



Win a \$500 cash grant to attend AAI IMMUNOLOGY2023™

Enter today ►



The Journal of
Immunology

RESEARCH ARTICLE | APRIL 01 2008

Protection against Influenza A Virus by Memory CD8 T Cells Requires Reactivation by Bone Marrow-Derived Dendritic Cells¹ **FREE**

Paola Castiglioni; ... et. al

J Immunol (2008) 180 (7): 4956–4964.

<https://doi.org/10.4049/jimmunol.180.7.4956>

Related Content

Bradykinin Stimulates IL-8 Production in Cultured Human Airway Smooth Muscle Cells: Role of Cyclooxygenase Products

J Immunol (September,1998)

Quantification of Repertoire Diversity of Influenza-Specific Epitopes with Predominant Public or Private TCR Usage

J Immunol (November,2006)

Temporal Segregation of 4-1BB Versus CD28-Mediated Costimulation: 4-1BB Ligand Influences T Cell Numbers Late in the Primary Response and Regulates the Size of the T Cell Memory Response Following Influenza Infection

J Immunol (April,2002)

Protection against Influenza A Virus by Memory CD8 T Cells Requires Reactivation by Bone Marrow-Derived Dendritic Cells¹

Paola Castiglioni,* De Shon Hall,* Erica L. Jacovetty,*[†] Elizabeth Ingulli,*[†] and Maurizio Zanetti^{2*‡}

Influenza A virus is the causative agent of an acute inflammatory disease of the airway. Although Abs can prevent infection, disease and death can be prevented by T cell-mediated immunity. Recently, we showed that protection against lethal influenza A (PR8/34) virus infection is mediated by central memory CD8 T cells (T_{CM}). In this study, using *relB*^{-/-} mice we began to investigate the role of bone marrow (BM)-derived dendritic cells (DCs) in the mechanism of protection. We found that in the absence of functional DCs, memory CD8 T cells specific for the nucleoprotein epitope (NP₃₆₆₋₃₇₄) fail to protect even after adoptive transfer into naive recipients. Through an analysis of Ag uptake, activation of memory CD8 T cells, and display of peptide/MHC complex by DCs in draining LNs and spleen early after virus infection, we established that lack of protection is associated with defective Ag presentation by BM-derived DCs and defective homing of memory T cells in the lymph nodes draining the airway tract. Collectively, the data suggest that protection against the influenza A virus requires that memory CD8 T cells be reactivated by Ag presented by BM-derived DCs in the lymph nodes draining the site of infection. They also imply that protection depends both on the characteristics of systemic adaptive immunity and on the coordinated interplay between systemic and local immunity. *The Journal of Immunology*, 2008, 180: 4956–4964.

Influenza virus infects humans by gaining entry through the airway. Vaccination with formalin-inactivated, whole virus influenza vaccines protect against infection by inducing Abs that intercept the virus at the portal of entry (1). Abs against the virus hemagglutinin Ag are involved in protection against infection, whereas Abs against the neuraminidase Ag decrease the amount of virus released from infected cells. However, because of antigenic changes in the virus, vaccines need to be reformulated periodically to compensate for antigenic shift and drift (2). In contrast, protection against disease is mediated by MHC class I-restricted CD8 T lymphocytes that eliminate virus-infected cells. CTLs are directed against peptides of the core nucleoprotein (NP)³ or the matrix (M1) protein on the inner side of the virus envelope (3–5). These Ags are more resistant to antigenic shift and drift and are generally conserved among different virus types. There exists ample epidemiological evidence that T cell immunity is important in protection against disease in humans (6, 7) and that protection is lost by antigenic drift that causes virus escape (8). However, whereas Abs protect against infection and CTLs protect against

disease, upon secondary virus challenge Abs can only neutralize viruses of the same type whereas CTLs may lyse cells infected with different virus types (9, 10).

In mice as in humans, influenza virus infection is an inflammatory disease of the airway (11). CTL responses are coordinately associated with attenuation of symptoms and protection from disease (12, 13). During natural infection the T cell response is initiated in the draining lymph nodes (LNs) of the airway tract (cervical and mediastinal) where dendritic cells (DCs) and macrophages traffic from inflamed lung tissue and present viral Ags to precursor T lymphocytes, causing their clonal expansion. This peaks at around days 7–10 (14) and then contracts while recovery from infection occurs and T cell memory is gradually generated and thereafter maintained. Memory T lymphocytes enable mice to mount a rapid and more potent response upon secondary virus challenge. The anamnestic response contains disease and prevents death. Presently, however, vaccination directed at inducing influenza virus-specific CD8 T cell responses is not an option for humans and is used experimentally to understand the pathodynamics of infection and the mechanisms of protection.

Vaccination with B lymphocytes genetically programmed to present a dominant CD8 T cell epitope of the influenza virus NP induces potent CD8-specific T cell responses in vivo (15) and provides complete protection against lethal challenge with influenza A virus (15) even if injected in low numbers (300/mouse) (16). Protection against lethal virus challenge in naive C57BL/6 mice is also obtained by the adoptive transfer of immune L-selectin (CD62L^{high}) CD8 T cells (16), suggesting that central memory CD8 T cells (T_{CM}) are the correlate of protection in this disease. This is consistent with the notion that CD8 T_{CM} cells possess greater Ag-driven proliferation potential (17) and greater ability to kill in vivo (18) compared with effector memory CD8 T cells and that T_{CM} cells are the correlate of protection in a number of diseases (19).

*The Laboratory of Immunology, Department of Medicine and Moores Cancer Center, [†]Department of Pediatrics, and [‡]Biomedical Science Program, University of California San Diego, La Jolla CA 92093

Received for publication July 6, 2007. Accepted for publication February 1, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health Grant RO1AI062894.

² Address correspondence and reprint requests to Dr. Maurizio Zanetti, University of California San Diego, 9500 Gilman Drive, Number 0815, La Jolla, CA 92093. E-mail address: mzanetti@ucsd.edu

³ Abbreviations used in this paper: NP, nucleoprotein; BM, bone marrow; BMC, BM chimera; DC, dendritic cell; EID₅₀, 50% egg infectious dose; LN, lymph node; T_{CM}, central memory T cell.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/\$2.00

Table I. Immunized *relB*^{-/-} mice are not protected against lethal challenge with A/PR8/34 influenza virus^a

	Virus Challenge i.n. (4 × 10 ⁶ PFU/ml)	Survival	Time to Death (Days)
C57BL/6 BMC	+	4/4	
<i>relB</i> BMC	+	0/7	7 ± 0.7
C57BL/6	+	4/4	10 ± 0.7

^a Mice were vaccinated with 5 × 10³ transgenic B lymphocytes expressing the NP₃₆₆ CTL epitope. Lethal challenge was given 21 days after priming.

In this study, to further understand the mechanism of protection against lethal influenza A virus infection by vaccine-induced CD8 T_{CM} cells, we undertook studies on the role of bone marrow (BM)-derived DCs. Herein, we investigated the ability of memory CD8 T cells to confer protection against lethal influenza A virus infection in DC-compromised *relB* BM chimeras (BMC). We found that lack of functional BM-derived DCs in LNs draining the airway tract abrogates protection by memory CD8 T cells. We found that defective presentation of viral Ag by DCs to CD8 T_{CM} cells in the draining LNs of the airway tract and poor homing of memory T cells have a negative effect on the protective memory T cell response. We conclude that re-presentation of Ag to memory T cells in draining LNs is a necessary event for protection against disease induced by influenza A virus to occur.

