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T Cell Responses Are Required for Protection from Clinical Disease and for Virus Clearance in Severe Acute Respiratory Syndrome Coronavirus-Infected Mice▽

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A dysregulated innate immune response and exuberant cytokine/chemokine expression are believed to be critical factors in the pathogenesis of severe acute respiratory syndrome (SARS), caused by a coronavirus (SARS-CoV). However, we recently showed that inefficient immune activation and a poor virus-specific T cell response underlie severe disease in SARS-CoV-infected mice. Here, we extend these results to show that virus-specific T cells, in the absence of activation of the innate immune response, were sufficient to significantly enhance survival and diminish clinical disease. We demonstrated that T cells are responsible for virus clearance, as intravenous adoptive transfer of SARS-CoV-immune splenocytes or *in vitro*-generated T cells to SCID or BALB/c mice enhanced survival and reduced virus titers in the lung. Enhancement of the number of virus-specific CD8 T cells by immunization with SARS-CoV peptide-pulsed dendritic cells also resulted in a robust T cell response, earlier virus clearance, and increased survival. These studies are the first to show that T cells play a crucial role in SARS-CoV clearance and that a suboptimal T cell response contributes to the pathological changes observed in SARS. They also provide a new approach to SARS vaccine design.

Severe acute respiratory syndrome (SARS), caused by a novel coronavirus (SARS-CoV), resulted in over 8,000 cases of respiratory disease, with 10% mortality, in 2002 and 2003 (18). Patients with severe disease developed acute lung injury (ALI), concomitant with neutrophilia, lymphopenia, and prolonged expression of several proinflammatory cytokines (4, 17, 18, 32). Virus was cleared slowly from these patients and could be detected in respiratory secretions for as long as 21 days postinfection (p.i.) (14, 17).

SARS has not recurred to a significant extent since 2003, so most studies have used animal infections to investigate the mechanism of severe disease. These studies have been facilitated by the isolation and characterization of SARS-CoV variants that cause severe respiratory disease in mice or rats after adaptation to these hosts by serial passage (15, 16, 22). One strain, isolated after 15 passages through the lungs of BALB/c mice, caused respiratory disease in young BALB/c mice (MA15 virus) (22). As in humans with severe disease, cytokine expression is elevated and prolonged in animals with severe disease, whether caused by the human Urbani or rodent-adapted strains of SARS-CoV, and is accompanied by delayed kinetics of virus clearance (1, 5, 15, 23, 26, 35). This elevated expression of proinflammatory mediators is believed to be a major contributory factor in the development of pneumonia and, subsequently, ALI.

These studies did not address the role of the antivirus T cell response in disease. T cell responses are critical for virus clearance and protection from clinical disease in mice infected with other coronaviruses, such as mouse hepatitis virus (MHV) (31), or other pulmonary pathogens, such as influenza A virus or Sendai virus (7, 29). Although some studies using the Urbani strain showed that virus clearance was T cell independent (5, 34), mice infected with this strain develop only mild pneumonitis, so that the requirement for a virus-specific T cell response for protection in SARS-CoV-infected animals remains unclear.

Young BALB/c mice infected with MA15 virus develop severe respiratory disease, with substantial mortality, dependent upon virus dosage (22). We recently showed that the pulmonary immune response is inefficiently activated in these mice at early times after infection, resulting in a barely detectable antivirus T cell response in mice with a lethal infection (35). Survival correlated with the development of a SARS-CoV-specific CD4 and CD8 T cell response. Poor activation of the immune response could be reversed by depletion of inhibitory alveolar macrophages with clodronate, a drug that depletes phagocytic cells (28, 30), or by treatment with poly(I-C) or CpG, both of which activate macrophages and dendritic cells (DCs). Disease could also be ameliorated by adoptive transfer of activated DCs, which are able to traffic to the lung draining lymph nodes (DLN) and prime the antivirus T cell response. These interventions effect two changes in the host immune response: they result in activation of the innate immune system and facilitate the development of a robust virus-specific T cell response. From these results, we could not determine the relative importance of these two limbs of the immune response in protection. Here, we examined whether a robust antivirus T cell response was sufficient to protect mice in the absence of interventions that activated the innate immune response. Our results indicate that virus-specific T cells are necessary and sufficient for virus clearance and for protection from clinical disease in MA15 virus-infected mice.

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MATERIALS AND METHODS

Mice, cells, and virus. Pathogen-free BALB/c, SCID (severe combined immunodeficiency; BALB/c background), and C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). RAG1$^{-/-}$ (recombination activation gene) mice were maintained in the animal care facility at the University of Iowa. All animal studies were approved by the University of Iowa Animal Care and Use Committee. African green monkey kidney-derived Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 25 mM HEPES and 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO). Mouse-adapted SARS-CoV (MA15) was a kind gift from Kanta Subbarao (NIH, Bethesda, MD) (22). Virus was passaged once on Vero E6 cells.

Peptides. A peptide library, covering all 4 structural proteins of SARS-CoV, was provided by BEI Resources (Manassas, VA). Virus-specific peptides were synthesized by BioSynthesis, Inc. (Lewisville, TX).

Virus infection and titration. Mice were lightly anesthetized with isoflurane and infected intranasally (i.n.) with 3×10^4 or 3×10^3 PFU (BALB/c and SCID mice) or 3×10^5 PFU (B6 and RAG1$^{-/-}$ mice) of MA15 virus in 50 μl of DMEM. Mice were monitored for weight loss and mortality daily. All work with MA15 virus was conducted in the University of Iowa biosafety level 3 (BSL3) Laboratory Core Facility. To obtain lungs for virus titers, animals were sacrificed at the time points postinfection (p.i.) indicated in the figures and lungs were removed into phosphate-buffered saline (PBS). Tissues were homogenized using a manual homogenizer, and titers were determined on Vero E6 cells. For plaque assays, cells were fixed with 10% formaldehyde and stained with crystal violet 3 days postinfection. Viral titers are expressed as PFU/g tissue.

Preparation of cells from lungs, DLN, and spleens. Cells were prepared from lungs, DLN, and spleens as previously described (35). Briefly, mice were euthanized with 100 μl pentobarbital (50 mg/ml; Lundbeck, Inc., Deerfield, IL) and perfused with 5 ml PBS. Lungs, DLN, and spleens were then removed. Lungs were minced and digested with 1 mg/ml collagenase D (Roche, Indianapolis, IN) and 0.1 mg/ml DNase (Roche) for 30 min at room temperature (RT). DLN and spleens were minced and pressed though a wire screen. Single cell suspensions were prepared and counted prior to use in assays.

To enhance numbers of virus-specific T cells in the lungs of BALB/c mice, alveolar macrophages were depleted by treatment with 75 μl of liposomes containing dichloromethylene bisphosphonate (clodronate) 1 day prior to infection, as described previously (35).

