Endogenous Matrix Metalloproteinases 2 and 9 Regulate Activation of CD4⁺ and CD8⁺ T cells

Heather L. Benson¹, Shahriar Mobashery², Mayland Chang², Farrah Kheradmand³, Jeong Soo Hong³, Gerald N. Smith⁴, Rebecca A. Shilling⁵, and David S. Wilkes⁶

¹Department of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana; ²Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana; ³Department of Medicine–Pulmonary and Critical Care, Baylor College of Medicine, Houston Texas; ⁴Department of Medicine–Rheumatology, Indiana University School of Medicine, Indianapolis, Indiana; ⁵Section of Pulmonary and Critical Care Medicine, Department of Medicine and Committee on Immunology, University of Chicago, Chicago, Illinois; and ⁶Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana

We reported that inhibiting matrix metalloproteinases (MMP), known to remodel the extracellular matrix, also down-regulated antigen-specific T-cell responses. However, the direct role of MMP2 and MMP9 in regulating intracellular function in T cells is unknown. Markers of cellular activation and cytokine profiles were examined in anti-CD3–stimulated wild-type C57BL/6 mouse–derived CD4⁺ or CD8⁺ T cells, or MMP2- or MMP9-deficient (–/–) mice. MMP-sufficient T cells were also treated with SB-3CT, a highly selective inhibitor of MMP2 and MMP9. The effect of MMP-specific inhibition on T cell–dependent, antigen-specific murine lung injury was examined *in vivo*. SB-3CT induced dose-dependent reductions in anti-CD3–stimulated T-cell proliferation. Although MMP2^{–/–} cells were reduced 20%, anti-CD3–induced proliferation was down-regulated 80–85% in MMP9^{–/–} or in SB-3CT–treated wild-type CD4⁺ and CD8⁺ T cells. Intracellular calcium flux was augmented in response to MMP inhibition or deficiency in the same cells, and IL-2 production was reduced in CD4⁺ and CD8⁺ MMP9^{–/–} T cells. SB-3CT–mediated MMP2 and MMP9 inhibition abrogated antigen-specific CD8⁺ T cell–mediated lung injury *in vivo*. MMPs, particularly MMP9, may function intracellularly to regulate T-cell activation. T cell–targeted MMP inhibition may provide a novel approach of immune regulation in the treatment of T cell–mediated diseases.

Keywords: matrix metalloproteinase 2; matrix metalloproteinase 9; T cells; SB-3CT

Matrix metalloproteinases (MMPs) constitute a family of over 25 secreted and transmembrane-bound, zinc-dependent endopeptidases involved in degradation of the components of the extracellular matrix and tissue remodeling. These proteolytic endopeptidases are also capable of degrading and processing other proteinases, adhesion molecules, chemokines, and cytokines, in addition to many newly identified nonmatrix substrates (2). MMPs are synthesized as inactive zymogens (proform), and require cleavage of their prodomain for activation (3, 4). Many reports have demonstrated MMP involvement in a multitude of biological processes, such as angiogenesis, wound

CLINICAL RELEVANCE

These studies identify that matrix metalloproteinases (MMPs) have novel roles in T-cell activation. MMP inhibition identifies a new class of immunosuppressant compounds.

healing, and embryogenesis, as well as in many pathological conditions, such as tumor invasion, myocardial infarction (5), and rheumatoid arthritis (6–9). It is believed that the exacerbation of many of these disease states is due to the unregulated elevation of MMP expression. In addition, other reports have highlighted the role of MMPs in a variety of lung diseases associated with T-cell activity, such as pulmonary fibrosis (10), emphysema, chronic obstructive pulmonary disease (11), and asthma (12), as well as in bronchiolitis obliterans syndrome (13), which results from autoimmune-mediated injury after lung allograft rejection (14, 15).

Interestingly, the gelatinases (MMP2 and MMP9) have been shown to play critical roles in T-cell infiltration into tissues, suggesting their importance in T cell–mediated injury (16). In this regard, studies have shown that CD4⁺ and CD8⁺ T cells, among others, have the ability to produce MMP2 and MMP9 upon stimulation (17), which implies that T cell–derived gelatinases may play a pivotal role in the pathogenesis of T cell–mediated lung injury. Prior reports have shown that gelatinase inhibition may have potential clinical relevance in that they may provide protective or anti-inflammatory effects in many disease states involving the unregulated elevation of MMPs. As such, one study reported that cardiac allograft rejection was inhibited in MMP2-deficient recipient mice that received wild-type allografts, and that this inhibition not only correlated with a decrease in mononuclear cellular infiltration into the allografts, but alloantigen-induced activation was diminished in MMP2-deficient T cells (14). A similar study reported differential lymphocyte cell activation in MMP9-deficient compared with MMP9-sufficient lymphocytes in a model of tracheal allograft obstructive airway disease (15). In addition, we reported that systemic MMP inhibition in rat lung transplant recipients abrogated expression of proinflammatory cytokines, and down-regulated allo- and auto-antigen-induced T-cell proliferation (18). Collectively, these studies clearly demonstrate a direct role for MMPs, and particularly MMP2 and/or MMP9, in T-cell activation. However, the specific roles of MMP2 and MMP9 in T-cell (CD4⁺ and CD8⁺) activation are unknown, and are examined in the current study.

MATERIALS AND METHODS

Animals

Female Balb/c and C57BL/6 mice (6–10 wk old), were purchased from Harlan (Indianapolis, IN). MMP2-deficient (MMP2^{–/–}), MMP9^{–/–}, and

(Received in original form April 7, 2010 and in final form June 18, 2010)

This work was supported by National Institutes of Health grants HL081350 to G.N.S. and D.S.W., HL067177 to D.S.W., CA122417 from the National Institutes of Health to M.C. and S.M., NIH/NIGMS, R25 GM079657 Indiana University Initiative for Maximizing Graduate Student Diversity to H.B., U19AI070973 to F.K., and NIAID K08 AI 059105 and American Society of Transplantation/Wyeth Basic Science Faculty Development Grant to R.S.