Materials and Methods

Mice and generation of BMCs

Eight- to 10-wk-old C57BL/6 (CD45.2⁺) mice were purchased from The Jackson Laboratory. Homozygous *relB*^{-/-} mice were bred in the animal facility of the University of California San Diego (La Jolla, CA). BMCs were generated by injecting i.v. 5 × 10⁶ BM cells from *relB*^{-/-} mice into lethally irradiated (1100 rad) heterozygous *relB*^{+/-} (-/- → +/-) or C57BL/6 (-/- → +/+) mice. As control, 5 × 10⁶ BM cells from C57BL/6 mice were injected into lethally irradiated C57BL/6 (+/+ → +/+) mice. Mice were used 5–6 wk after BM transfer. OVA TCR OT-I CD45.1⁺RAG^{-/-} transgenic mice were bred in the animal facility of the University of California San Diego. OVA TCR OT-II transgenic mice were a gift from Dr. M. Croft (La Jolla Institute for Allergy and Immunology, La Jolla, CA).

Plasmid DNA, proteins, and viruses

Plasmid γ 1NV²NP³ was engineered as described (20). To allow for detection of transgenic cells by FACS, the gene for enhanced GFP (EGFP) was inserted at the C terminus of the γ 1 constant region. Plasmid DNA were purified using a Qiagen MegaPrep kit (Qiagen) and stored at -20°C until use. OVA (grade VII) was purchased from Sigma-Aldrich. FITC-conjugated OVA was purchased from Molecular Probes. A/PR8/34 (A/Puerto Rico/8/34 strain) influenza virus (2 × 10⁸ PFU/ml) was a gift from Dr. A. Garcia-Sastre (Mount Sinai School of Medicine, New York, NY). A/HKx31-OVA (H3N2) influenza virus (10⁷ EID₅₀/ml; EID₅₀ is 50% egg infectious dose) and the A/HKx31 (H3N2) control influenza virus were gifts from Dr. P. G. Thomas (St. Jude Children's Research Hospital, Memphis, TN).

For protection experiments, mice were challenged intranasally (i.n.) with 50 μ l of A/PR8/34 virus at 4 × 10⁶ PFU/ml (lethal dose). Mice survival was monitored daily. Unprotected mice die between days 10 and 12. For experiments shown in Figs. 2 and 7, mice were challenged i.n. with 30 μ l of A/HKx31-OVA virus at 3.33 × 10⁶ EID₅₀/ml (sub-lethal dose). Mice were sacrificed on days 1 and 3 after virus challenge.

In vivo immunization and Ag administration

Spontaneous transgenesis was performed as described (15). Briefly, spleen cells were harvested, washed with PBS, and incubated with 25 μ g of plasmid DNA for 1 h at 37°C together with the pMACS Kk plasmid (Miltenyi Biotec) coding for a truncated mouse H-2 K^k molecule as a selectable cell surface marker. After overnight incubation, transgenic cells were magnetically sorted and analyzed by flow cytometry for enhanced GFP expression on a FACScalibur apparatus (BD Biosciences). Mice were immunized by single injection via the tail vein with a suspension of spleen lymphocytes containing 5,000 transgenic B lymphocytes (determined by FACS analysis). OVA and FITC-conjugated OVA were diluted in sterile PBS to a final concentration of 10 mg/ml. Mice were lightly anesthetized with isoflurane (Abbott Laboratories) and administered i.n. with 50 μ l of Ag solution.

Preparation of LN single cells suspension and sorting of DCs

Airway and nonairway LNs, pooled from 2–6 mice per group depending on the experiment, were harvested 24 h after the administration of FITC-OVA or 48 h after the administration OVA. LNs were digested in collagenase D (1 mg/ml; Roche Molecular Biochemicals) and DNase (0.02 mg/ml, grade II bovine pancreatic; Boehringer) for 30 min at 37°C. LNs were then minced and passed through a cell strainer. Due to the photosensitivity of the FITC material, LNs from FITC-OVA-treated mice were protected from direct light throughout the manipulation. Positive selection of DCs from LNs was done by magnetic cell sorting after incubation with anti-CD11c magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Purified DCs from FITC-OVA-administered mice were stained with biotin-conjugated mAb against mouse CD11c (clone HL3) (BD Pharmingen). After 20–30 min at 4°C, cells were washed with PBS containing 0.5% BSA and 0.05% NaN₃, and stained with CyChrome-streptavidin and PE-conjugated anti-IA^b (clone AF6–120.1). Cells were analyzed by flow cytometry on a FACScalibur apparatus (Becton Dickinson) and gates were set to select for viable DCs.

In vitro proliferation and cytokine assay

Airway and nonairway LN DCs pooled from 2–6 mice per group depending on the experiment were cultured (0–10⁵/well) with 2 × 10⁵ OVA-specific CD8 T cells from OT-I mice or with 2 × 10⁵ OVA-specific CD4 T cells from OT-II mice. OT-I and OT-II splenocytes were depleted of APCs by using a mixture of mAbs plus rabbit complement. The mAbs M5114 anti-I-A, CA4 anti-class II, RA3.6.B2 anti-B220, PK136 anti-NK, M1/70 anti-CD11b, 3.155 anti-CD8, and GK1.5 anti-CD4 were added to OT-II and OT-I splenocytes for CD8 and CD4 depletion, respectively. [³H]Thymidine was added at 1 μ Ci/well and the cells were incubated for 16–18 h at 37°C. Cells were harvested onto glass fiber filter mats using a TomTec cell harvester and the radioactivity was measured in a liquid scintillation counter (Betaplate; Wallac). Results are expressed as means ± SD of the counts per minute of triplicate cultures. Supernatants for cytokine detection were harvested after 40 h and stored at -20°C. IL-2 and IFN- γ were measured using the Opt EIA mouse set (BD Pharmingen).

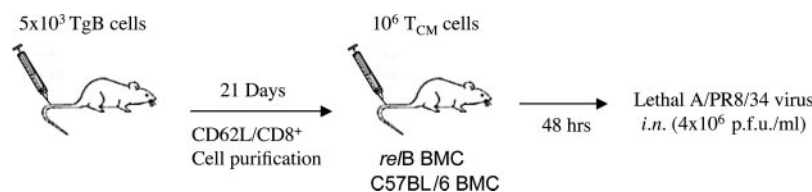


FIGURE 1. Experimental design of the adoptive transfer of memory CD62L^{high} CD8 T cells in BMC mice. Memory CD62L^{high} CD8 T cells were purified from spleens of C57BL/6 mice immunized with 5 × 10³ B lymphocytes transgenic for γ 1NV²NP³. The purity of the sorted populations was determined by FACS analysis. Purified CD62L^{high} memory CD8 T cells were transferred into naive *relB* BMC and C57BL/6 BMC (1 × 10⁶ cells/mouse). After 48 h mice were challenged with a lethal dose of A/PR8/34 virus and subsequently monitored daily for 3 wk after challenge. TgB cells, Transgenic B cells.

Table II. *The adoptive transfer of CD8 TCM (CD62L^{high}) cells fails to protect *relB*^{-/-} BMC^a*

	Transfer of CD62L ^{high} T Cells			Survival ^b
	Immune (1 × 10 ⁶ /mouse)	Naive (1 × 10 ⁶ /mouse)	Virus Challenge i.n. (4 × 10 ⁶ PFU/ml) ^a	
C57BL/6	–	–	+	0/2
C57BL/6 BMC	+	–	+	4/4
<i>relB</i> ^{-/-} BMC	+	–	+	0/6
C57BL/6	–	+	+	0/5

^a Lethal challenge was given 48 h after the adoptive transfer of 10⁶ CD62L^{high} CD8 T cells from immune or naive C57BL/6 mice.

^b Survival was recorded over a period of 3 wk.