Flow cytometry. The following monoclonal antibodies were used for these studies: rat anti-mouse CD4 (RM4-5), rat anti-mouse CD8β (53-6.7), rat anti-mouse CD16/32 (2.4G2), rat anti-mouse interleukin-2 (IL-2) (JES6-5H4), rat anti-mouse IL-10 (JES5-16E3), rat anti-mouse IL-17A (TC11-18H10), all from BD Biosciences (San Diego, CA), and rat anti-mouse gamma interferon (IFN-γ) (XMG1.2) and rat anti-mouse tumor necrosis factor alpha (TNF-α) (MP6-XT22), both from eBioscience (San Diego, CA).

For intracellular cytokine staining (ICS), cells were cultured at 1×10^6 per 96-well plate at 37°C for 5 h or for the time periods indicated in the figures in the presence of brefeldin A (BD Biosciences). Cells were then labeled with surface antibodies, fixed and permeabilized with cytofix/cytoperm solution (BD Biosciences), and labeled with anti-IFN-γ antibody. All flow cytometry data were acquired on a BD FACSCalibur (BD Biosciences) flow cytometer and were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

***In vivo* cytotoxicity assays.** *In vivo* cytotoxicity assays were performed on day 6 after MA15 virus infection, as previously described (3, 35). Briefly, splenocytes from naive mice were costained with PKH26 (Sigma-Aldrich, St. Louis, MO) and either 1 μM or 100 nM carboxyfluorescein diacetate succinimidyl ester ([CFSE] Molecular Probes, Eugene, OR) and then pulsed with the peptides (3 μM) indicated in the figures at 37°C for 1 h. Cells (5×10^5) from each group were mixed together (1×10^6 cells in total) and transferred i.n. into mice. At 12 h after transfer, total lung cells were isolated. Target cells were identified on the basis of PKH26 staining and were distinguished from each other by differential CFSE staining. After gating on PKH26-positive cells, the percent killing was calculated as previously described (35).

Generation of SARS-CoV-specific T cell lines. DLN or spleens were removed from mice 7 to 9 days after immunization with DCs coated with S366 peptide (CD8 T cell line) or with N353 peptide in complete Freund's adjuvant (CD4 T cell line), and single cell suspensions were prepared in RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) (Atlanta Biologicals, Lawrenceville, GA). Following washing, cells (4×10^6/well) were seeded in 24-well Costar plates (Nunc, Roskilde, Denmark) supplemented with 1 μM peptides as indicated in the figures. After 5 days of culture at 37°C, cells were harvested and viable cells (4×10^5/well) recultured in the presence of peptides and recombinant human IL-2 (rhIL-2) (5 to 10 IU/ml) (NIH AIDS Research and Reference Reagent Program, Germantown, MD) in the presence of irradiated (3,000 rad) syngeneic splenocytes (4×10^6/well). T cell lines were maintained on a 10- to 14-day refeed cycle.

Generation of BMDCs and BMDC/peptide immunization. Bone marrow-derived DCs (BMDCs) were generated as previously described (35). Briefly, bone marrow cells were red blood cell depleted and plated at a density of 1×10^6/ml in X-vivo 15 medium (Lonza Walkersville, Walkersville, MD) supplemented with recombinant granulocyte-macrophage colony-stimulating factor (1,000 U/ml; BD Pharmingen) and recombinant interleukin-4 (50 U/ml; eBioscience). After 6 days, BMDCs were stimulated with 1 μg/ml lipopolysaccharide (LPS) for 18 h and further incubated with or without 1 μg/ml S366 peptide for 2 h at 37°C. CD11c$^+$ DCs were purified using CD11c microbeads and a Miltenyi autoMACS magnetic cell sorter (Miltenyi Biotec, Cologne, Germany). Purity was confirmed by flow cytometry. BALB/c mice were then injected with 5×10^5 BMDCs in 200 μl PBS intravenously (i.v.).

Statistical analysis. Student's *t* test was used to analyze differences in mean values between groups. All results are expressed as means ± standard errors of the means (SEM). *P* values of <0.05 were considered statistically significant.

RESULTS

Identification and characterization of SARS-CoV T cells by using lung-derived MA15 virus-infected BALB/c and B6 lymphocytes. Initially, we identified virus-specific CD4 and CD8 T cell epitopes recognized in three mouse strains, BALB/c, B6, and C3H. Epitopes were initially identified using a matrix-based screening strategy (6) and a panel of overlapping peptides that covered the four virus structural proteins (envelope protein [E], transmembrane protein [M], nucleocapsid protein [N], and spike glycoprotein [S]). Minimal CD8 T cell epitopes were established by alignment with well-described consensus motifs (19–21) for those epitopes not previously identified in published reports and confirmed subsequently using peptides corresponding to these minimal epitopes. Data for B6 mice are shown in Fig. 1A since this strain was used herein; epitopes recognized in BALB/c mice were reported previously (35).

We identified virus-specific cells by using intracellular IFN-γ expression assays. We identified three CD8 (S366-374 [amino acid positions 366 to 374 of the S protein], S521-529, and S1061-1071) and one CD4 (N353-370) T cell epitope in BALB/c mice (35) and seven CD8 (S436-443, S525-532, S497-504, S627-642, S641-658, N219-228, and M173-180) but no CD4 T cell epitopes in B6 mice (Fig. 1A). Some of these epitopes were previously identified (8, 12, 36), while others were not previously described. Further, some epitopes shown to be targets for SARS-CoV-specific T cells in these studies were not recognized by T cells in our assays. These differences occurred most likely because we used lung-derived T cells in our assays; it is possible that these other epitopes were present at levels insufficient to induce a detectable T cell response.

To further assess the functionality of these cells, we measured TNF, IL-2, IL-17A, and IL-10 expression. TNF is produced by most IFN-γ-positive cells during acute viral infections, while coexpression of IL-2 is indicative of optimal effector function (24). IL-17A is expressed by Th17 cells (9), while IL-10, an anti-inflammatory cytokine, is expressed in the influenza A virus-infected lung (27) and may contribute to the poor T cell activation that we observed in BALB/c mice. For these experiments, BALB/c mice were infected with a sublethal dose of virus (3×10^3 PFU). In both BALB/c and B6 mice, the majority of virus-specific CD4 and CD8 T cells dually expressed IFN-γ and TNF (shown in Fig. 1B for BALB/c CD8