Correspondence and requests for reprints should be addressed to David S. Wilkes, M.D., Department of Medicine, Indiana University School of Medicine, Fairbanks Hall, Suite 6200, Indianapolis, IN 46202. E-mail: dwilkes@iupui.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 44, pp 700–708, 2011

Originally Published in Press as DOI: 10.1165/rncmb.2010-0125OC on July 16, 2010

Internet address: www.atsjournals.org

MMP2/MMP9 double-deficient (MMP2/9^{-/-}) mice (C57BL/6 background) (Baylor College of Medicine, Houston, TX), CC10-ovalbumin (OVA) mice (C57BL/6 background), and OT-1 TCR transgenic mice

(C57BL/6-Thy1.1 background) were also used (16, 19). All mouse studies were conducted in accordance with institutional animal care and usage guidelines.

Figure 1. Differential matrix metalloproteinase (MMP) 9 mRNA and protein expression in CD4⁺ and CD8⁺ T cells. Pure splenic (A) CD4⁺ and (B) CD8⁺ T cells were cultured in the absence or presence of anti-CD3 antibody (Ab; 1 μg/ml) for 72 hours. RNA was isolated, cDNA synthesized, and mRNA expression levels measured by quantitative RT-PCR. Data were normalized to β-actin. Data are representative of two separate experiments performed in triplicate. (C) Gelatin zymogram analysis of CD4⁺ and CD8⁺ T-cell lysates and supernatant. Data are representative of one of four separate experiments. (A) *P = 0.01; (B) *P = 0.003. MW, molecular weight; RQ, relative quantification.

Figure 2. Broad-spectrum and specific MMP inhibition abrogated anti-CD3-induced T-cell proliferation. Pure splenic CD4⁺ T cells were treated with (A) CD4⁺, and (B) CD8⁺ T cells were treated with SB-3CT (5–25 μM), and cultured in the presence of anti-CD3 Ab (0.5 μg/ml) for 72 hours. T-cell proliferation was measured by 3H thymidine incorporation. Data are representative of the mean (±SD) of three experiments performed in triplicate (*P < 0.001). (C) Cell viability (annexin V and propidium iodide [PI]) assessed in CD4⁺ and CD8⁺ T cells after 6-hour treatment with SB-3CT.

Selective MMP Inhibitor

SB-3CT, a specific mechanism-based MMP2/9 inhibitor, was a generous gift from Dr. Shahriar Mobashery (University of Notre Dame, Notre Dame, IN).

T-Cell Proliferation Assays

CD4⁺ or CD8⁺ T cells were isolated from wild-type Balb/c or C57BL/6 mice (1×10^6 /ml) and incubated with the indicated concentrations of MMP inhibitor or vehicle control for 6 hours. In additional studies, CD4⁺ or CD8⁺ T cells isolated from MMP2^{-/-}, MMP9^{-/-}, MMP2/9^{-/-} mice were used. The treated cells were cultured (1×10^5 /well) in cRPMI in the presence of anti-CD3 antibody (Ab) (0.5–1 µg/ml; BD Biosciences, San Jose, CA) at 37°C for 72 hours, and harvested as previously reported (20). This generalized protocol was used following the various isolation methods and treatment conditions indicated.

Intracellular Calcium Flux

Calcium flux was measured in CD4⁺ and CD8⁺ wild-type or MMP9^{-/-} or SB-3CT-treated (10 µM) T cells using the Fluo-4 NW Calcium Assay kit (Molecular Probes, Carlsbad, CA) in accord with the manufacturer's protocol. Cells were then stimulated with anti-CD3 Ab (10 µg/ml) and read in real time on a FlexStation I (Molecular Devices, Sunnyvale, CA) for 300 seconds.

Activation of OT-I Thy1.1⁺ CD8⁺ T Cells and Adoptive Transfer into CC10-OVA Mice

OT-I Thy1.1⁺ CD8⁺ T cells were treated with 10 µM of SB-3CT or the corresponding vehicle control for 6 hours. γ-Irradiated wild-type splenocytes (5×10^7) were cultured in 30 ml of 10% Dulbecco's modified Eagle's medium supplemented with 0.7 µg/ml of OVA peptide (SIINFEKL), followed by the addition of OT-I Thy1.1⁺ CD8⁺ T cells, anti-CD28 Ab (2 µg/ml), IL-2 (132.02 U/ml), and IL-12 (10 ng/ml). On Day 5, cells were resuspended in PBS, and 7.5×10^5 cells were intratracheally instilled into the lungs of CC10-OVA mice.

Identification of OT-I Thy1.1⁺CD8⁺ T Cells in the Lung of CC10-OVA Mice after Adoptive Transfer

The lungs of CC10-OVA mice were perfused and excised 10 days after adoptive transfer of SB-3CT- or vehicle-treated OT-I Thy1.1⁺ CD8⁺ T cells. Cells were resuspended in FACS buffer (10% BSA in PBS) and analyzed immediately on a FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ). FCS Express (DeNovo Software, Los Angeles, CA) was used for further analysis.

Histology

Lungs were perfused, inflated, and fixed with neutral buffered formalin. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Images were acquired at 20× using an Olympus microscope and DP12 digital camera (Olympus, Center Valley, PA). Histology was scored in a blinded manner, as previously reported (21).

Statistical Analysis

Data were analyzed by either two-way ANOVA with paired *t* tests or nonparametric *t* tests using Prism 4 (GraphPad Software for Windows; GraphPad Inc., San Diego, CA) or Microsoft Office Excel 2007 (Microsoft Corp., Seattle, WA).