Sorting and transfer of CD62L^{high} (T_{CM}) CD8 T cells

For protection experiments, C57BL/6 mice were immunized i.v. with 5 × 10³ B lymphocytes transgenic for plasmid γ 1NV²NP³. Three weeks later mice were sacrificed and single cell suspensions were prepared from the spleens. CD8 T cells were isolated by negative selection using a mixture of biotin-conjugated mAbs as the primary labeling reagent and anti-biotin mAbs conjugated to microbeads as the secondary labeling reagent (CD8 T cell isolation kit; Miltenyi Biotec). CD62L^{high} (T_{CM}) cells were sorted from the enriched CD8 T cell fraction using anti-CD62L magnetic beads (Miltenyi Biotec). Cells were then washed twice in PBS and 1 × 10⁶ CD62L^{high} CD8 T cells were injected i.v. into *relB* BMC, C57BL/6 BMC, and C57BL/6 recipients. Mice were lethally challenged 48 h later. As a control, CD62L^{high} CD8 T cells were sorted from C57BL/6 naive mice and injected i.v. into C57BL/6 recipients. In DC reconstitution experiments (see Table III), DCs were prepared from the BM of C57BL/6 mice as described previously (21). Briefly, cells were cultured in Iscove's complete medium supplemented with 10% FCS (HyClone), recombinant mouse GM-CSF (1000 U/ml; BD Pharmingen), and recombinant mouse IL-4 (4 ng/ml; R&D Systems). On day 7 of culture nonadherent cells were washed, resuspended at 5 × 10⁶ cells/ml, and pulsed with the NP₃₆₆ peptide (5 μ g/ml) for 1 h at 37°C. Cells were then washed and injected i.v. (5 × 10⁵ DCs per mouse) into *relB* BMC. Mice were subsequently challenged with a lethal dose of A/PR8/34 virus.

For adoptive transfer experiments, spleen and LN CD8 T cells were harvested from OT-I mice. Spleen and LN cell suspension was prepared and 10⁷ cells per mouse were injected i.v. into C57BL/6 mice (7–10 mice were injected per experiment). Twenty-four hours later mice were immunized i.p. with the OVA peptide SIINFEKL (100 μ g/mouse) together with the helper peptide TPPAYRPPNAPIL (60 μ g/mouse) of the hepatitis B virus core (HBVc) Ag in IFA (Difco). Three weeks later, mice were sacrificed and a single cell suspension was prepared from the spleens and LNs. CD8 T cells were isolated by negative selection using a CD8 T cell isolation kit (Miltenyi Biotec) and EasySep CD8 T cell enrichment kit (Stem-Cell Technologies). Enriched CD8 cells were stained using anti-CD8 (PerCP) (clone 53-6.7), anti-CD45.1 (FITC) (clone A20), anti-CD44 (PE) (clone IM7), anti-CD62L (PE) (clone MEL-14), and anti-CD25 (PE) (clone PC61) mAbs (BD Pharmingen). Enriched CD8 T cells were labeled with 10 μ M CFSE (Molecular Probes) for 10 min at room temperature. Approximately 1.5 × 10⁴ CD8/CD45.1 T_{CM} cells were injected i.v. into *relB* BMC or C57BL/6 recipients. Mice were infected 24 h later with 30 μ l of A/HKX31-OVA virus 3.33 × 10⁶ EID₅₀/ml.

Flow cytometry analysis

Isolated airway LNs were harvested on days 1 and 3 after infection. Cells were stained with anti-CD8 PerCP, anti-CD45.1 allophycocyanin (clone A20), and anti-SIINFEKL/K^b Ab 25-D1.1b. DC number and phenotype were assessed by staining with mAb anti-CD11c (clone HL3), CD86 (clone GL-1), and MHC class II (clone AF6-120.1). For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences) and stained with anti-CD8 PerCP, anti-CD45.1 allophycocyanin, and anti-IFN- γ PE (clone XMG1.2).

Immunohistochemistry

Tissue was processed as previously described (22). Briefly, LNs and spleens were removed, embedded in Tissue-Tek (Sakura Finetek), snap frozen in precooled isopentane, and stored at –80°C. Cryostat sections (10 μ m thick) were fixed in acetone for 10 min, rehydrated in PBS, and treated with avidin/biotin blocking reagent (Vector Laboratories) and anti-FcR (clone 2.4G2) to block nonspecific binding. Sections were processed in a humidified chamber using the Tyramide Signal Amplification (TSA) systems according to the manufacturer's instructions (PerkinElmer) using biotin-labeled 25-D1.16, HRP-labeled streptavidin, biotin-labeled tyramide and streptavidin-Cy3, and FITC-labeled anti-B220 (eBioscience). The slides were then washed three times in PBS and mounted in Vectashield (Vector Laboratories). Digitized images were acquired using a Nikon imaging station and processed with Photoshop Software (Adobe Systems).

Results

*Vaccinated *relB* BMCs are not protected from lethal influenza virus infection*

Homozygous *relB*^{-/-} mice have an atrophic thymic medulla, possess no LNs, and lack BM-derived DCs (23), although they possess a population of CD8 α ⁺ lymphoid DCs in the spleen (24). BMCs generated by transferring homozygous (–/–) *relB* BM cells into lethally irradiated (1100 rad) hemizygous (+/–) *relB* recipients carry the same DC defect as *relB*^{-/-} mice but have a longer life span (25). Notably, in the absence of the severe inflammation characteristic of homozygous mice, BM DCs from *relB*^{-/-}

Table III. *The adoptive transfer of CD8 TCM (CD62L^{high}) cells protects *relB*^{-/-} BMC injected with peptide-pulsed DCs^a*

	Transfer of CD62L ^{high} T cells (1 × 10 ⁶ /mouse)	DC Reconstitution ^b	Virus Challenge i.n. (4 × 10 ⁶ PFU/ml)	Survival ^c
C57BL/6 BMC	+	–	+	4/4
<i>relB</i> BMC	+	+	+	5/5
C57BL/6	+	–	+	0/4

^a Mice were adoptively transferred with 10⁶ CD62L^{high} CD8 T cells from immune C57BL/6 mice.

^b BM-derived C57BL/6 DCs pulsed with NP₃₆₆ peptide were injected 24 h after adoptive transfer of memory CD8 T cells and were allowed to park in vivo 10 days prior to virus challenge.

^c Survival was recorded over a period of 3 wk.