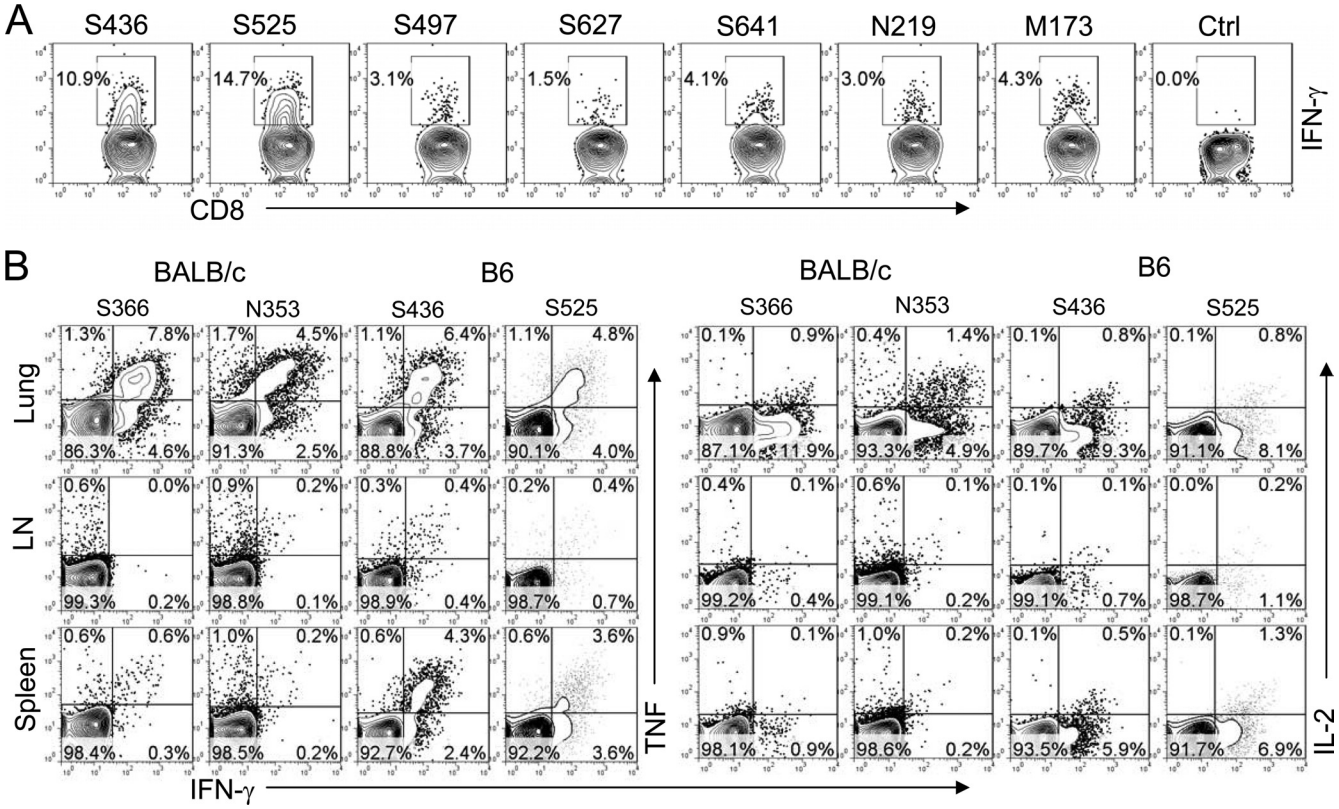

FIG. 1. T cell epitope identification and cytokine production in MA15 virus-infected BALB/c and B6 mice. (A) B6 mice (6 to 8 weeks old) were infected intranasally with 3×10^5 PFU MA15 virus in 50 μl DMEM. BALB/c mice were treated with clodronate, because in its absence, most BALB/c mice do not mount a virus-specific T cell response after infection with this virus dosage (35). At day 8, single cell suspensions were prepared from lungs and stimulated with 1 μM SARS-CoV-specific CD8 or CD4 T cell peptides for 5 h in the presence of brefeldin A. Frequencies of MA15 virus-specific T cells (determined by IFN-γ intracellular staining) are shown. Data are representative of three independent experiments, with 5 to 8 mice/group. (B) BALB/c or B6 mice (6 to 8 weeks old) were infected intranasally with 3×10^3 PFU or 3×10^5 PFU MA15 virus, respectively, in 50 μl DMEM. At day 7, single cell suspensions were prepared from lungs, LNs, and spleens and stimulated with SARS-CoV CD8 or CD4 T cell peptides for 5 h in the presence of brefeldin A. IL-2, TNF-α, and IFN-γ expression levels are shown. Data are summarized in Fig. 2A.

[S366] and CD4 [N353] and B6 CD8 [S436 and S525] T cell epitopes). A small proportion of these cells also expressed IL-2, but virtually none produced IL-10 or IL-17 (data not shown). Lastly, virus-specific CD8 T cells from both strains of mice were equivalently cytolytic, as assessed in *in vivo* cytotoxicity assays (Fig. 2B).

While there were greater total numbers of virus-specific CD8 T cells in B6 mice than in BALB/c mice, the distributions of virus-specific CD4 and CD8 T cells were also different in the two strains (Fig. 2A). Comparable numbers of virus-specific CD8 T cells were present in the lungs and spleens of BALB/c mice, whereas 10- to 15-fold more virus-specific CD8 T cells were present in the spleens than in the lungs of infected B6 mice. These differences likely reflected differences in clinical illness, i.e., a greater proportion of virus-specific T cells trafficked to the site of infection in BALB/c mice, which develop symptomatic respiratory disease.

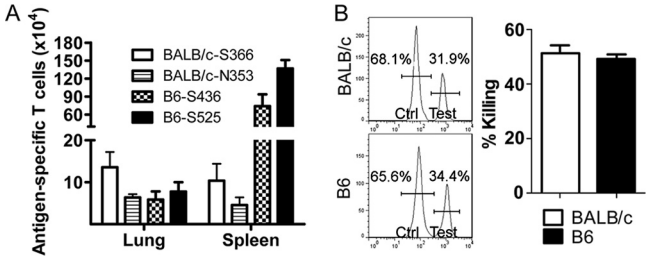

FIG. 2. Characterization of T cell response in MA15 virus-infected BALB/c and B6 mice. (A) Numbers of MA15 virus-specific T cells are shown. Data are representative of two independent experiments, with 4 mice/group. (B) *In vivo* cytotoxicity assays were performed on day 6 p.i. as described in Materials and Methods. Data are representative of three independent experiments, with 3 or 4 mice/group.

Transferred SARS-CoV-specific T cells clear virus and ameliorate clinical disease in both immunocompetent and immunocompromised mice. To directly assess the ability of virus-specific T cells to clear virus and improve clinical outcomes, we transferred cells from the spleens or lungs of infected BALB/c mice to SCID mice, which lack T and B cells, and then infected them with MA15 virus. Splenocyte transfer enhanced survival and virus clearance in these infected SCID mice (Fig. 3A and B). To examine the roles of CD4 versus CD8 T cells in this process, we purified CD4 or CD8 T cells from the lungs of lethally infected mice that were pretreated with clodronate to deplete alveolar macrophages. This treatment resulted in an increased frequency of virus-specific T cells in the lungs (35).