RESULTS

MMP9 Is Expressed in CD4⁺ and CD8⁺ T Cells

To begin to address the role of MMPs in T-cell activation, we measured the mRNA and protein expression pattern of MMP9 in cell lysates and conditioned media of resting and anti-CD3-stimulated CD4⁺ and CD8⁺ T cells by means of quantitative RT-PCR and substrate zymography, respectively. As shown in Figure 1, there were detectable levels of MMP9 mRNA expression in unstimulated CD4⁺ (Figure 1A; $P = 0.01$) and CD8⁺ (Figure 1B;

$P = 0.003$) T cells. Interestingly, after anti-CD3 Ab stimulation, MMP9 mRNA transcript levels were increased in both cell populations, although CD8⁺ transcript levels were more pronounced. Analysis of MMP9 protein expression by gelatin zymography (Figure 1C) revealed constitutive expression of pro-MMP9 in untreated CD4⁺ and CD8⁺ T-cell lysates. After stimulation with anti-CD3 Ab, pro-MMP9 expression was slightly diminished in the T-cell lysates, and increased in the T-cell supernatant. These data confirm prior reports showing inducible MMP9 expression in stimulated T cells (22–26).

Broad-Spectrum and Specific MMP Inhibition Abrogates Anti-CD3-Induced T-Cell Proliferation

In addition to other studies demonstrating the effects of MMP inhibition in transplantation, recently, our laboratory demonstrated that broad-spectrum pharmacologic inhibition of MMPs by the chemically modified tetracycline, COL-3, abrogated alloantigen-induced T-cell proliferation (18). Effects of broad-spectrum MMP inhibitors, such as COL-3, may not be MMP specific, and have been shown to alter other non-MMP-related biochemical activities (27). To circumvent these limitations in our current studies, a highly selective MMP2 and MMP9 inhibitor, SB-3CT,

Figure 3. MMP2- and MMP9-deficient (–/–) T cells display altered proliferative ability. Wild-type (Wt) and (A) MMP2^{-/-} CD4⁺, (B) MMP9^{-/-} CD4⁺, and (C) MMP9^{-/-} CD8⁺ T cells were cultured in the presence of anti-CD3 Ab (0.5 µg/ml) for 72 hours. T-cell proliferation was measured by ³H thymidine incorporation. Data are representative of the mean (±SD) of three separate experiments performed in triplicate. # $P = 0.02$; ** $P < 0.001$.

was used. This inhibitor is transformed in an enzyme-dependent process in the active sites of MMP2 and MMP9 (28, 29), leading to tight-binding inhibition (30). To investigate the effects of MMP2 and/or MMP9 inhibition, anti-CD3-stimulated CD4⁺ and CD8⁺ T cells were treated with SB-3CT, followed by an assessment of the proliferative response. Compared with untreated cells, SB-3CT suppressed proliferation in both CD4⁺ and CD8⁺ T cells in a dose-dependent manner (Figures 2A and 2B; $P < 0.05$ and $P < 0.001$, respectively). In addition, SB-3CT treatment decreases gelatinolytic activity in T-cell lysates before and after stimulation with anti-CD3 as compared with untreated cell lysates. However, SB-3CT treatment does not completely abrogate gelatinolytic activity in T-cell supernatant after stimulation with anti-CD3 (data not shown). The inhibitory effect of SB-3CT was unaffected in SB-3CT-treated cells (Figure 2C). To confirm that the effects of SB-3CT were mediated via MMP2 and/or MMP9,

we next examined anti-CD3-induced proliferation in CD4⁺ T cells from MMP2^{-/-} or MMP9^{-/-} mice. As compared with wild-type T cells, MMP2^{-/-} CD4⁺ T cells only exhibited a 20% decrease in proliferation (Figure 3A; $P = 0.02$). Strikingly, MMP9 deficiency resulted in more than 80% reduction in proliferation (Figure 3B; $P < 0.001$). Significant reductions in proliferation were also observed in CD8⁺ MMP9^{-/-} T cells (Figure 3C; $P < 0.001$). In addition, our observation of MMP2/9^{-/-} T cells displayed a proliferative trend toward that of MMP9^{-/-} T cells, further demonstrating the importance of MMP9 in T-cell proliferation.

Anti-CD3 Ab-Induced Calcium Flux Is Increased In MMP9-Deficient and SB-3CT-Treated T Cells

We next sought to determine the intracellular events affected by MMP inhibition or deficiency. Because increased intracellular calcium flux is one of the very early events after T-cell

Figure 4. MMP deficiency or inhibition increases calcium flux. (A) CD4⁺ or (B) CD8⁺ T cells isolated from wild-type and MMP9^{-/-} mice. (C) CD8⁺ T cells were treated with SB-3CT (10 µM) or vehicle (DMSO + polyethylene glycol [PEG], diluted similarly in cRPMI). (A–C) Cells were cultured in calcium-free media and stimulated with anti-CD3 Ab (10 µg/ml). calcium flux was measured for 100 seconds in real time. Data are representative of one of three separate experiments performed in triplicate.

receptor-mediated T-cell activation via anti-CD3 (31, 32), we next examined the effect of MMP inhibition on intracellular calcium release from the endoplasmic reticulum. Because MMP9 deficiency had the greatest effect on the proliferative response, we focused these studies on MMP9^{-/-} cells. Compared with wild-type cells, intracellular flux was up-regulated in MMP9^{-/-} CD4⁺ and CD8⁺ T cells (Figures 4A–4B). Studies conducted in parallel examining SB-3CT-treated cells yielded similar results (Figure 4C). These results demonstrate that, under normal conditions, MMP9 down-regulates intracellular calcium flux in response to T-cell activation via the T-cell receptor. These data also suggest that the effects of MMP9 deficiency on T-cell proliferation, a late event in T-cell activation, are downstream to the anti-CD3-induced release of intracellular calcium.