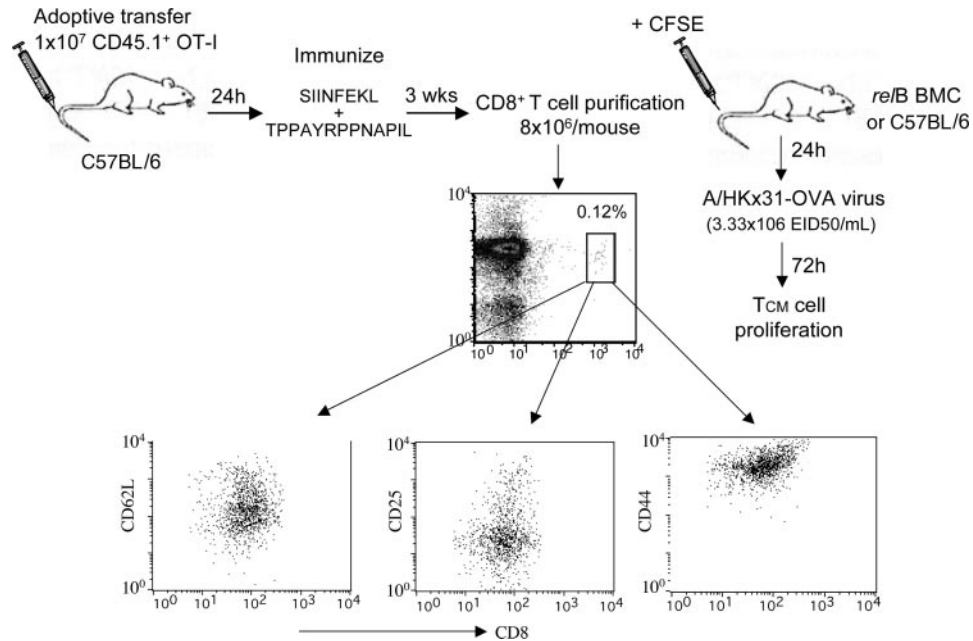


FIGURE 2. Experimental design for the generation and adoptive transfer of SIINFEKL-specific memory CD8 T cells in *re/B* BMC and C57BL/6 mice. Splens and LN cells (10^7) from OT-1 TCR transgenic CD45.1 Rag^{-/-} mice were injected i.v. into 7–10 C57BL/6 recipients and allowed to reach homeostatic distribution in different tissues compartments for 24 h. Mice were then injected i.p. with OVA_{257–264} (SIINFEKL; 100 μg/mouse) together with the hepatitis B virus core (HBVc) helper peptide in IFA. Three weeks later mice were sacrificed and a single cell suspension was prepared from the splens and LNs. CD8 T cells were isolated by negative selection, CFSE-labeled and adoptively transferred ($\sim 8 \times 10^6$ purified CD8 T cells/mouse) into *re/B* BMC and C57BL/6 mice. After negative selection and before injection, the cell suspension was analyzed and found to contain a small (0.12%) number of CD45.1⁺ OT-I CD8 T cells (*inset*). A phenotypic analysis of cells harvested on day 21 showed that these cells are CD62L^{int}CD44^{high}/CD25^{low} (where int is intermediate). Mice were then infected with 30 μl of A/HKx31-OVA virus (3.33×10^6 EID₅₀/ml). Draining LNs and/or splens were harvested on days 3 and 8 after infection.

mice are able to populate tissues when a normal radioresistant infrastructure already exists within the tissue (26). Although in *re/B*^{-/-} mice spleen DCs are unable to prime T cells (27, 28), we previously showed that *re/B* BMCs immunized with transgenic B lymphocytes as APCs prime CD4 and CD8 T cells (15). Thus, this model is ideally suited to investigate the role of BM-derived DCs in the mechanism of protection against lethal influenza A virus infection.

In this study, we asked the question as to whether memory CD8 T cells induced by immunization with transgenic B lymphocytes expressing the dominant CTL epitope of the virus nucleoprotein

(NP₃₆₆) (4) could protect *re/B* BMC from lethal virus challenge. C57BL/6 BMCs served as a control for the effects of irradiation and cell reconstitution. We found that while transgenic B lymphocyte-immunized C57BL/6 BMCs were protected, none of the *re/B* BMC or naive C56BL/6 mice used as a control survived the lethal virus challenge (Table I). Notably, time to death in *re/B* BMC mice was shorter than that in unprotected C57BL/6 mice used as controls (7 days vs 10 days). This prompted studies on the role of

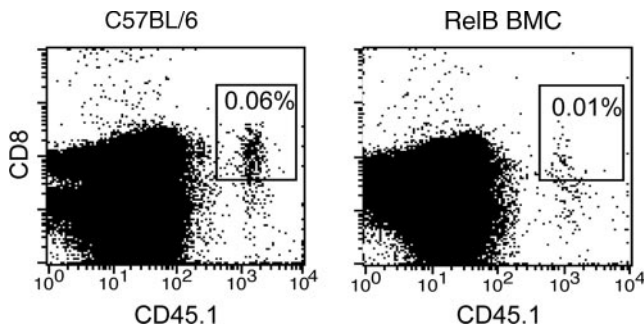


FIGURE 3. Adoptively transferred memory CD8 T cells have impaired homing to secondary lymphoid organs in *re/B* BMC. CFSE-labeled CD45.1⁺ CD8 memory T cells were adoptively transferred into *re/B* BMC and C57BL/6 mice. Mice were infected with 30 μl of A/HKx31-OVA virus (3.33×10^6 EID₅₀/ml). Draining LNs were harvested on day 3 after infection. Live cells were analyzed for CD45.1 and CD8 expression by flow cytometry. Numbers indicate the percentage of memory CD8 T cells that migrated and home the draining LNs. Data are representative of two experiments.

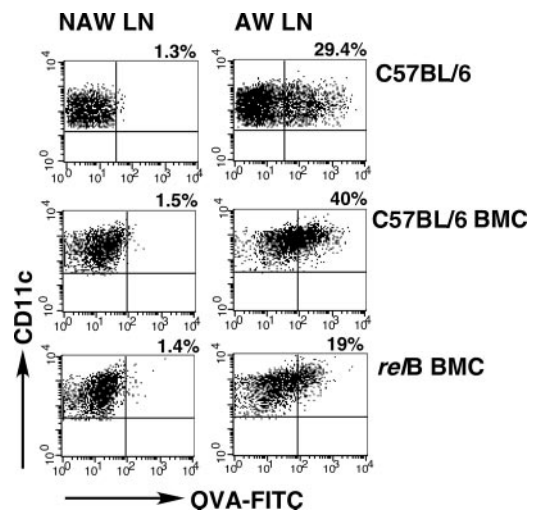
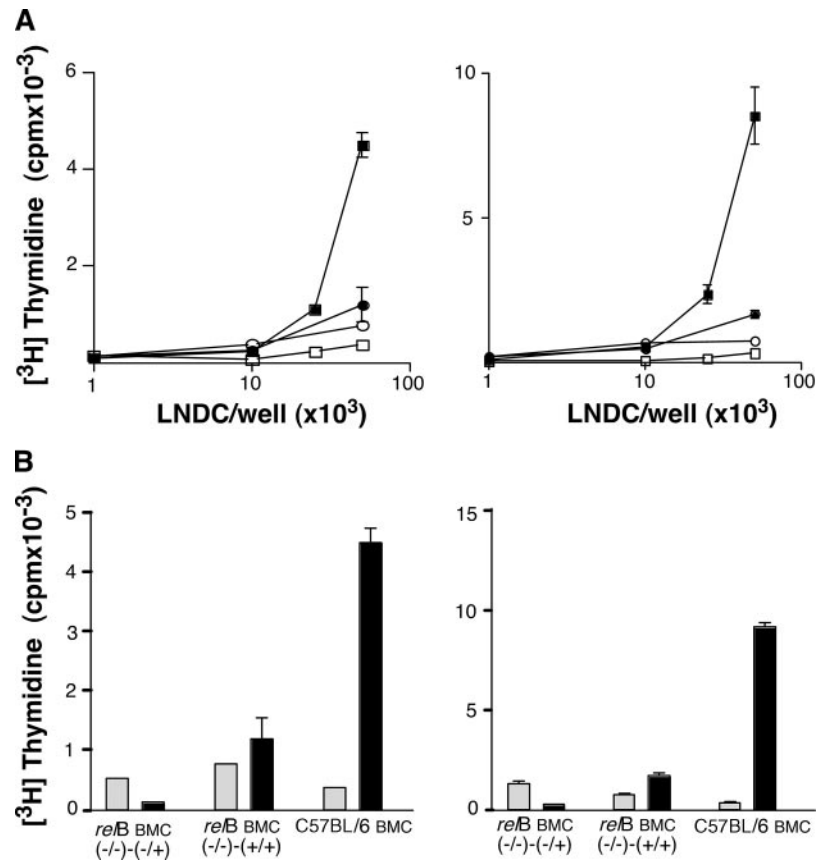


FIGURE 4. Ag uptake by residual lung DCs in *re/B* BMCs. Mice were injected i.n. with 50 μl of FITC-conjugated OVA (10 mg/ml). DCs were isolated 24 h later from pools of airway (AW) and nonairway (NAW) LNs, stained with an anti-CD11c mAb, and analyzed by flow cytometry. The percentage of FITC-positive DCs is shown in each panel.