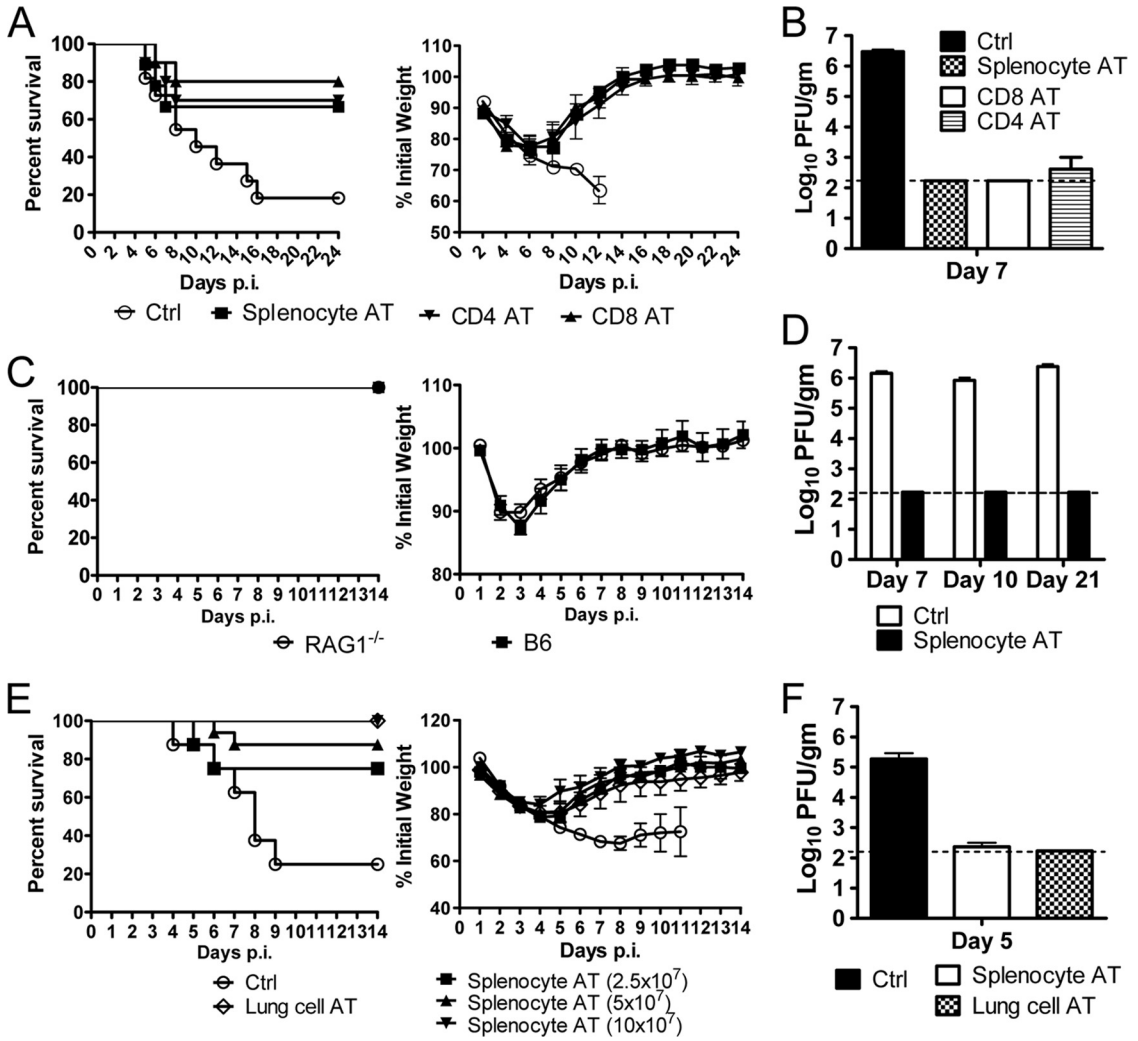

FIG. 3. SARS-CoV-specific T cell transfer cleared virus and ameliorated clinical disease in both immunocompromised and immunocompetent mice. Donor BALB/c mice were treated with clodronate 1 day prior to infection with 3×10^4 PFU MA15 virus. Donor B6 mice were infected with 3×10^5 PFU MA15. Mice were sacrificed 8 days p.i. and cells harvested from the infected lungs and spleens. Recipient SCID (A and B), B6 and RAG1$^{-/-}$ (C and D), or BALB/c (E and F) mice (6 to 8 weeks old) received 2.5×10^7, 5×10^7, or 10×10^7 splenocytes, 2×10^7 total lung cells, or 5×10^6 lung-derived CD4 or CD8 T cells at day -1 or no cells and were then infected intranasally with 3×10^4 PFU (SCID and BALB/c mice) or 3×10^5 PFU (B6 and RAG1$^{-/-}$ mice) MA15 virus in 50 μl DMEM at day 0. Mortality and weight were monitored daily. (A) Recipient SCID mice. Eleven mice were in the control group, 9 mice received 5×10^7 total splenocytes, and 10 mice received lung-derived CD4 or CD8 T cells. Survival was significantly greater in recipients of CD4 ($P < 0.05$) or CD8 ($P < 0.01$) T cell transfer and nearly significantly greater after total splenocyte transfer ($P = 0.06$). (C) B6 and RAG1$^{-/-}$ mice. Eight mice were in each group. B6 and RAG1$^{-/-}$ mice showed mild weight loss but remained asymptomatic after MA15 virus infection even in the absence of adoptively transferred cells. (E) BALB/c mice. Eight mice were in the control group; 8, 16, or 8 mice received 2.5×10^7, 5×10^7, or 10×10^7 splenocytes, respectively; and 8 mice received total lung cells. Survival was significantly greater after transfer of 5×10^7 or 10×10^7 splenocytes ($P < 0.007$ for both) or lung cells ($P < 0.007$). For virus titers, infected SCID (B), RAG1$^{-/-}$ (D), or BALB/c (F) mice received adoptively transferred cells or no cells and were sacrificed at the indicated time points. Lung homogenates were prepared, and titers were determined on Vero E6 cells. Viral titers are expressed as PFU/g tissue. There were 4 mice/group. AT, adoptive transfer. The horizontal line denotes the limit of detection.

Cells were then transferred into infected SCID mice. Lungs were used as the source for transferred cells because SARS-CoV-specific T cells in BALB/c mice were present at this site in greater numbers and higher frequency than in the spleen (Fig. 2A). As shown in Fig. 3A and B, CD4 or CD8 T cells, in the absence of the other subset, enhanced survival and resulted in accelerated kinetics of virus clearance in recipient SCID mice. Although RAG1$^{-/-}$ mice, like the parental B6 mice, remain asymptomatic after MA15 virus infection (Fig. 3C), virus is not cleared by day 9 (25). Thus, to further evaluate a role for SARS-CoV-specific T cells in virus clearance, we transferred splenocytes from SARS-CoV-immune B6 mice to infected RAG1$^{-/-}$ mice. As expected, virus titers were unchanged for up to 21 days p.i. in untreated RAG1$^{-/-}$ mice but were below the limit of detection by day 7 p.i. in mice that received transferred cells (Fig. 3D).