Cytokine Transcript and Protein Expression Is Impaired in MMP9^{-/-} and SB-3CT-Treated Wild-Type CD4⁺ or CD8⁺ T Cells

Increased intracellular calcium flux is associated with translocation of the transcription factor, nuclear factor of activated T-cells (NFATc1), and NFATc1-mediated production of IL-2. In addition,

autocrine production of IL-2 has a key role in anti-CD3-induced T-cell proliferation. Therefore, we next determined the expression of NFATc1 and IL-2 in MMP^{-/-} or SB-3CT-treated cells. Interestingly, NFATc1 transcript expression was down-regulated in MMP2^{-/-} or MMP9^{-/-} CD4⁺ T cells, and in the same wild-type cells treated with SB-3CT (Figures 5A and 5B, respectively). Data showing diminished NFATc1 expression would also suggest low levels of expression of IL-2 protein, which is NFAT dependent. As expected, IL-2 protein was diminished in MMP^{-/-} and SB-3CT-treated cells (Figures 5C and 5D, respectively). Consistent with these findings, expression of CD25 transcripts, the IL-2 receptor, which is NFATc1 dependent, was also down-regulated (Figures 5E and 5F, respectively). In addition, surface expression of CD25 protein was also down-regulated in MMP^{-/-} cells (see Figure E2 in the online supplement). Lower levels of CD25 expression could suggest that exogenous IL-2 would not recover anti-CD3-induced T-cell proliferation in MMP^{-/-} or SB-3CT-treated cells. Indeed, exogenous IL-2 did not recover proliferation of these cells (data not shown). Translocation of NFATc1 is also important for expression of forkhead box P3 (FOXP3) a transcriptional regulator known to have key functions in CD4⁺ regulatory T cells (Tregs). Data showing low levels of NFATc1 could

Figure 5. MMP deficiency or inhibition alters cytokine transcript and protein expression. CD4⁺ T cells were isolated from wild-type, MMP2^{-/-}, or MMP9^{-/-} mice. (B, D, and F) CD4⁺ T cells were treated with SB-3CT (5–20 μM). Cells were cultured in the presence or absence of anti-CD3 Ab (1 μg/ml). (A–B) nuclear factor of activated T cells (NFATc1), (C–D) IL-2, and (E–F) CD25 expression levels were measured by quantitative RT-PCR. (C–D) IL-2 protein expression was measured by cytometric bead assay. Data are representative of three separate experiments performed in triplicate. #*P* < 0.05; *P < 0.001.

Figure 6. SB-3CT-treated, antigen-specific T cells (OT-I) display impairment in proliferative ability. (A) OT-I transgenic CD8⁺ T cells were treated with SB-3CT (5–20 μ M) or vehicle (DMSO + PEG, diluted similarly in CRPMI), and cultured in the presence of ovalbumin (OVA)-pulsed antigen-presenting cells (APCs) for 72 hours. Data are representative of two separate experiments performed in triplicate ($^{\#}P < 0.05$; $^{*}P < 0.001$). (B) At 7 days after adoptive transfer, bronchoalveolar lavage (BAL) fluid from the CC10-OVA (CC10) or nontransgenic (B6) mice was analyzed, and total cells present in the BAL quantitated. (C) Neutrophils were stained with GR1 and analyzed by means of flow cytometry. $^{**}P < 0.01$ as compared with stimulated wild-type cells ($n = 10$ mice [CC10] per treatment group; $n = 5$ control mice [B6] per treatment group).

suggest low levels of Foxp3 in MMP-inhibited CD4⁺ T cells. Interestingly, FOXP3 transcripts were up-regulated in MMP^{-/-} cells and those cells treated with SB-3CT (Figures E1A and E1B, respectively). Data showing increased FOXP3 expression in MMP^{-/-} cells could suggest that blockade or absence of MMPs could have induced cells with regulatory function.

To directly examine if MMP inhibition induced Treg function, we used suppressor assays in which CD4⁺25⁺ T cells were treated with SB-3CT and cocultured at varying ratios with untreated CD4⁺25⁺ T cells in the presence of irradiated antigen-presenting cells (APCs) for 72 hours. As shown in Figure E1C, SB-3CT treatment at each ratio inhibited T-cell proliferation by 50%. However, as the ratio of SB-3CT-treated cells increased, T-cell proliferation also increased, suggesting that SB-3CT treatment does not induce Treg function.

To determine if Treg function was affected in response to SB-3CT treatment, CD4⁺25⁺ T cells (Tregs) were treated with SB-3CT and cocultured at varying ratios, as shown previously here in the suppressor assay. Interestingly, CD4⁺25⁺ T cells retained their suppressive function (Figure E1D). Worth noting, however, is that SB-3CT-treated CD4⁺25⁺ T cells displayed a somewhat altered suppressive ability, requiring more treated cells to exhibit their suppressive nature.

MMP9 Deficiency Alters CD4⁺ and CD8⁺ T-Cell Phenotypes in Response to Anti-CD3

To characterize further the role of T cell-derived MMP9, surface phenotype studies were performed. MMP9^{-/-} or corresponding wild-type CD4⁺ or CD8⁺ T cells were stimulated with anti-CD3, and a panel of surface markers was assessed. As expected, analysis of wild-type CD4⁺ T cells revealed increased surface expression levels of all of the T-cell activation markers, CD25, CD69, CD62L, CD44, cytotoxic T-lymphocyte antigen 4 (CTLA-4), CD40L, and CD45RO (Figures E2 and E3). In comparison, analysis of CD4⁺ T cells from MMP9^{-/-} T cells revealed increased surface expression levels of CD62L, CTLA-4, and CD45RO. CD44 and CD40L expression levels decreased slightly compared with wild-type cells. CD25 and CD69 expression levels were both significantly diminished. These data show

that, as compared with wild-type CD4⁺ T cells, MMP9^{-/-} CD4⁺ T cells have significantly lower levels of cell surface CD25 and CD69, while expressing higher levels of CD45RO and CTLA-4.