FIGURE 5. *relB*^{-/-} DCs in LNs draining the airway tract fail to prime CD4 and CD8 T cell responses against OVA. **A**, *relB* BMC (open and filled circles) and C57BL/6 BMC (open and filled squares) were injected i.n. with 50 μ l of OVA (10 mg/ml). Forty-eight hours after injection, DC were purified from a pool of airway (filled circles and squares) and nonairway (open circles and squares) lymph nodes and cultured at different concentrations (0–10⁵ cells/well) with 2 \times 10⁵ OVA specific OT-II (left panel) or OT-I (right panel) T cells. **B**, *relB* BMC (-/- \rightarrow +/-) or (-/- \rightarrow +/+) and C57BL/6 BMC were injected i.n. with 50 μ l of OVA (10 mg/ml). Forty-eight hours after injection, DC were purified from a pool of airway (filled bars) and nonairway lymph nodes (open bars) and cultured at 5 \times 10⁴ cells/well with 2 \times 10⁵ OVA specific OT-II (left panel) or OT-I (right panel) T cells. [³H]Thymidine incorporation was measured after 72 h. Results are expressed as means \pm SD of counts per minute of triplicate cultures.



BM-derived DCs in protection mediated by memory CD8 T cells against the influenza A virus.

Adoptively transferred immune CD62L^{high} CD8 T cells fail to protect *relB* BMCs

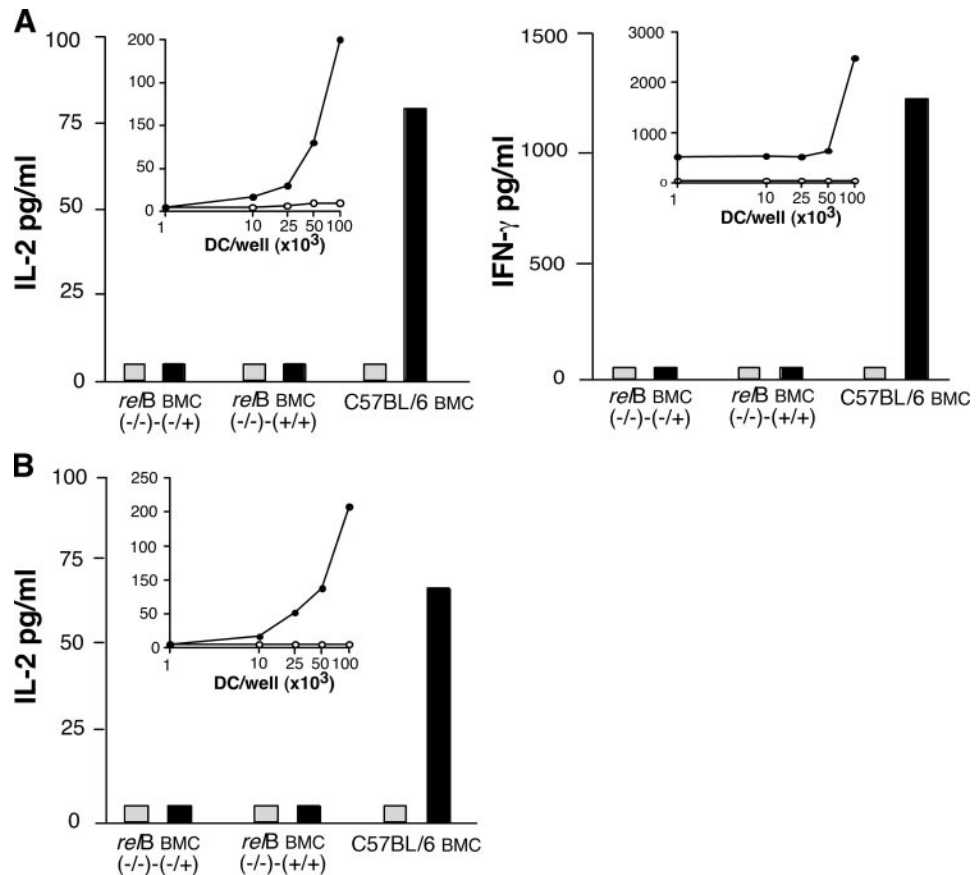
Previously, we showed that in this murine model of infection CD8 T_{CM} cells are the correlate of protection from disease and death (16). We also demonstrated that CD4 and CD8 T cell responses can be induced in *relB* BMCs by vaccination with Ag-presenting transgenic B lymphocytes (15). Therefore, we reasoned that lack of protection in immunized *relB* BMCs could result from a defective reactivation of memory CD8 T cells in the LNs draining the lung rather than an impaired T cell response. To test this hypothesis, CD62L^{high} CD8 memory T cells from immune C57BL/6 mice were adoptively transferred in *relB* BMCs or in C57BL/6 BMCs as a control (Fig. 1). Consistent with our previous data (16), C57BL/6 BMCs given immune CD62L^{high} CD8 T cells were fully protected (4/4) whereas none (0/6) of the *relB* BMC survived the virus challenge (Table II). To verify that protection was mediated only by adoptively transferred memory T cells and not by naive T cells cotransferred within the memory T cell pool, CD62L^{high} CD8 T cells from naive mice were injected into C57BL/6 recipients. After lethal virus challenge, none of the mice that had received CD62L^{high} CD8 naive T cells were protected (Table II). This is consistent with data from Cerwenka et al. (29) who showed that the transfer of as many as 10⁷ naive hemagglutinin TCR Tg CD8 T cells failed to protect mice. Furthermore, it raised the possibility that adoptively transferred memory CD8 T cells failed to protect due to inadequate reactivation by *relB*^{-/-} DCs in the draining LNs. Finally, to corroborate the idea that protection requires representation of Ag by BM-derived DCs, we sought to rescue the inability of *relB* BMCs to resist lethal virus challenge upon adop-

tive transfer of T_{CM} cells by injecting 5 \times 10⁵ C57BL/6 BM-derived DCs pulsed with the NP₃₆₆ peptide before virus challenge. As indicated in Table III, *relB* BMCs given both T_{CM} cells and NP₃₆₆ peptide-pulsed competent DCs were protected from lethal A/PR8/34 virus challenge.

Impaired homing of memory CD8 T cells in the draining LNs of *relB* BMCs

In light of the above results we decided to test the possibility that the reactivation of memory CD8 T cells by DCs in the draining LNs is necessary for protection. To test this hypothesis, new experiments were designed in which memory CD8 T cells generated in DC-competent mice were adoptively transferred into *relB* BMC. To enhance the resolution of the experiment, we used CD8 T cells TCR transgenic for a MHC class I-restricted epitope (SIINFEKL) of OVA and the recombinant A/HKx31-OVA influenza virus that codes for OVA (30) so that MHC-restricted presentation of SIINFEKL could be easily monitored in the context of viral infection. The experimental design is depicted in Fig. 2. A total of 8 \times 10⁶ CD8 T cells that contained \sim 9.6 \times 10⁴ memory OT-I cells (0.12%; Fig. 2 inset) as estimated from a phenotypic analysis of CD45.1⁺ cells (Fig. 2 inset) were CFSE-labeled and immediately injected into three *relB* BMC or three C57BL/6 recipients (for a total of 1.5 \times 10⁴ memory T cells per mouse). The experiment was performed twice. Twenty-four hours after adoptive transfer, mice were infected i.n. with a sublethal dose (3.3 \times 10⁶ EID₅₀/ml) of A/HKx31-OVA influenza virus. Three days after virus challenge mice were sacrificed and the draining LNs were removed and analyzed. Fig. 3 shows that adoptively transferred memory OT-I cells accumulated within the draining LNs of C57BL/6 mice where they represented 0.06% of the total LN population.