Finally, the protective ability of virus-specific T cells in lethally infected BALB/c mice was determined under conditions in which approximately 70% of mice succumb to the infection. For these experiments, we transferred 2.5×10^7, 5×10^7, or

10×10^7 splenocytes or 2×10^7 lung-derived lymphocytes from infected mice. Approximately 15% of spleen cells in the infected mouse are $CD8^+$ and 1% are epitope S366 specific (Fig. 1B), resulting in transfer of approximately 3.75×10^4, 7.5×10^4, or 15×10^4 epitope S366-specific CD8 T cells when mice received 2.5×10^7, 5×10^7, or 10×10^7 splenocytes, respectively. A total of 2×10^7 lung-derived cells contains approximately 20×10^4 epitope S366-specific CD8 T cells. One hundred percent of mice that received 10×10^7 splenocytes or 2×10^7 lung-derived cells survived the infection, while 75 to 90% were protected from lethal disease after transfer of lower numbers of splenocytes. Mice that received transferred lung-derived lymphocytes or splenocytes developed milder clinical disease than mice that did not receive cells (Fig. 3E). Virus titers diminished from 10^5 to undetectable after adoptive transfer of lung-derived cells (Fig. 3F). With numbers of epitope S366-specific CD8 T cells used as a surrogate marker for the total T cell response, these results suggest that transfer of 10×10^4 to 20×10^4 epitope S366-specific CD8 T cells is sufficient for protection.

Similar results were obtained when *in vitro*-cultured virus-specific CD8 T cells were transferred to infected BALB/c mice. These cells were harvested from SARS-CoV-immune BALB/c spleens and lymph nodes, stimulated with peptide S366, and propagated *in vitro* as described in Materials and Methods. Most of the CD8 T cells produced IFN-γ after stimulation with S366 peptide (Fig. 4A). Epitope S366-specific CD8 T cells were cytolytic as measured by an *in vivo* cytotoxicity assay (Fig. 4B). Transfer of these cells to infected BALB/c mice resulted in a change in survival from 20% to >90% (Fig. 4C), reduced clinical disease (Fig. 4C), and more-rapid virus clearance (Fig. 4D). We detected transferred CD8 T cells (Fig. 4E) in both the lungs and the spleens of infected mice as early as days 1 and 2 p.i., although the frequency of virus-specific cells was 20- to 30-fold higher in the lungs, the site of infection. The majority of these cells expressed IFN-γ (Fig. 4E). Remarkably, these transferred cells, by effecting virus clearance, also reversed the poor immune response detected in untreated BALB/c mice. Thus, we detected host-derived CD8 T cells that responded to epitope S366 (Fig. 4F) at day 6 p.i. at levels equivalent to those previously detected after treatment with clodronate or poly(I-C) (35). Transfer of *in vitro*-cultured virus-specific CD4 T cells also resulted in protection (8/11 survived, compared to 2/8 control mice; $P = 0.07$) (Fig. 4C). Collectively, these data show that transferred SARS-CoV-immune T cells, in the absence of activation of the innate immune response, enhanced survival and virus clearance and enabled activation of the endogenous T cell response.

Immunization with DC/peptide S366 complexes induced protective CD8 T cell responses. Immunization with DCs coated with peptide rapidly induces a potent antipeptide CD8 T cell response, with cells entering the memory T cell pool by 7 days p.i. (2). DC immunization also has the advantage of not causing an inflammatory reaction, so only the CD8 T cell limb of the immune response is activated. To determine whether DC immunization could protect BALB/c mice from lethal MA15 virus-induced disease, DC/peptide S366 complexes were prepared and administered to mice 1 week prior to infection. Control mice received no DCs or uncoated DCs. DC/peptide S366 immunization prior to MA15 virus infection resulted in the rapid ingress of peptide-specific cells into the lung, so that they comprised over 35% of the CD8 T cells by day 5 p.i. The majority of these cells expressed both IFN-γ and TNF (Fig. 5A). A significant, but lower, number of peptide S366-specific CD8 T cells was also present in the spleen (Fig. 5A and B). DC immunization resulted in nearly complete protection from MA15 virus-induced fatal disease and greatly diminished weight loss in these mice compared to results for controls (Fig. 5C). Further, the kinetics of virus clearance was more rapid in mice that received peptide S366-coated DCs than in controls (Fig. 5D).

DISCUSSION

Most previous studies of patients with SARS or animals experimentally infected with SARS-CoV have focused on the innate immune response. This approach was based on observations that humans developed severe disease during the process of virus clearance, were lymphopenic, and exhibited evidence of dysregulated cytokine and chemokine responses (4, 14, 17, 18, 32). Although one study showed that a robust anti-SARS-CoV antibody response developed in recovered patients (4), the role of the adaptive immune response in the initial host response to the infection is largely unexplored. In our previous paper, we showed that the components of the innate immune response required for an antivirus T cell response are not activated in MA15 virus-infected BALB/c mice, resulting in a virtual absence of these cells at the site of infection (35). This absence precluded studies of the role of T cells in protection and pathogenesis in these infected mice.

For a normal T cell response to a pulmonary pathogen, inhibitory alveolar macrophages and dendritic cells must be activated and DCs must migrate to DLN (10, 33). We previously showed that dendritic cells are not activated and do not migrate to DLN in the MA15 virus-infected BALB/c lung. However, depletion of inhibitory macrophages facilitated respiratory DC activation and migration, resulting in a potent anti-SARS-CoV T cell response, more-rapid virus clearance, and protection from clinical disease. Similar effects occurred if DCs were activated by poly(I-C) or CpG or if mice received adoptively transferred activated DCs (35). From these initial results, it was impossible to determine the contribution of the T cell response to this improvement in clinical disease. Here, using several approaches, we show that the virus-specific T cell response is required for virus clearance and is able to mediate protection from clinical disease, even in the absence of exogenous activation of the innate immune response. Delivery of either MA15 virus-immune splenocytes or epitope-specific *in vitro*-cultured CD8 T cells significantly improved survival (Fig. 3A and 4C). Immunization with DCs coated with peptide S366, which also protected mice (Fig. 5C), does not induce an inflammatory response (2), so the effect of this intervention should be only to prime a CD8 T cell response, with minimal activation of the innate immune response.