Analysis of cell surface expression in wild-type CD8⁺ T cells revealed increases in CD25, CD62L, and CD69 (Figures E2 and E3). In addition, CD40L, CD44, and CTLA-4 were expressed, although the percent expression was less than or equal to 20%. CD45RO was also expressed at very low levels, not exceeding 5%. Analysis of MMP9^{-/-} CD8⁺ T cells compared with wild-type CD8⁺ T cells revealed low expression levels of CD69, CD25, and CD62L. CD45RO and CD44 surface expression levels remained the same as in wild-type cells. Interestingly, CTLA-4 and CD40L surface expression show slight elevation compared with wild-type cells (Figures E2 and E3). Consistent with the lack of induction of NFATc1 expression, CD25 expression did not increase in response to anti-CD3 stimulation in MMP9^{-/-} T cells. Taken together, these data show that CD4⁺ and CD8⁺ T cells display differential cell surface expression in the absence of MMP9.

Gelatinase Inhibition Abrogates Antigen-Specific CD8⁺ T Cell-Induced Lung Injury

Our data have demonstrated that, compared with CD4⁺ cells, CD8⁺ T cells express higher levels of MMP9 in response to anti-CD3 stimulation, and that MMP inhibition or deficiency down-regulates cellular function. We next determined the role of CD8⁺ T cell-derived MMP2 and MMP9 in a model of CD8⁺ T cell-dependent lung injury *in vivo*. Medoff and colleagues (33) reported that instilling OVA-specific CD8⁺ T cells into the lungs of mice genetically engineered to overexpress OVA by the epithelium in the distal airways results in peribronchiolar and perivascular inflammation. In our studies, these mice, termed CC10-OVA mice, received intrapulmonary instillations of activated CD8⁺ T cells that express the T-cell receptor specific for OVA peptide, SIINFEKL, bound to the class I major histocompatibility complex H-2K^b (OT-1 cells, Thy 1.2⁺) (33). Parallel studies were conducted in which these cells were treated with SB-3CT before intrapulmonary instillation, followed by an assessment of lung injury (34, 35).

As shown in Figure 6A, untreated or vehicle-treated OT-I transgenic CD8⁺ T cells proliferated in response to OVA peptide-pulsed APCs. Interestingly, SB-3CT treatment of OT-I T cells completely abrogated the proliferative response to OVA-pulsed APCs. Examination of CD4⁺ T cells from OT-II transgenic mice revealed a similar trend (data not shown). These data demonstrate that, similar to polyclonal activation via anti-CD3, highly selective inhibition of MMP2 and MMP9 also abrogates antigen-specific proliferation of CD8⁺ T cells.

Analysis of total cell accumulation in bronchoalveolar lavage 7 days after intrapulmonary instillation revealed no differences in the quantity of total bronchoalveolar lavage cells recovered in the SB-3CT-treated (MMP inhibitor) and vehicle groups (Figure 6B). However, the quantity of neutrophils (Gr-1⁺), a marker of injury in this model (33), was decreased significantly in the SB-3CT-treated group (Figure 6C; $P < 0.01$). The OT-I transgenic mice were Thy1.1⁺ and, therefore, provided a means of tracking the transferred cells in the CC10-OVA mice, which were in a Thy1.2⁺ background. We next determined if there was a difference in the accumulation of CD8⁺ Thy1.1⁺ T cells in the lung between the two CC10-OVA-treated groups (vehicle or SB-3CT). Interestingly, treatment with SB-3CT resulted in significantly fewer CD8⁺ Thy1.1⁺ (donor) cells in lung parenchyma (Figure 7A; $P < 0.01$). Moreover, fewer of these cells expressed the activation marker, CD25 (Figure 7B; $P < 0.01$).

Fewer neutrophils and donor-derived CD8⁺ T cells in lungs of CC10-OVA mice that received gelatinase-inhibited cells suggests less severe lung injury. Indeed, gelatinase inhibition of OT-I T cells before adoptive transfer abrogated the development of perivascular and peribronchiolar inflammation (Figure 7C). This is further confirmed by blinded histological scoring demonstrating a significant decrease in scores of the SB-3CT-treated group as compared the vehicle-treated group (Figure 7D; $P = 0.03$).

DISCUSSION

Although prior studies confirmed that MMP2 and MMP9 are expressed in T cells (36), data from the current study reveal that, although MMP2 and MMP9 are both induced, MMP9, in particular, plays a key role in regulating T-cell activation. This conclusion is derived from data showing that MMP9 deficiency significantly impairs the activation of CD4⁺ and CD8⁺ T cells. However, it is notable that MMP9 is induced greatly in activated CD8⁺ compared with CD4⁺ T cells. In the current study, we report that broad-spectrum MMP inhibition, MMP9-specific inhibition, as well as genetic deficiency of MMP9 all result in down-regulation of polyclonal and antigen-specific activation-induced proliferation in CD4⁺ and CD8⁺ T cells. However, MMP deficiency or inhibition was associated with increases in intracellular calcium release in response to polyclonal stimulation via

Figure 7. Murine model of antigen-specific CD8⁺ effector T cell-mediated lung injury. (A) CD8⁺Thy1.1⁺ T cells were isolated from the lungs of mice after the adoptive transfer of SB-3CT (10 μ M) or vehicle (DMSO + PEG, diluted similarly in CRPMI). (B) CD25 expression in CD8⁺Thy1.1⁺ T cells from the lungs of CC10-OVA mice ($*P < 0.01$; $n = 9$ mice [CC10] per treatment group; $n = 5$ control mice [B6] per treatment group). (C) CD8⁺ T cells were isolated from OT-1 transgenic mice and treated with SB-3CT or vehicle. Histology of the lung was evaluated by hematoxylin and eosin staining (magnification, 20 $\times$; $n = 6$ mice [CC10] per group). Panels 1a and 1b are representative of two fields of a CC10 mouse lung after adoptive transfer of vehicle-treated OT-I CD8⁺ T cells. Panels 2 and 3 are representative of two different CC10 mouse lung samples after adoptive transfer of SB-3CT-treated OT-I CD8⁺ T cells. (D) Histological scoring was performed blinded for all groups. $**P = 0.03$ as compared with vehicle-treated (CC10) group (unpaired t test with Welch's correction).

anti-CD3 (Figure E4). NFATc1 and CD25 gene expression were down-regulated, whereas *foxp3* gene expression was elevated. Analysis of IL-2 expression revealed down-regulation of protein expression in response to MMP9 inhibition and MMP9 deficiency. We also demonstrated, in an *in vivo* model, that MMP9 inhibition impaired the degree of T cell-mediated lung injury. Collectively, these data clearly indicate a role for T cell-derived MMP9 in the process of T-cell activation.