FIGURE 6. *relB*^{-/-} DCs in LNs draining the airway tract fail to prime CD4 and CD8 T cell responses against OVA. **A**, *relB* BMC (-/- → +/-) or (-/- → +/+) and C57BL/6 BMC were injected i.n. with 50 μl of OVA (10 mg/ml). Forty-eight hours after injection, DC were purified from a pool of airway (filled bars) and non-airway lymph nodes (open bars) and cultured at 5 × 10⁴ cells/well with 2 × 10⁵ OVA-specific CD8 T cells. The supernatants for IL-2 and IFN-γ detection were collected after 40 h. **B**, *relB*^{-/-} BMC (-/- → +/-) or (-/- → +/+) and C57BL/6 BMCs were injected i.n. with 50 μl of OVA (10 mg/ml). Forty-eight hours after injection, DC were purified from a pool of airway (filled bars) and non-airway (open bars) LNs and cultured at 5 × 10⁴ cells/well with 2 × 10⁵ OVA-specific CD4 T cells. The supernatants for IL-2 detection were collected after 40 h. The insets refer to cytokine production in cultures where OVA-specific T cells were seeded with different concentrations (0–10⁵ cells/well) of airway LN DCs from *relB* BMC (open circle) or C57BL/6 BMC (filled circle).



However, the percentage of memory OT-I cells detected in *relB* BMC was substantially lowered (0.01%), indicating a limited migration and homing to the regional LNs (Fig. 3). These results raised the possibility that the homing of memory CD8 T cells may play a role in protection against a lethal influenza A virus infection.

Defective Ag uptake by airway *relB*^{-/-} DCs

To gain further insights, we probed DCs lining the airway mucosa in *relB* BMC for their ability to capture Ag and migrate to draining LNs by using soluble OVA as the Ag (28). Briefly, OVA-FITC was administered (500 μg/mouse) to *relB* BMC, C57BL/6 BMC, or C57BL/6 mice by inhalation, hence mimicking the natural route of infection by the influenza virus. Draining LNs were harvested 24 h later and the percentage of CD11c⁺FITC⁺ cells was assessed by FACS analysis. Fig. 4 shows that whereas OVA-FITC uptake in C57BL/6 or C57BL/6 BMC varied between 30 and 40%, the percentage of CD11c⁺ FITC⁺ cells in *relB* BMC was lower (19%). This cannot be explained on the basis of defective repopulation, because the average number of CD11c⁺ cells in the airway LN pools was ~80,000 for *relB* BMC (*n* = 12), ~140,000 for C57BL/6 BMC (*n* = 10), and ~93,000 for C57BL/6 mice (*n* = 7), suggesting no obvious repopulation defect in *relB* BMC, a finding in agreement with Crowley and Lo (26).

Defective Ag presentation by *relB*^{-/-} DCs in airway-draining LNs after inhalation of soluble Ag

Next, we probed the ability of DCs in LNs draining the airway tract of *relB* BMC to process and present OVA. OVA was administered (500 μg/mouse) by inhalation and the draining LNs were harvested 48 h later. In this experimental design, we allowed Ag

uptake and Ag processing to occur in vivo. After 48 h we assessed the priming of OVA-specific CD8 (OT-I) and CD4 (OT-II) T lymphocytes in vitro. Fig. 5A shows that only DCs isolated from the draining LNs of C57BL/6 BMCs induced a specific proliferation of OT-I (*right panel*) or OT-II (*left panel*) lymphocytes. In contrast, upon stimulation with DCs isolated from the draining LNs of *relB* BMCs no proliferation occurred in either OT-I or OT-II lymphocytes, irrespective of the DC:T cell ratio studied.

We reasoned that lack of T cell activation could be due to an intrinsic defect of DC function in the LNs of *relB* BMC. To examine this issue, studies were repeated using in addition *relB* BMCs generated by transferring homozygous *relB*^{-/-} BM cells into irradiated C57BL/6 recipients (-/- → +/+), considering that in these mice the defect intrinsic to *relB*^{-/-} DCs would be the only defect present and that abnormalities in the stromal architecture of the LNs would be minimal. Fig. 5B shows that neither activation of OT-I (*left panel*) nor that of OT-II (*right panel*) lymphocytes was rescued in *relB* (-/-) → +/+ BMCs. These results suggest that the LN environment does not contribute in a demonstrable way to the functional defect of *relB*^{-/-} DCs in the draining LNs of the lung. To corroborate these findings, we measured the production of cytokines in the supernatant of T cell cultures seeded with DCs isolated from the draining LNs. Fig. 6A shows that only OT-I lymphocytes seeded with DCs isolated from the draining LNs of C57BL/6 BMCs produced IL-2 (*left panel*) and IFN-γ (*right panel*). Similarly, Fig. 6B shows IL-2 production only in cultures of OT-II lymphocytes seeded with DCs isolated from the draining LNs of C57BL/6 BMC. Taken together, these results demonstrate that *relB*^{-/-} DCs in the draining LNs are intrinsically defective in Ag presentation to T cells.

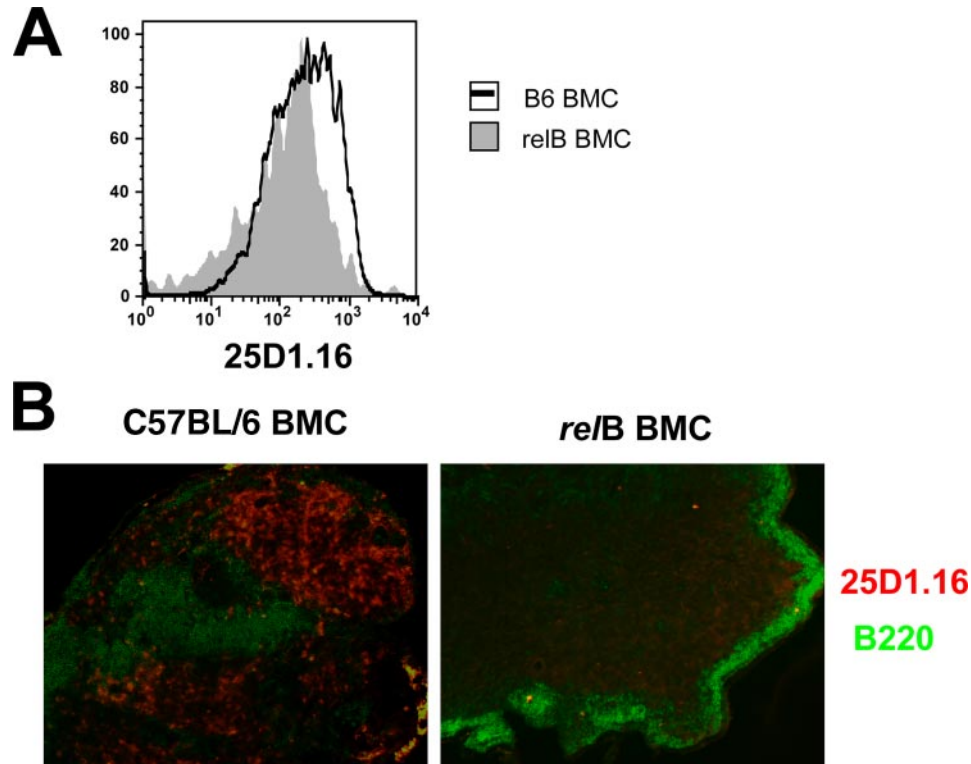


FIGURE 7. Ag presentation by DCs in LNs draining the airway tract. Memory CD8 TCR transgenic OT-I cells were adoptively transferred into C57BL/6 mice or *reIB* BMCs and mice were challenged 24 h later with a sublethal dose of A/HKx31-OVA influenza virus. Twenty-four hours after infection, draining LNs and spleens were removed, digested, and stained for DCs (CD11c⁺) and presentation of SIINFEKL/K^b (clone 25.D-1.16). *A*, CD11c⁺ cells presenting the SIINFEKL peptide complex with the K^b molecule are shown. Samples from C57BL/6 mice that were left uninfected or infected with A/HKx31 virus were used as negative controls (data not shown). *B*, Immunofluorescence analysis on frozen tissue sections from airway-draining LN sections were performed using biotin-labeled 25-D-1.16 followed by sequential incubation in HRP-labeled streptavidin, biotin-labeled tyramide, and streptavidin-Cy3. Costaining with FITC-conjugated anti-B220 Ab was used to identify B cell areas. Digitized images were acquired using a Nikon imaging station and processed using Photoshop software.