An effective and well-regulated innate immune response, critical for the development of a protective T cell response, is not mounted efficiently in MA15 virus-infected BALB/c mice. However, transfer of antivirus T cells reverses this process, by at least two likely mechanisms. First, the transferred cells directly augment the kinetics of virus clearance, thereby decreas-

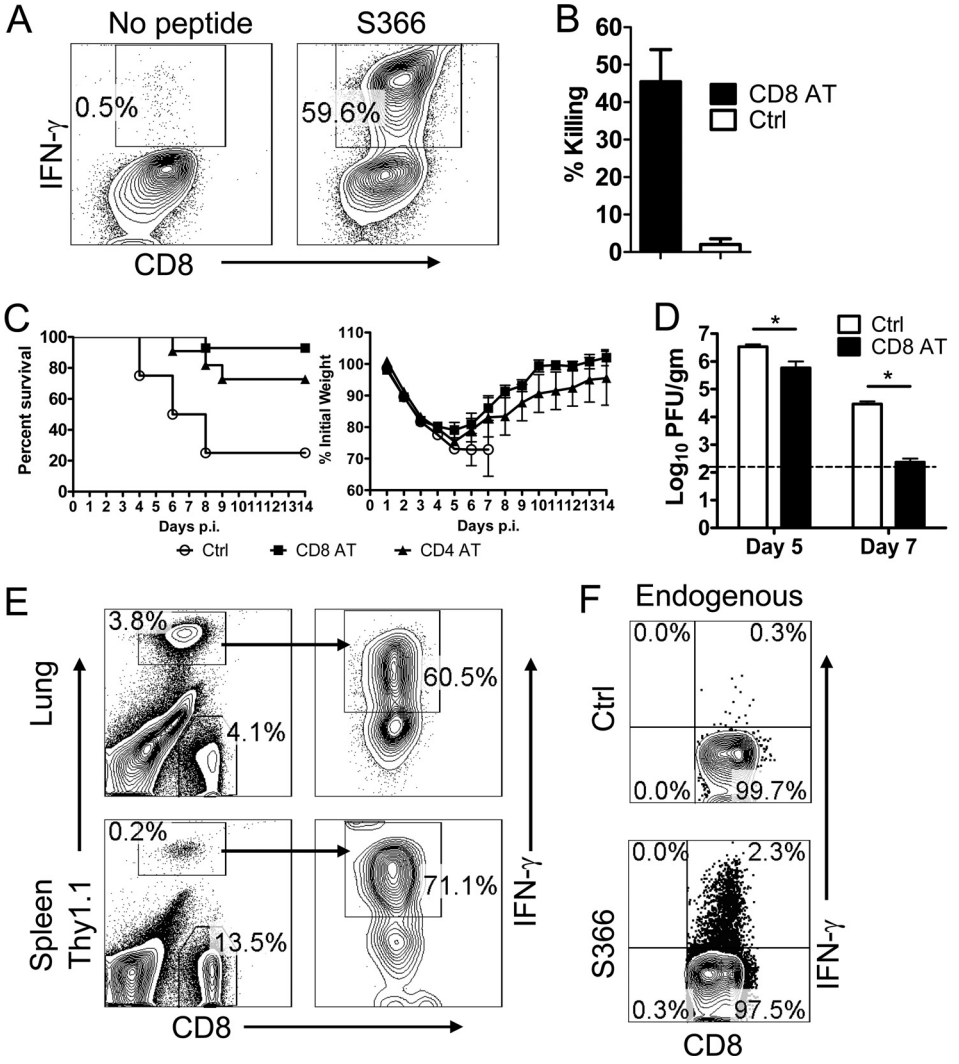

FIG. 4. Transfer of SARS-CoV-specific CD8 T cell lines decreased virus titers and ameliorated clinical disease in BALB/c mice. Epitope S366-specific CD8 or epitope N353-specific CD4 T cell lines (TCLs) were propagated *in vitro* as described in Materials and Methods. (A) TCLs were stimulated with SARS-CoV CD8 peptide for 5 h in the presence of brefeldin A and examined for expression of IFN-γ. (B) *In vivo* cytotoxicity assays were performed 1 day after transfer of CD8 T cells as described in Materials and Methods. Data are representative of two independent experiments, with 3 or 4 mice/group. (C) CD8 or CD4 T cells (1×10^7) were transferred to 6- to 8-week-old BALB/c mice 1 day prior to i.n. infection with 3×10^4 PFU MA15 virus. Mortality was monitored daily. Eight mice were in the control group, 14 mice received CD8 T cells ($P = 0.002$), and 11 mice received CD4 T cells ($P = 0.07$). (D) For virus titers, lungs were homogenized at the indicated time points and titers were determined on Vero E6 cells. Viral titers are expressed as PFU/g tissue. There were 4 mice/group. The horizontal line indicates the limit of detection. *, $P < 0.05$. (E) Single cell suspensions were prepared from the lungs and spleens of infected BALB/c mice that had received 1×10^7 CD8 T cells. Cells were stimulated with SARS-CoV-specific CD8 T cell peptide S366 for 5 h in the presence of brefeldin A. IFN-γ expression frequencies of transferred T cells are shown. (F) Endogenous CD8 T cell responses at day 6 p.i. in recipients of transferred CD8 T cells were determined by IFN-γ intracellular staining. Data are representative of two independent experiments, with 4 mice/group.

ing the stimulus for the development of a dysregulated immune response. Second, expression of cytokines such as IFN-γ, TNF, and IL-2 by the transferred cells after exposure to infected cells in the recipient animal modifies the inflammatory milieu of the lungs, countering a putative dysregulated immune response. Release of these cytokines resulted in activation of respiratory DCs and, subsequently, an enhanced endogenous T cell response (Fig. 4F). In support of this role for T cell-expressed cytokines, treatment of SARS-CoV-infected BALB/c mice with IFN-γ protected animals from lethal disease (15; our unpublished results).

However, the T cell response must act coordinately with other arms of the immune response. Thus, the T cell response may, under some circumstances, also contribute to immunopathological disease in MA15 virus-infected mice. Although most infected SCID mice die after MA15 virus infection, the time to death is greater than that for BALB/c mice (compare Fig. 3A and E). Further, the few SCID mice that die after T cell transfer die at earlier times p.i. than untreated SCID mice (Fig. 3A). A likely explanation for these results is that the suboptimal T cell response detected in lethally infected BALB/c mice or in some recipients of transferred T cells is not