In our investigation of the intracellular T-cell signaling events, the data show that intracellular calcium release was increased in response to polyclonal activation in the absence of MMP9, or when MMP2 and MMP9 were inhibited by SB-3CT. These findings suggest that, in response to MMP inhibition or MMP9 deficiency, the increase in calcium influx may be a mechanism by which a cell attempts to compensate for the lack of effective distal activation events. Accordingly, MMP9, in particular, may function as a tonic down-regulator of calcium-mediated events.

Intracellular calcium flux precedes the translocation of NFATc1, which regulates the expression of the IL-2 receptor- α (CD25), and multiple cytokine genes, including IL-2. Data showing increased intracellular calcium flux should be associated with up-regulated NFATc1-related events. Surprisingly, we observed a decrease in NFATc1, IL-2, and CD25 expression. These findings strongly suggest that activity of MMP2 and/or MMP9 is required for NFATc1 activation and subsequent expression of IL-2 and CD25. These data may also explain why addition of exogenous IL-2 did not recover the proliferative response in SB-3CT-treated cells. Because our results suggested that MMP2 or MMP9 inhibition may cause the T cells to exhibit Treg function, we investigated markers that are characteristically found in Tregs. Unexpectedly, we observed that *foxp3* expression was elevated in SB-3CT-treated and MMP9^{-/-} T cells, despite low expression of NFATc1 transcripts and low expression of IL-2 and CD25.

In our investigation of specific MMP inhibition *in vivo*, we reported a significant decrease in the percentage of CD8⁺ Thy1.1⁺ T cells in the lung of CC10-OVA mice, suggesting that MMP inhibition may affect T-cell migration and/or decrease cellular activation. Further analysis of CD25 surface expression on CD8⁺ Thy1.1⁺ T cells in the lung revealed a dramatic decrease in CD25 surface expression, suggesting decreased cellular activation. These results are similar to the *in vitro* data demonstrating a significant decrease in CD25 mRNA and cell surface expression in response to MMP inhibition. Histological analysis of lung sections collected from the lungs of CC10-OVA mice demonstrated increased perivascular and perinuclear infiltrates after the transfer of vehicle-treated OT-1 cells. In contrast, after the adoptive transfer of SB-3CT-treated OT-1 cells, the mononuclear cellular infiltration was minimal, suggesting that MMP9 inhibition attenuated the degree of inflammation within the lung, thus significantly impairing the degree of T cell-mediated lung injury. In addition, we showed that SB-3CT treatment decreased neutrophil influx into the lung. One possible explanation for these findings may be that SB-3CT inhibited neutrophil chemoattractants, leading to the decrease in neutrophils influx. Hardison and colleagues (37) recently described a matrix-derived neutrophil chemoattractant, proline-glycine-proline, which has been correlated with elevated levels of MMP-9 found in clinical samples after lung transplantation. It is therefore possible that, in addition to the inhibition of MMP9, SB-3CT treatment may have inhibited proline-glycine-proline expression, thereby causing the decrease in neutrophils recruitment.

Collectively, the data from the current study suggest that the action of MMPs, if occurring intracellularly, is downstream of pathways involved in intracellular calcium flux, and proximal to

NFATc1-related events, such as expression of IL-2 and CD25. This hypothesis is counter to the known activities of MMP2 and MMP9 in the extracellular milieu. A peptide fragment of aggrecan (the interglobular domain), identified as VDIPEN, has been identified as a cleavage site for MMP9 and five other MMPs (MMP1, -2, -3, -7, and -8) (38). In an attempt to locate an intracellular substrate for MMP2 or MMP9, we used a VDIPEN Ab that specifically recognizes this cleavage site and performed Western blot analysis on lysates from activated T cells. Although an intracellular substrate was not identified in these studies (data not shown), these data do not exclude an intracellular substrate for MMP2 or MMP9. Indeed, Kwan and colleagues (39) reported the presence of an active form of MMP2 within the nucleus of cardiac myocytes. Si-Tayeb and colleagues (40) reported the presence of an active form of MMP3 in the nucleus of a human hepatocellular carcinoma cell line (HepG2) and samples from patients with hepatocellular carcinoma, and identified a nuclear localization signal. Alternatively, the effects of MMP deficiency could be explained by extracellular events leading to the signaling alterations observed in the current study. Although we are not able to prove intracellular activity, the results shown are less likely due to an extracellular event. We suggest this because intracellular calcium flux—a very rapid event after T-cell receptor activation—was up-regulated, but NFATc1-dependent functions, which are initially dependent on intracellular calcium flux, were down-regulated. However, we cannot rule out the possibility that another potential mechanism may be that MMP9 either directly cleaves a cell surface signaling receptor or activates another protease that may cleave a receptor to increase responsiveness of T-cell receptor signaling.

Perhaps the most novel aspects of these data are the potential clinical implications. The current studies are an extension of our work showing that inhibiting MMP2 and MMP9 during lung transplantation suppressed both allo- and autoimmune-associated T-cell responses (18). Direct evidence of T cell-suppressive effects in that study may be best exemplified by the observation that these rodent lung transplant recipients developed lesions consistent with the clinical condition known as post-transplant proliferative disorder, which may be associated with profound suppression of T-cell activity (18). Although those data provided indirect evidence that inhibiting MMP2 and/or MMP9 suppressed T-cell activation, the current study reveals a critical role for these MMPs in activating CD4⁺ and CD8⁺ T cells. Accordingly, the current study and our prior report suggest that inhibiting MMP9, in particular, could be a novel approach to immunosuppression for the treatment of T cell-dependent diseases, such as organ allograft rejection and autoimmune diseases. The current data could also help to explain the limited effects of MMP inhibition in the treatment of various cancers (41, 42). Although metastases may have been suppressed in those reports, a key target of MMP activity in cancer, the effects on tumor burden were limited. This could have been due to MMP inhibition-mediated suppression of antitumor cellular immunity (T cell dependent), which may be required for reduction in tumor burden. These questions will be addressed in future studies.