*Defective Ag presentation by *reIB*^{-/-} DCs in airway draining LNs after virus infection*

Based on the above results, it became important to monitor Ag presentation by *reIB*^{-/-} DCs in LNs draining the airway tract after intranasal virus challenge because the inflammatory component of virus infection could not be mimicked by inhalation of OVA-FITC. Briefly, using an experimental design similar to that shown in Fig. 2, C57BL/6 mice and *reIB* BMC were challenged i.n. with a sublethal (3.3×10^6 EID₅₀/ml) dose of A/HKx31-OVA influenza virus. DCs were isolated from the draining LNs 24 h after virus infection and stained using mAb 25.D-1.16, which recognizes the SIINFEKL peptide complexed with the K^b molecule (31). FACS analysis showed marked Ag presentation in DCs from C57BL/6 BMCs but not from *reIB* BMCs (Fig. 7*A*). We also sought SIINFEKL/K^b complex staining in frozen sections of LNs harvested 24 h after virus infection (Fig. 7*B*). In the draining LNs of C57BL/6 mice, Ab 25.D-1.16 produced a diffuse and intense staining in the T cell area (red), suggesting active Ag presentation by BM-derived DCs. Minimal SIINFEKL/K^b complex staining was observed in LN sections of *reIB* BMC (Fig. 7*B*).

Discussion

In this study we show that protection against lethal influenza A virus infection by memory CD8 T cells requires that DCs in the LNs draining the site of infection re-present viral Ag to memory CD8 T cells. Because lack of the *reIB* gene severely impairs the Ag-presenting function of BM-derived DCs (28), our findings

suggest that DCs play a key role in the mechanism of protection against influenza A virus infection. Upon infection, the path to presentation of viral Ags can be divided into two steps: 1) Ag uptake by DCs; and 2) Ag processing and presentation to T cells in the T cell zone. Our data suggest that *reIB*^{-/-} DCs in the LNs draining the airway tract are defective at both levels. This implies that protective memory CD8 T cells require reactivation in the LNs draining the airway tract by BM-derived DCs.

The key finding of this study is that memory CD8 T cells fail to protect if the Ag-presenting function of DCs in the draining LNs of the airway is impaired. Several lines of evidence support this conclusion. First, lung DCs isolated from the draining LNs of *reIB* BMCs, unlike DCs from C57BL/6 BMC mice, showed a markedly decreased uptake of soluble Ag (Fig. 4). Second, Ag presentation by *reIB* DCs in the draining LNs harvested within 24 h after virus infection was reduced compared with DCs from C57BL/6 mice. This was true whether DCs were analyzed as cell suspensions or as frozen tissue by direct immunofluorescence (Fig. 7, *A* and *B*). Third, *reIB*^{-/-} DCs in the draining LNs of the airway tract failed to prime CD8 or CD4 T cells in vitro (Fig. 5, *A* and *B*), a finding consistent with a previous report from this laboratory that residual splenic DCs in *reIB* BMC are unable to prime CD4 T cells against soluble Ag whether Ag uptake occurs in vivo or in vitro (28). Fourth, analysis of the LNs draining the airway tract showed poor homing of memory CD8 T cells in *reIB* BMCs and, hence, insufficient reactivation of memory CD8 T cells compared with C57BL/6 mice (Fig. 3). Of note, reconstitution experiments with C57BL/6 BM-derived DCs pulsed with the NP₃₆₆ peptide

restored protection in *relB* BMC (Table III). Collectively, the present data point to a critical role by BM-derived DCs in reactivating memory CD8 T cells in protection against lethal virus challenge. Because an abnormal stromal environment postirradiation and reconstitution did not apparently play a role (Fig. 5B), a tempting hypothesis would then be that the defect of *relB*^{-/-} DCs is linked to a defect in signaling through the lymphotoxin- β receptor, which was found to regulate the homeostasis of myeloid DCs (32).

The present results stress the importance of local immunity in protection against influenza A virus. Failure of adoptively transferred CD8 T_{CM} cells to protect *relB*^{-/-} BMCs clearly suggests that vaccine-induced, T cell-mediated protection against disease rests on at least two prerequisites. One is the induction of systemic T cell immunity with the generation of CD8 T_{CM} cells (19). The other is the necessity for memory T cells to undergo Ag reactivation in the LNs draining the site of infection. Previous studies established that memory CD8 T cells localize to the lung (33) and mediate protection by accelerating the clearance of the influenza virus in the lung (34). Zammit et al. (35) demonstrated that the interaction with DCs is a major mechanism for reactivation of memory CD8 T cells in airway LNs following influenza virus infection. Similarly, Belz et al. (36) showed that T cell memory responses against influenza virus depend on BM-derived DCs for expansion in the spleen and in LNs 10 days after viral challenge. Interestingly, presentation of viral Ag in the draining LNs is also required for the maintenance of virus-specific CD8 T cells (37). Similarly, our data suggest that reactivation of memory CD8 T cells by BM-derived DCs in the draining LNs is critically necessary for protection against lethal influenza virus infection. Due to the fact that activation of memory CD8 T cells may require a longer time than one would anticipate (36) and that lethality by the virus occurs within 7–10 days, we suggest that the DC insufficiency in *relB* BMCs renders the host unable to take advantage of protective memory T cells. Notwithstanding this interpretation, we cannot rule out the possibility that *relB* BMCs may also have a defect in innate immunity that is known to be at play against influenza A virus infection (38). For instance, innate immunity could aid in the early control of viral load and this would facilitate the antiviral effect of memory CTL.

Current vaccines against influenza A virus infection induce Abs that block infection by intercepting the virus at the portal of entry but are subject to antigenic shift and antigenic drift. Vaccines that induce CD8 T cell responses against conserved viral Ags are candidates for protection against disease. Based on this study and recently published work (33–37), vaccine-induced, T cell-mediated protection against influenza A virus requires the induction of protective memory CD8 T cells (16) and their migration to the draining LNs to be sampled and reactivated by virus laden DCs (39). In this sequential program, qualitative events (i.e., the preferential induction of CD8 T_{CM} cells) and geographical considerations (i.e., migration to draining LNs and local restimulation by DCs) need to occur coordinately. Our analysis in vivo using mice with defective DC function places emphasis on the interplay between these various phases and suggests that the characteristics of both systemic and local immunity play an important role. This understanding may facilitate the design of T cell protective vaccines against influenza A virus infection.

Acknowledgments

We thank Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine, New York, NY) for the gift of the A/PR8/34 virus and Dr. Paul Thomas (St. Jude Children's Research Hospital, Memphis, TN) for the gift of the A/HKx31 and A/HKx31-OVA viruses.

Disclosures

The authors have no financial conflict of interest.

