late and early transferred HA-specific CD4+ T cells were sorted from the spleens and lungs on day 7 postransfer and analyzed for IFN-γ receptor 1 and 2. Relative to the level in naive cells, the induction of IFN-γ receptor 1 was decreased in the late transferred cells when compared with the early transferred cells (Fig. 4B). The induction of IFN-γ receptor 2 in the late and early transferred cells, however, was similar (Fig. 4B).

**An increase in Foxp3+ Treg is not responsible for the decoupling of proliferation and effector cytokine response**

Both the late and early transferred HA-specific CD4+ T cells responded with coupled proliferation and IFN-γ production on day 4 postransfer (Figs. 2, 4A). Production of IFN-γ and other effector

![FIGURE 2. Disarmament of IFN-γ production in activation of influenza-specific CD4+ T cells after viral eradication. Naive HA-specific CD4+ T cells were adoptively transferred into syngeneic C57/B10.D2 recipient mice on day 14 after influenza virus infection (Late). Similarly, HA-specific naive CD4+ T cells were transferred on day 0 with infection (Early). (A) IFN-γ- and (B) IL-2–producing late (●) and early (▲) transferred HA-specific CD4+ T cells from the stated organ were compared on mentioned days after transfer by intracellular staining (mean ± SD; *p < 0.01, ***p < 0.0001, n = 6/group). (C) In vivo expansion of late and early transferred HA-specific CD4+ T cells from the stated organ was compared on day 7 by their CFSE profile.](http://www.jimmunol.org/Download)
cytokines was reduced thereafter in late transferred cells. This could be caused by the active suppression of Treg. We analyzed the natural Treg population by definition of Foxp3 expression in CD4+ T cells on days 4 and 7 after adoptive transfer. Foxp3+ adoptively transferred cells were comparable between naive, late, and early transferred HA-specific CD4+ T cells. The native Foxp3+ HA-specific CD4+ T cells were analyzed from donor cells transferred in uninfected mice (Fig. 5A, 5B). Foxp3+ endogenous CD4+ T cells were elevated in infected mice in general, but the percentages of FOXP3+ CD4+ T cells were comparable between the groups with late or early transferred cells (Fig. 5A, 5B).

On day 7 after adoptive transfer, the clonotypic and endogenous CD4+ T cells were analyzed for subsets expressing CD25, CTLA4, LAG3, or GITR, as these subsets have shown regulatory activity in several experimental systems. The CD4+ T cell subsets with expression of CD25, CTLA4, or LAG3, or GITR, as these subsets have shown regulatory activity in several experimental systems. The CD4+ T cell subsets with expression of CD25, CTLA4, or LAG3 were not increased in late transfer group when compared with the early transfer group (Fig. 5C, 5D). Compared to early transfer group, the GITR+ clonotypic CD4+ T cells were not increased in late transfer group as well. The GITR+ endogenous CD4+ T cells were fewer in lungs of late transfer group too. However, in the spleen, the percentage of GITR+ endogenous CD4+ T cells was higher in the late transfer group than the early transfer group (Fig. 5C, 5D).

Altered relative expression, but not impeded induction of transcription factors in the phenomenon of decoupling

We analyzed the intracellular expression of T-bet and IFN-γ in the HA-specific CD4+ T cells. The percentage of T-bet–expressing late transferred cells was more than T-bet+ early transferred cells on day 4 posttransfer. However, IFN-γ production was comparable between late and early transferred cells at this time point (Fig. 6A). T-bet induction was increased with time, as >90% of the late and early transferred cells stained positive for T-bet on day 7 posttransfer (Fig. 6A). Despite comparable T-bet induction, IFN-γ production was almost silenced in the late transferred HA-specific CD4+ T cells on day 7 posttransfer (Fig. 6A). The naive HA-specific CD4+ T cells expressed basal levels of T-bet and IFN-γ at these time points after being adoptively transferred into uninfected mice (dotted line, Fig. 6A).

We sorted late and early transferred cells on day 7 posttransfer and analyzed the mRNA levels of T-bet and IFN-γ to confirm the findings at the protein level. The induction of T-bet in the late and early transferred cells, relative to the level in naive cells, was similar as well (Fig. 6B). IFN-γ mRNA was not detected in the late transferred cells, whereas a significant increase in IFN-γ transcript was detected in the early transferred cells (Fig. 6A).
According to transcript levels, induction of GATA-3 was elevated in the late transferred cells compared with the early transferred cells. IL-4 mRNA was increased in the early transferred cells, but not detected in the later transferred cells or the naive cells (Fig. 6B). The induction of ROR-γt was elevated in the late transferred cells. Finally, IL-17a transcripts were comparable between late and early transferred cells (Fig. 6B).

For the early transferred cells, expression of T-bet was dominant over GATA-3. For the late transferred cells, the dominance of T-bet was decreased and the relative expression between T-bet and GATA-3 was significantly less (Fig. 6C). The ratio of relative expression between T-bet and ROR-γt was altered as well (Fig. 6C). The GATA-3/ROR-γt ratio did not change between late and early transferred cells (data not shown).