Author Disclosure: D.S.W. is a cofounder and chief scientific officer of ImmuneWorks Inc. (less than \$1,000), has received industry-sponsored grants from ImmuneWorks Inc. (\$50,001–\$100,000) and Quark Biotech (more than \$100,000), has a patent pending with ImmuneWorks Inc. for the use of type V collagen to prevent lung transplant rejection, and holds stock in ImmuneWorks Inc. (no value assigned to ImmuneWorks stock); H.L.B. has received sponsored grants from the National Institutes of Health and National Institute of General Medicine (\$10,001–\$50,000); R.A.S. has received an industry-sponsored grant from Wyeth (\$10,001–\$50,000), and sponsored grants from NIH NIAID (more than \$100,000), the American Society of Transplantation (\$10,001–\$50,000), and the Louis Block Family Fund (\$10,001–\$50,000); S.M. has received lecture fees from AstraZeneca (less than \$1,000); none of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: to the authors thank Drs. Matthias Clauss and Alexander Obukhov for their support and analysis of the calcium data, and Dr. John Mort for the VDIEN antibody.

References

- Greenlee KJ, Corry DB, Engler DA, Matsunami RK, Tessier P, Cook RG, Werb Z, Kheradmand F. Proteomic Identification of *in vivo* substrates for matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. *J Immunol* 2006;177:7312–7321.
- Goetzl E, Banda M, Leppert D. Commentary: matrix metalloproteinases in immunity. *J Immunol* 1996;156:1–4.
- Nagase H, Woessner JFJ. Matrix metalloproteinases. *J Biol Chem* 1999;274:21491–21494.
- Rosenblum G, Meroueh S, Toth M, Fisher JF, Fridman R, Mobashery S, Sagi I. Molecular structures and dynamics of the stepwise activation mechanism of a matrix metalloproteinase zymogen: challenging the cysteine switch dogma. *J Am Chem Soc* 2007;129:13566–13574.
- Creemers EEJM, Cleutjens JPM, Smits JFM, Daemen MJAP. Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? *Circ Res* 2001;89:201–210.
- Shapiro SD, Senior RM. Matrix metalloproteinases: matrix degradation and more. *Am J Respir Cell Mol Biol* 1999;20:1100–1102.
- Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001;17:463–516.
- Opdenakker G, Van-den-Steen P, Van-Damme J. Gelatinase B: a tuner and amplifier of immune functions. *Trends Immunol* 2001;22:571–579.
- Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, Proost P, Van Damme J. Gelatinase B functions as regulator and effector in leukocyte biology. *J Leukoc Biol* 2001;69:851–859.
- Crouch E. Pathobiology of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 1990;259:L159–L184.
- Cosio MG, Guerassimov A. Chronic obstructive pulmonary disease: inflammation of small airways and lung parenchyma. *Am J Respir Crit Care Med* 1999;160:S21–S25.
- Belleguic C, Corbel M, Germain N, Léna H, Boichot E, Delaval P, Lagente V. Increased release of matrix metalloproteinase 2 and 9 in the plasma of acute severe asthmatic patients. *Clin Exp Allergy* 2002;32:217–223.
- Dell'Agli M, Galli GV, Bosio E, D'Ambrosio M. Inhibition of NF- κ B and metalloproteinase-9 expression and secretion by parthenolide derivatives. *Bioorg Med Chem Lett* 2009;19:1858–1860.
- Campbell LG, Ramachandran S, Liu W, Shipley JM, Itohara S, Rogers JG, Moazami N, Senior RM, Jaramillo A. Different roles for matrix metalloproteinase-2 and matrix metalloproteinase-9 in the pathogenesis of cardiac allograft rejection. *Am J Transplant* 2005;5:517–528.
- Fernandez FG, Campbell LG, Liu W, Shipley JM, Itohara S, Patterson GA, Senior RM, Mohanakumar T, Jaramillo A. Inhibition of obliterative airway disease development in murine tracheal allografts by matrix metalloproteinase-9 deficiency. *Am J Transplant* 2005;5:671–683.
- Corry DB, Kiss A, Song L-Z, Song L, Xu J, Lee S-H, Werb Z, Kheradmand F. Overlapping and independent contributions of MMP2 and MMP9 to lung allergic inflammatory cell egression through decreased CC chemokines. *FASEB J* 2004;18:995–997.
- Graesser D, Mahooti S, Madri JA. Distinct roles for matrix metalloproteinase-2 and α 4 integrin in autoimmune T cell extravasation and residency in brain parenchyma during experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2000;109:121–131.
- Yoshida S, Iwata T, Chiyo M, Smith GN, Foresman BH, Mickler EA, Heidler KM, Cummings OW, Fujisawa T, Brand DD, et al. Metalloproteinase inhibition has differential effects on alloimmunity, autoimmunity, and histopathology in the transplanted lung. *Transplantation* 2007;83:799–808.
- Shilling RA, Clay BS, Tesciuba AG, Berry EL, Lu T, Moore TV, Bandukwala HS, Tong J, Weinstock JV, Flavell RA, et al. CD28 and ICOS play complementary non-overlapping roles in the development of Th2 immunity in vivo. *Cell Immunol* 2009;259:177–184.
- Sumpter TL, Payne KK, Wilkes DS. Regulation of the NFAT pathway discriminates CD4+CD25+ regulatory T cells from CD4+CD25-helper T cells. *J Leukoc Biol* 2008;83:708–717.