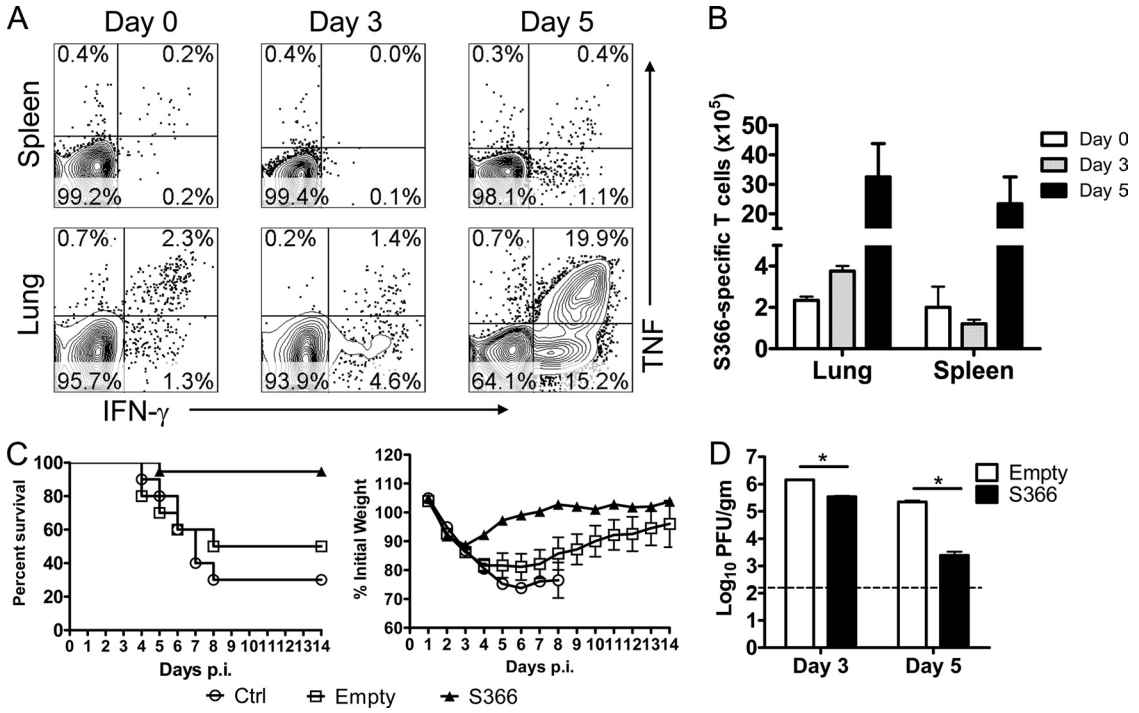

FIG. 5. Immunization with DC/peptide S366 complexes induced protective CD8 T cell responses. BALB/c mice (6 to 8 weeks old) were immunized with BMDCs pulsed with peptide S366. Seven days later, mice were infected intranasally with 3×10^4 PFU MA15 virus. Single cell suspensions were prepared from lungs and spleens at the indicated time points and stimulated with SARS-CoV S366 peptide for 5 h in the presence of brefeldin A. TNF-α and IFN-γ expression frequencies (A) and numbers (B) of SARS-CoV-specific T cells are shown. (C) Mortality was monitored daily. Ten mice were in the control group, and 10 and 19 mice were immunized with uncoated BMDCs (empty) and peptide S366-coated BMDCs, respectively. Survival was significantly greater in recipients of S366-coated BMDCs than in those receiving no (control) or empty BMDCs ($P < 0.02$). (D) For virus titers, lungs were homogenized at the indicated time points and titers were determined on Vero E6 cells. Viral titers are expressed as PFU/g tissue. There were 4 mice/group. The horizontal line denotes the limit of detection.

able to clear virus but rather causes an immunopathological disease resulting in an earlier death than that which occurs in SCID mice lacking any T cell response.

SARS has not recurred in the last 5 years, but SARS-like CoVs continue to circulate in bats and other wild animals (11, 13). Thus, recurrence of SARS or another coronavirus-mediated disease remains a possibility. When the disease was still present, vaccine development was considered a high priority and was heavily influenced by the observation that antivirus neutralizing antibodies were protective. While passively administered antibody can be used to protect small populations of exposed individuals, our results suggest that immunogens used for mass vaccination should induce a potent T cell response, both to affect virus clearance and to negate the effects of a dysregulated innate immune response.

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REFERENCES

1. **Baas, T., A. Roberts, T. H. Teal, L. Vogel, J. Chen, T. M. Tumpey, M. G. Katze, and K. Subbarao.** 2008. Genomic analysis reveals age-dependent innate immune responses to severe acute respiratory syndrome coronavirus. J. Virol. **82:**9465–9476.
2. **Badovinac, V. P., K. A. Messingham, A. Jabbari, J. S. Haring, and J. T. Harty.** 2005. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. Nat. Med. **11:**748–756.
3. **Barber, D. L., E. J. Wherry, and R. Ahmed.** 2003. Cutting edge: rapid in vivo killing by memory CD8 T cells. J. Immunol. **171:**27–31.
4. **Cameron, M. J., L. Ran, L. Xu, A. Danesh, J. F. Bermejo-Martin, C. M. Cameron, M. P. Muller, W. L. Gold, S. E. Richardson, S. M. Poutanen, B. M. Willey, M. E. DeVries, Y. Fang, C. Seneviratne, S. E. Bosinger, D. Persad, P. Wilkinson, L. D. Greller, R. Somogyi, A. Humar, S. Keshavjee, M. Louie, M. B. Loeb, J. Brunton, A. J. McGeer, S. R. N. Canadian, and D. J. Kelvin.** 2007. Interferon-mediated immunopathological events are associated with atypical innate and adaptive immune responses in patients with severe acute respiratory syndrome. J. Virol. **81:**8692–8706.
5. **Chen, J., Y. F. Lau, E. W. Lamirande, C. D. Paddock, J. H. Bartlett, S. R. Zaki, and K. Subbarao.** 2010. Cellular immune responses to severe acute respiratory syndrome coronavirus (SARS-CoV) infection in senescent BALB/c mice: CD4+ T cells are important in control of SARS-CoV infection. J. Virol. **84:**1289–1301.
6. **Hoffmeister, B., F. Kiecker, L. Tesfa, H. D. Volk, L. J. Picker, and F. Kern.** 2003. Mapping T cell epitopes by flow cytometry. Methods **29:**270–281.
7. **Hou, S., P. C. Doherty, M. Zijlstra, R. Jaenisch, and J. M. Katz.** 1992. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. J. Immunol. **149:**1319–1325.
8. **Huang, J., Y. Cao, J. Du, X. Bu, R. Ma, and C. Wu.** 2007. Priming with SARS CoV S DNA and boosting with SARS CoV S epitopes specific for CD4+ and CD8+ T cells promote cellular immune responses. Vaccine **25:**6981–6991.
9. **Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo.** 2009. IL-17 and Th17 cells. Annu. Rev. Immunol. **27:**485–517.
10. **Legge, K. L., and T. J. Braciale.** 2003. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. Immunity **18:**265–277.
11. **Li, W., Z. Shi, M. Yu, W. Ren, C. Smith, J. H. Epstein, H. Wang, G. Crameri, Z. Hu, H. Zhang, J. Zhang, J. McEachern, H. Field, P. Daszak, B. T. Eaton, S. Zhang, and L. F. Wang.** 2005. Bats are natural reservoirs of SARS-like coronaviruses. Science **310:**676–679.
12. **Lien, S. P., Y. P. Shih, H. W. Chen, J. P. Tsai, C. H. Leng, M. H. Lin, L. H. Lin, H. Y. Liu, A. H. Chou, Y. W. Chang, Y. M. Chen, P. Chong, and S. J. Liu.** 2007. Identification of synthetic vaccine candidates against SARS CoV infection. Biochem. Biophys. Res. Commun. **358:**716–721.