FIGURE 5. Decoupling of proliferation and cytokine production was not associated with increased Treg. Naive HA-specific CD4+ T cells were adoptively transferred into syngeneic C57/B10.D2 recipient mice on day 14 after influenza virus infection (decoupled; late). Similarly, HA-specific naive CD4+ T cells were transferred on day 0 with infection (coupled; early). (A) FOXP3 expression in HA-specific CD4+ T cells was compared between late (black) and early (gray) transferred cells on day 7 by flow cytometry. (B) At the same time, FOXP3-expressing endogenous CD4+ T cells were compared between the groups of mice (late, black; and early, gray) (mean ± SD; n = 6/group). Naive HA-specific CD4+ T cells were transferred into uninfected mice, and their FOXP3 expression (-----) was presented as control. (C) The percentages of CD25+, LAG3+, CTLA4+, and GITR+ HA-specific CD4+ T cells were compared between late (black) and early (gray) transferred cell on day 7 by flow cytometry. (D) At the same time, CD25+, LAG3+, CTLA4+, and GITR+ endogenous CD4+ T cells were compared between the groups of mice with late (black) and early (gray) transferred cells (mean ± SD; ***p < 0.0001; n = 6/group).

The phenomenon of decoupling is a process that provides better memory response

Our studies show that the late transferred cells responded with the phenomenon of decoupling. To explore this further, we studied the impact of decoupling on memory response. Upon reinfection with influenza virus, both the late and early transferred cells facilitated the eradication of virus when compared with their naive counterparts. When compared with the early cells, the late transferred cells conferred better viral clearance. No virus was detectable in the lungs of mice with late transferred cells on day 2 postreinfection, whereas there was virus in the lungs of mice with early transferred cells at the same time. We could not spot any virus on day 7 postreinfection in the lungs of mice with either late or early transferred cells. However, a significant amount of virus could be measured in the lungs of mice with adoptively transferred naive cells at these time points (Fig. 7A). The clonotypic percentage of late transferred cells was higher when compared with early transferred cells on day 7 postreinfection in the lungs, mediastinal lymph nodes, and spleens (Fig. 7B). The production of both IFN-γ and IL-2 was greater by these late transferred cells after the reinfection (Fig. 7C). The late transferred cells produced significant amounts of IFN-γ upon reinfection despite their previous disarmament of IFN-γ production (Fig. 7C). The IFN-γ production by late transferred cells upon reinfection was
Decoupled proliferation/effector function of the late transferred HA-specific CD4+ T cells provided better memory response than the early transferred cells. It may have been caused by the more mature influenza immunity resulting from the primary infection, instead of the decoupled clonotypic CD4+ T cells themselves. Additional control experiments were performed with heterologous influenza virus strains for the primary infection; these resulted in mature influenza immunity in general, but not the decoupled clonotypic CD4+ T cell response. Naive 6.5 CD4+ T cells were adoptively transferred 14 d after the first challenge with PR8, PR8-H3N2, or WSN. On day 7 after adoptive transfer, endogenous CD8+ T cells was measured by intracellular staining and was comparable on stated days by flow cytometry with intracellular staining. IFN-γ production by late (■) and early (□) transferred cells was measured by intracellular staining and was compared on stated days. Dotted line represents basal levels of T-bet expression or IFN-γ production by naive HA-specific CD4+ T cells into uninfected mice. (B) On day 7 after transfer, RNA was isolated from the sorted late (■) and early (□) transferred HA-specific CD4+ T cells retrieved from the spleen and lung. Genomic expression of mentioned transcription factors and cytokines was measured by qRT-PCR. Fold changes in the transcripts of these genes compared with that in the naive cells are presented as mentioned in Materials and Methods (*p < 0.01, **p < 0.001, ***p < 0.0001, n = 6/group, UD, Undetectable). (C) Relative expression of transcription factors in late (■) and early (□) transferred HA-specific CD4+ T cells from spleens and lungs is presented. Dotted line represents relative expression of the mentioned transcription factors in naive HA-specific CD4+ T cells into uninfected mice.

Discussion
Our data show that influenza HA-specific CD4+ T cell responses contribute effector cytokines that lead to lung inflammation in acute influenza. The effector cytokine production is restrained by HA-specific CD4+ T cells when they are activated by residual influenza virus-derived Ag. However, the proliferation of these cells remains preserved in the response to residual Ag. Impairment
of T cell function, especially the effector cytokine IFN-\(\gamma\) response in proliferating T cells, has also been shown in systems of transplantation and autoimmunity (15–17). We previously observed that T cell division is not strictly linked to effector function (18, 19). Preserved T cell proliferation despite disarmament of effector cytokine secretion implies a possible mechanism for restrained cytokine response in autoimmunity (20). The same can be implied as a mechanism for controlling the cytokine storm in influenza virus infection.