- Bobadilla JL, Love RB, Jankowska-Gan E, Xu Q, Haynes LD, Braun RK, Hayney MS, Munoz del Rio A, Meyer K, Greenspan DS, et al. Th-17, monokines, collagen type V, and primary graft dysfunction in lung transplantation. *Am J Respir Crit Care Med* 2008;177:660–668.
- Owen JL, Iragavarapu-Charyulu V, Gunja-Smith Z, Herbert LM, Grosso JF, Lopez DM. Up-regulation of matrix metalloproteinase-9 in T lymphocytes of mammary tumor bearers: role of vascular endothelial growth factor. *J Immunol* 2003;171:4340–4351.
- Leppert D, Waubant E, Galardy R, Bunnett N, Hauser S. T cell gelatinases mediate basement membrane transmigration in vitro. *J Immunol* 1995;154:4379–4389.
- Montgomery AMSH, Reisfeld RA. Production and regulation of gelatinase B by human T-cells. *Biochim Biophys Acta* 1993;1176:265–268.
- Weeks BS, Schnaper HW, Handy M, Holloway E, Kleinman HK. Human T lymphocytes synthesize the 92 kDa type IV collagenase (gelatinase B). *J Cell Physiol* 1993;157:644–649.
- Zhou H, Bernhard EJ, Fox FE, Billings PC. Induction of metalloproteinase activity in human T-lymphocytes. *Biochim Biophys Acta* 1993;1177:174–178.
- Sandler C, Ekoski E, Lindstedt KA, Vainio PJ, Finel M, Sorsa T, Kovanen PT, Golub LM, Eklund KK. Chemically modified tetracycline (CMT)-3 inhibits histamine release and cytokine production in mast cells: possible involvement of protein kinase C. *Inflamm Res* 2005;54:304–312.
- Brown S, Bernardo MM, Li ZH, Kotra LP, Tanaka Y, Fridman R, Mobashery S. Potent and selective mechanism-based inhibition of gelatinases. *J Am Chem Soc* 2000;122:6799–6800.
- Toth M, Bernardo MM, Gervasi DC, Soloway PD, Wang Z, Bigg HF, Overall CM, DeClerck YA, Tschesche H, Cher ML. Tissue inhibitor of metalloproteinase (TIMP)-2 acts synergistically with synthetic matrix metalloproteinase (MMP) inhibitors but not with TIMP-4 to enhance the (membrane type 1)-MMP-dependent activation of pro-MMP-2. *J Biol Chem* 2000;275:41415–41423.
- Forbes C, Shi Q, Fisher JF, Lee M, Hesk D, Llarrull LI, Toth M, Gossing M, Fridman R, Mobashery S. Active site ring-opening of a thirane moiety and picomolar inhibition of gelatinases. *Chem Biol Drug Des* 2009;74:527–534.
- Hall SR, Rhodes J. Schiff base-mediated co-stimulation primes the T-cell-receptor-dependent calcium signalling pathway in CD4 T cells. *Immunology* 2001;104:50–57.
- Zitt C, Strauss B, Schwarz EC, Spaeth N, Rast G, Hatzelmann A, Hoth M. Potent inhibition of Ca^{2+} release-activated Ca^{2+} channels and T-lymphocyte activation by the pyrazole derivative BTP2. *J Biol Chem* 2004;279:12427–12437.
- Medoff BD, Seung E, Wain JC, Means TK, Campanella GSV, Islam SA, Thomas SY, Ginns LC, Grabie N, Lichtman AH, et al. BLT1-mediated T cell trafficking is critical for rejection and obliterative bronchiolitis after lung transplantation. *J Exp Med* 2005;202:97–110.
- Stripp B, Sawaya P, Luse D, Wikenheiser K, Wert S, Huffman J, Lattier DL, Singh G, Katyal SL, Whitsett JA. cis-acting elements that confer lung epithelial cell expression of the CC10 gene. *J Biol Chem* 1992;267:14703–14712.
- Carbone F, Bevan M. Induction of ovalbumin-specific cytotoxic T cells by *in vivo* peptide immunization. *J Exp Med* 1989;169:603–612.
- Oviedo-Orta E, Bermudez-Fajardo A, Karanam S, Benbow U, Newby AC. Comparison of MMP-2 and MMP-9 secretion from T helper 0, 1 and 2 lymphocytes alone and in coculture with macrophages. *Immunology* 2008;124:42–50.
- Hardison MT, Galin FS, Calderon CE, Djekic UV, Parker SB, Wille KM, Jackson PL, Oster RA, Young KR, Blalock JE, Gaggari A. The presence of a matrix-derived neutrophil chemoattractant in bronchiolitis obliterans syndrome after lung transplantation. *J Immunol* 2009;182:4423–4431.
- Hughes CE, Caterson B, Fosang AJ, Roughley PJ, Mort JS. Monoclonal antibodies that specifically recognize neopeptide sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism in situ and *in vitro*. *Biochem J* 1995;305:799–804.
- Kwan JA, Schulze CJ, Wang W, Leon H, Sariahmetoglu M, Sung M, Sawicka J, Sims DE, Sawicki G, Schulz R. Matrix metalloproteinase-2 (MMP-2) is present in the nucleus of cardiac myocytes and is capable of cleaving poly (ADP-ribose) polymerase (PARP) *in vitro*. *FASEB J* 2004;18:690–692.
- Si-Tayeb K, Monvoisin A, Mazzocco C, Lepreux S, Decossas M, Cubel G, Taras D, Blanc JF, Robinson DR, Rosenbaum J. Matrix metalloproteinase 3 is present in the cell nucleus and is involved in apoptosis. *Am J Pathol* 2006;169:1390–1401.
- Lokeshwar BL. MMP inhibition in prostate cancer. *Ann NY Acad Sci* 1999;878:271–289.
- Zervos EE, Shafii AE, Rosemurgy AS. Matrix metalloproteinase (MMP) inhibition selectively decreases type II MMP activity in a murine model of pancreatic cancer. *J Surg Res* 1999;81:65–68.