13. **Liu, L., Q. Fang, F. Deng, H. Wang, C. E. Yi, L. Ba, W. Yu, R. D. Lin, T. Li, Z. Hu, D. D. Ho, L. Zhang, and Z. Chen.** 2007. Natural mutations in the receptor binding domain of spike glycoprotein determine the reactivity of cross-neutralization between palm civet coronavirus and severe acute respiratory syndrome coronavirus. J. Virol. **81:**4694–4700.
14. **Mazzulli, T., G. A. Farcas, S. M. Poutanen, B. M. Willey, D. E. Low, J. Butany, S. L. Asa, and K. C. Kain.** 2004. Severe acute respiratory syndrome-associated coronavirus in lung tissue. Emerg. Infect. Dis. **10:**20–24.
15. **Nagata, N., N. Iwata, H. Hasegawa, S. Fukushi, A. Harashima, Y. Sato, M. Saijo, F. Taguchi, S. Morikawa, and T. Sata.** 2008. Mouse-passaged severe acute respiratory syndrome-associated coronavirus leads to lethal pulmonary edema and diffuse alveolar damage in adult but not young mice. Am. J. Pathol. **172:**1625–1637.
16. **Nagata, N., N. Iwata, H. Hasegawa, S. Fukushi, M. Yokoyama, A. Harashima, Y. Sato, M. Saijo, S. Morikawa, and T. Sata.** 2007. Participation of both host and virus factors in induction of severe acute respiratory syndrome (SARS) in F344 rats infected with SARS coronavirus. J. Virol. **81:**1848–1857.
17. **Peiris, J. S., C. M. Chu, V. C. Cheng, K. S. Chan, I. F. Hung, L. L. Poon, K. I. Law, B. S. Tang, T. Y. Hon, C. S. Chan, K. H. Chan, J. S. Ng, B. J. Zheng, W. L. Ng, R. W. Lai, Y. Guan, K. Y. Yuen, and HKU/UCH SARS Study Group.** 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet **361:** 1767–1772.
18. **Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. K. Yee, W. W. Yan, M. T. Cheung, V. C. Cheng, K. H. Chan, D. N. Tsang, R. W. Yung, T. K. Ng, K. Y. Yuen, and SARS Study Group.** 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet **361:** 1319–1325.
19. **Rammensee, H., J. Bachmann, N. P. Emmerich, O. A. Bachor, and S. Stevanovic.** 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics **50:**213–219.
20. **Rammensee, H. G., T. Friede, and S. Stevanoviic.** 1995. MHC ligands and peptide motifs: first listing. Immunogenetics **41:**178–228.
21. **Reche, P. A., J. P. Glutting, H. Zhang, and E. L. Reinherz.** 2004. Enhancement to the RANKPEP resource for the prediction of peptide binding to MHC molecules using profiles. Immunogenetics **56:**405–419.
22. **Roberts, A., D. Deming, C. D. Paddock, A. Cheng, B. Yount, L. Vogel, B. D. Herman, T. Sheahan, M. Heise, G. L. Genrich, S. R. Zaki, R. Baric, and K. Subbarao.** 2007. A mouse-adapted SARS-coronavirus causes disease and mortality in BALB/c mice. PLoS Pathog. **3:**e5.
23. **Rockx, B., T. Baas, G. A. Zornetzer, B. Haagmans, T. Sheahan, M. Frieman, M. D. Dyer, T. H. Teal, S. Proll, J. van den Brand, R. Baric, and M. G. Katze.** 2009. Early upregulation of acute respiratory distress syndrome-associated cytokines promotes lethal disease in an aged-mouse model of severe acute respiratory syndrome coronavirus infection. J. Virol. **83:**7062–7074.
24. **Seder, R. A., P. A. Darrah, and M. Roederer.** 2008. T-cell quality in memory and protection: implications for vaccine design. Nat. Rev. Immunol. **8:**247–258.
25. **Sheahan, T., T. E. Morrison, W. Funkhouser, S. Uematsu, S. Akira, R. S. Baric, and M. T. Heise.** 2008. MyD88 is required for protection from lethal infection with a mouse-adapted SARS-CoV. PLoS Pathog. **4:**e1000240.
26. **Smits, S. L., A. de Lang, J. M. van den Brand, L. M. Leijten, W. F. van Ijcken, M. J. Eijkemans, G. van Amerongen, T. Kuiken, A. C. Andeweg, A. D. Osterhaus, and B. L. Haagmans.** 2010. Exacerbated innate host response to SARS-CoV in aged non-human primates. PLoS Pathog. **6:**e1000756.
27. **Sun, J., R. Madan, C. L. Karp, and T. J. Braciale.** 2009. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. Nat. Med. **15:**277–284.
28. **Thepen, T., N. Van Rooijen, and G. Kraal.** 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. J. Exp. Med. **170:**499–509.
29. **Topham, D. J., R. A. Tripp, and P. C. Doherty.** 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J. Immunol. **159:** 5197–5200.
30. **Van Rooijen, N., and A. Sanders.** 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Methods **174:**83–93.
31. **Williamson, J. S., and S. A. Stohlman.** 1990. Effective clearance of mouse hepatitis virus from the central nervous system requires both CD4+ and CD8+ T cells. J. Virol. **64:**4589–4592.
32. **Wong, R. S., A. Wu, K. F. To, N. Lee, C. W. Lam, C. K. Wong, P. K. Chan, M. H. Ng, L. M. Yu, D. S. Hui, J. S. Tam, G. Cheng, and J. J. Sung.** 2003. Haematological manifestations in patients with severe acute respiratory syndrome: retrospective analysis. BMJ **326:**1358–1362.
33. **Xia, W., C. E. Pinto, and R. L. Kradin.** 1995. The antigen-presenting activities of Ia+ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. J. Exp. Med. **181:**1275–1283.
34. **Yang, Z. Y., W. P. Kong, Y. Huang, A. Roberts, B. R. Murphy, K. Subbarao, and G. J. Nabel.** 2004. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature **428:**561–564.
35. **Zhao, J., N. Van Rooijen, and S. Perlman.** 2009. Evasion by stealth: inefficient immune activation underlies poor T cell response and severe disease in SARS-CoV-infected mice. PLoS Pathog. **5:**e1000636.
36. **Zhi, Y., G. P. Kobinger, H. Jordan, K. Suchma, S. R. Weiss, H. Shen, G. Schumer, G. Gao, J. L. Boyer, R. G. Crystal, and J. M. Wilson.** 2005. Identification of murine CD8 T cell epitopes in codon-optimized SARS-associated coronavirus spike protein. Virology **335:**34–45.