Preserved T cell proliferation despite disarmament of effector cytokine response has been described as an intermediate stage of development for Treg in systems of autoimmunity (16–19). However, it appears that decoupling proliferation and effector cytokine expression help memory T cell generation in viral infection, particularly in influenza (our data) (13). With disarmed effector function, the level of exhaustion decreases in decoupled influenza-specific CD4\(^+\) T cells. Preserved proliferation and less contraction enable a higher frequency of Ag-experienced cells to survive. Compared with naive, these cells become trained for better eradication of virus to provide resistance to reinfection.

Upon acute infection with influenza virus, proliferation and cytokine expression by HA-specific CD4\(^+\) T cells have been linked together in our experiments and shown in several other systems as well (21, 22). These cells produce more IFN-\(\gamma\), TNF-\(\alpha\), and IL-2 than IL-4, with dominant expression of T-bet over GATA-3. T-bet dominance is known to repress the binding of GATA-3 to its target DNA and block IL-4 production (23). In contrast, cytokine production is disarmed in proliferating HA-specific CD4\(^+\) T cells in response to residual influenza virus-derived Ag. The cells with decoupled proliferation and effector cytokine response show increased expression of GATA-3. Overexpression of GATA-3 alters the relative expression between T-bet and GATA-3 and perhaps suppresses cytokine production by decoupled HA-specific CD4\(^+\) T cells, in ways similar to the known suppression of IFN-\(\gamma\) production in T-bet–expressing CD4\(^+\) T cells by GATA-3 overexpression (24, 25). However, the reason for suppression of IL-4 production in GATA-3–overexpressing decoupled HA-specific CD4\(^+\) T cells is not clear.

The Th1/Th2 paradigm has recently shifted to the Th1/Th2/Th17/Treg hypothesis, a multilineage commitment from the same Th precursor cells (26, 27). Consequently, the relationship between the transcription factors T-bet, GATA-3, ROR-\(\gamma\)t, and Foxp3 has become important in determining the commitment of the cells. In our experiments, more IFN-\(\gamma\) production than IL-17a by HA-specific CD4\(^+\) T cells is with T-bet dominance over ROR-\(\gamma\)t in coupled proliferation and effector cytokine production during an initial infection.

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coincides with the decoupling of proliferation and cytokine response in our experiments. The alteration does not affect IL-17a production, but a serious impairment of IFN-γ production has been observed. The relationship between ROR-γt and T-bet is perhaps similar to the relationship between GATA-3 and T-bet. For the fourth factor of the Th1/Th2/Th17/Treg hypothesis, Foxp3+ and other Treg are comparable between decoupled and coupled proliferation and effector cytokine response in our experiments.

Strong T-bet expression leads to high expression of the receptor for IFN-γ (33), a factor that may be important in T cell contraction (34, 35). Compared with the cells responding with effector cytokines, T-bet dominance over GATA-3 and ROR-γt was altered in decoupled influenza-specific CD4+ T cells in our experiments. The expression of IFN-γ receptor 1 was less in the decoupled influenza-specific CD4+ T cells as well. Loss of T-bet dominance with decreased IFN-γ receptor 1 expression, not decreased expression of T-bet, perhaps facilitated survival of a higher frequency of decoupled influenza-specific CD4+ T cells.

The thymus releases mature naive T cells to the periphery through a continuous process. Various waves of naive T cells encounter Ag differentially during the course of an influenza virus infection. Our results describe the response by naive T cells that appears in the later phase of an influenza infection. Whereas there is a decoupling of proliferation and effector cytokine response, these cells significantly contribute to the generation of memory T cells. Mechanistically, the disarmament of effector cytokines in proliferating influenza-specific CD4+ T cells appears with an alteration of relative expressions, but not impeded induction, of transcription factors. These results provide a mechanism for restraining overwhelming acute-phase reactions without impeding the generation of memory T cells. Perhaps methods to alter T-bet dominance may

FIGURE 8. Efficient viral clearance with restrained IFN-γ production by decoupled HA-specific CD4+ T cells provides better memory response during reinfection with influenza virus. Wild-type C57/B10.D2 mice were infected with 2.5 × 10^3 PFU PR8 or PR8-H3N2 or WSN strains of influenza A virus and adoptively transferred with 2.5 × 10^6 influenza HA-specific naive CD4+ T cells on day 14 postinfection. (A) On day 7 after adoptive transfer, the percentages of clonotypic and endogenous T cells in the lungs were compared ex vivo (Naive: uninfected mice with synchronized adoptive transfer). The IFN-γ- and IL-2-producing clonotypic and endogenous T cells were compared by intracellular staining. (B) On day 7 after adoptive transfer, all mice were infected with 2.5 × 10^3 PFU PR8 strain of influenza virus. Naive mice receiving an adoptive transfer of 2.5 × 10^6 naive HA-specific CD4+ T cells and infection of 2.5 × 10^3 PFU PR8 virus served as the control. On day 7 after secondary infection, viral titer in the lungs, Ab response, percentages of T cells, and the IFN-γ- and IL-2-producing T cells were compared. Data represented as mean ± SD (***p < 0.0001, n = 6/group).
be a solution for controlling the damaging cytokine storm seen in the clinical setting of severe influenza infections.

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Disclosures
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References