References

- Salk, J., and D. Salk. 1977. Control of influenza and poliomyelitis with killed virus vaccines. *Science* 195: 834–847.
- World Health Organization. 2002. Influenza vaccines. World Health Organization position paper. *Weekly Epidemiological Record* 77: 230–238.
- Townsend, A. R. M., F. M. Gotch, and J. Davey. 1985. Cytotoxic T cells recognize fragments of influenza nucleoprotein. *Cell* 42: 457–467.
- Bastin, J., J. Rothbard, J. Davey, I. Jones, and A. Townsend. 1987. Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I-restricted cytotoxic T lymphocytes. *J. Exp. Med.* 165: 1508–1523.
- Bednarek, M. A., S. Y. Sauma, M. C. Gammon, G. Porter, S. Tamhankar, A. R. Williamson, and H. J. Zweerink. 1991. The minimum peptide epitope from the influenza virus matrix protein: extra and intracellular loading of HLA-A2. *J. Immunol.* 147: 4047–4053.
- Rimmelzwaan, G. F., and A. D. Osterhaus. 1995. Cytotoxic T lymphocyte memory: role in cross-protective immunity against influenza? *Vaccine* 13: 703–705.
- Nguyen, H. H., Z. Moldoveanu, M. J. Novak, F. W. van Ginkel, E. Ban, H. Kiyono, J. R. McGhee, and J. Mestecky. 1999. Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8⁺ cytotoxic T lymphocyte responses induced in mucosa-associated tissues. *Virology* 254: 50–60.
- Gog, J. R., G. F. Rimmelzwaan, A. D. Osterhaus, and B. T. Grenfell. 2003. Population dynamics of rapid fixation in cytotoxic T lymphocyte escape mutants of influenza A. *Proc. Natl. Acad. Sci. USA* 100: 11143–11147.
- Ada, G. L., and P. D. Jones. 1986. The immune response to influenza infection. *Curr. Top. Microbiol. Immunol.* 128: 1–54.
- Thomas, P. G., R. Keating, D. J. Hulse-Post, and P. C. Doherty. 2006. Cell-mediated protection in influenza infection. *Emerg. Infect. Dis.* 12: 48–54.
- Allan, W., Z. Tabi, A. Cleary, and P. C. Doherty. 1990. Cellular events in the lymph node and lung of mice with influenza: consequences of depleting CD4⁺ T cells. *J. Immunol.* 144: 3980–3986.
- Lukacher, A. E., V. L. Braciale, and T. J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* 160: 814–826.
- Epstein, S. L., C. Y. Lo, J. A. Misplon, and J. R. Bennink. 1998. Mechanism of protective immunity against influenza virus infection in mice without antibodies. *J. Immunol.* 160: 322–327.
- Doherty, P. C., and J. P. Christensen. 2000. Accessing complexity: the dynamics of virus-specific T cell responses. *Annu. Rev. Immunol.* 18: 561–592.
- Gerloni, M., M. Rizzi, P. Castiglioni, and M. Zanetti. 2004. T cell immunity using transgenic B lymphocytes. *Proc. Natl. Acad. Sci. USA* 101: 3892–3897.
- Castiglioni, P., M. Gerloni, and M. Zanetti. 2004. Genetically programmed B lymphocytes are highly efficient in inducing anti-virus protective immunity by central memory CD8 T cells. *Vaccine* 23: 699–708.
- Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4: 225–234.
- 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.
- Zanetti, M., and G. Franchini. 2006. T cell memory and protective immunity by vaccination: is more better? *Trends Immunol.* 27: 511–517.
- Billetta, R., G. Filaci, and M. Zanetti. 1995. Major histocompatibility complex class I-restricted presentation of influenza virus nucleoprotein peptide by B lymphoma cells harboring an antibody gene antigenized with the virus peptide. *Eur. J. Immunol.* 25: 776–783.
- Castiglioni, P., A. Martin-Fontecha, G. Milan, V. Tomajer, F. Magni, J. Michaelsson, C. Rugarli, A. Rosato, and M. Bellone. 2002. Apoptosis-dependent subversion of the T-lymphocyte epitope hierarchy in lymphoma cells. *Cancer Res.* 62: 1116–1122.
- Filatenkov, A. A., E. L. Jacovetty, U. B. Fischer, J. M. Curtsinger, M. F. Mescher, and E. Ingulli. 2005. CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. *J. Immunol.* 174: 6909–6917.
- Lo, D., H. Quill, L. Burkly, B. Scott, R. D. Palmiter, and R. L. Brinster. 1992. A recessive defect in lymphocyte or granulocyte function caused by an integrated transgene. *Am. J. Pathol.* 141: 1237–1246.
- Wu, L., A. D'Amico, K. D. Winkel, M. Suter, D. Lo, and K. Shortman. 1998. RelB is essential for the development of myeloid-related CD8 α ⁻ dendritic cells but not of lymphoid-related CD8 α ⁺ dendritic cells. *Immunity* 9: 839–847.
- Burkly, L., C. Hession, L. Ogata, C. Reilly, L. A. Marconi, D. Olson, R. Tizard, R. Cate, and D. Lo. 1995. Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* 373: 531–536.
- Crowley, M. T., and D. Lo. 1998. *Targeted Gene Knockouts: Insights into Dendritic Cell Biology*. Academic Press, London.
- Castiglioni, P., E. M. Janssen, K. R. Prilliman, M. Gerloni, S. Schoenberger, and M. Zanetti. 2002. Cross-priming is under control of the relB gene. *Scand. J. Immunol.* 56: 219–223.
- Castiglioni, P., C. Lu, D. Lo, M. Croft, P. Langlade-Demoyen, M. Zanetti, and M. Gerloni. 2003. CD4 T cell priming in dendritic cell-deficient mice. *Int. Immunol.* 15: 127–136.
- Cerwenka, A., T. M. Morgan, and R. W. Dutton. 1999. Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection:

- homing properties rather than initial frequencies are crucial. *J. Immunol.* 163: 5535–5543.
30. Jenkins, M. R., R. Webby, P. C. Doherty, and S. J. Turner. 2006. Addition of a prominent epitope affects influenza A virus-specific CD8⁺ T cell immunodominance hierarchies when antigen is limiting. *J. Immunol.* 177: 2917–2925.
 31. Porgador, A., J. W. Yewdell, Y. Deng, J. R. Bennink, and R. N. Germain. 1997. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* 6: 715–726.
 32. Kabashima, K., T. A. Banks, K. M. Ansel, T. T. Lu, C. F. Ware, and J. G. Cyster. 2005. Intrinsic lymphotoxin- β receptor requirement for homeostasis of lymphoid tissue dendritic cells. *Immunity* 22: 439–450.
 33. Topham, D. J., M. R. Castrucci, F. S. Wingo, G. T. Belz, and P. C. Doherty. 2001. The role of antigen in the localization of naive, acutely activated, and memory CD8⁺ T cells to the lung during influenza pneumonia. *J. Immunol.* 167: 6983–6990.
 34. Christensen, J. P., P. C. Doherty, K. C. Branum, and J. M. Riberdy. 2000. Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8⁺ T-cell memory. *J. Virol.* 74: 11690–11696.
 35. Zammit, D. J., L. S. Cauley, Q. M. Pham, and L. Lefrancois. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity* 22: 561–570.
 36. Belz, G. T., N. S. Wilson, C. M. Smith, A. M. Mount, F. R. Carbone, and W. R. Heath. 2006. Bone marrow-derived cells expand memory CD8⁺ T cells in response to viral infections of the lung and skin. *Eur. J. Immunol.* 36: 327–335.
 37. Zammit, D. J., D. L. Turner, K. D. Klonowski, L. Lefrancois, and L. S. Cauley. 2006. Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity* 24: 439–449.
 38. Tamura, S., and T. Kurata. 2004. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Jpn. J. Infect. Dis.* 57: 236–247.
 39. Oh, S., G. T. Belz, and M. C. Eichelberger. 2001. Viral neuraminidase treatment of dendritic cells enhances antigen-specific CD8⁺ T cell proliferation, but does not account for the CD4⁺ T cell independence of the CD8⁺ T cell response during influenza virus infection. *Virology* 286: 403–411.