Inhibition of Allergic Bronchial Asthma by Thrombomodulin Is Mediated by Dendritic Cells

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***Rationale*: Bronchial asthma is caused by inappropriate acquired immune responses to environmental allergens. It is a major health problem, with a prevalence that is rapidly increasing. Curative therapy is not currently available.**

***Objectives*: To test the hypothesis that thrombomodulin (TM) inhibits allergic bronchial asthma by inducing tolerogenic dendritic cells (DCs).**

***Methods*: The protective effect of TM was evaluated using a murine asthma model. Asthma was induced in mice by exposure to chicken egg ovalbumin, and the effects of inhaled TM or TM-treated DCs were assessed by administering before ovalbumin exposure.**

***Measurements and Main Results*: Treatment with TM protects against bronchial asthma measured as improved lung function and reduced IgE and cells in alveolar lavage fluid by inducing tolerogenic dendritic dells. These are characterized by high expression of surface TM (CD141/TM$^+$) and low expression of maturation markers and possess reduced T-cell costimulatory activity. The CD141/TM$^+$ DCs migrate less toward chemokines, and after TM treatment there are fewer DCs in the draining lymph node and more in the lungs. The TM effect is independent of its role in coagulation. Rather, it is mediated via the TM lectin domain directly interacting with the DCs.**

***Conclusions*: The results of this study show that TM is a modulator of DC immunostimulatory properties and a novel candidate drug for the prevention of bronchial asthma in atopic patients.**

Keywords: asthma; coagulation; dendritic cells; tolerance

Bronchial asthma affects 8% of adults and 20% of children worldwide (1). Curative therapy is not currently available (2). Inhaled glucocorticoids, the most effective and widely used therapy for asthma, control the inflammation but do not alter the immune dysregulation that underlies the disease. Moreover, inhaled glucocorticoids have several long-term side effects. Relative resistance to glucocorticoids in some patients leads to use of high doses of the drug, increasing the risk of side effects and underlining the need to develop novel therapies capable of modulating the abnormal immune reactivity in asthma.

(*Received in original form January 24, 2010; accepted in final form August 12, 2010*)

Supported by Grants-in-Aid (18590846, 17590788) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Japan Society for the Promotion of Science scholarship L8020 (J.M.); and EMC is an adjunct scientist with the Canadian Blood Services, holds a Canada Research Chair in Endothelial Cell Biology and a CSL-Behring Research Chair.

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

Am J Respir Crit Care Med Vol 183. pp 31–42, 2011
Originally Published in Press as DOI: 10.1164/rccm.201001-0107OC on August 13, 2010
Internet address: www.atsjournals.org

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Curative therapy is not available for bronchial asthma. Inhaled glucocorticoids control inflammation, but they do not cure the underlying immune dysregulation.

What This Study Adds to the Field

This study provides evidence that thrombomodulin is a modulator of dendritic cells' immunostimulatory properties and a candidate drug for the prevention of bronchial asthma in atopic patients.

Airway hyperresponsiveness (AHR) is one of the major clinical features of allergic airway diseases, including allergic asthma. There is a central role for respiratory tract dendritic cells (DCs) in the induction of AHR through the generation of lung-homing, allergen-specific effector T cells (3). In other circumstances, these DCs become tolerogenic, suppressing the bronchial asthma (4, 5). The signal(s) that causes the DCs to change from an immunogenic to a tolerogenic phenotype was unknown.

DCs are antigen-presenting cells that are generated in the bone marrow and migrate to organs, where they interrogate foreign antigens (6). After the antigens are phagocytosed, DCs move to the draining lymph node, where they present the antigen to cognate T cells to stimulate killing (7). There is no marker that can be used to uniquely identify all DCs while excluding all other cell types (8). DCs are subdivided into plasmacytoid (pDCs) and myeloid or conventional DCs (mDCs) based on their possession of the relevant lineage markers (9, 10).

Proteins from the coagulation cascade have recently been demonstrated to influence both innate (11) and acquired immunity (12). Fibrin generated by thrombin cleavage of fibrinogen has been linked to asthma (13, 14). Thrombomodulin (TM), which is also known as CD141 or blood dendritic cell antigen (BDCA)-3, is a cell surface protein that binds thrombin, the key effector enzyme of the coagulation cascade, converting it from a procoagulant to an anticoagulant and antifibrinolytic enzyme (15). Thrombin bound to TM is inhibited from cleaving fibrinogen, protease-activated receptor (PAR)-1, and other procoagulant substrates, but activates protein C (PC) to the anticoagulant and antiinflammatory activated protein C (aPC) and thrombin-activatable fibrinolysis inhibitor (TAFI) to the antifibrinolytic and antiinflammatory activated TAFI. In addition, TM exhibits antiinflammatory properties that do not

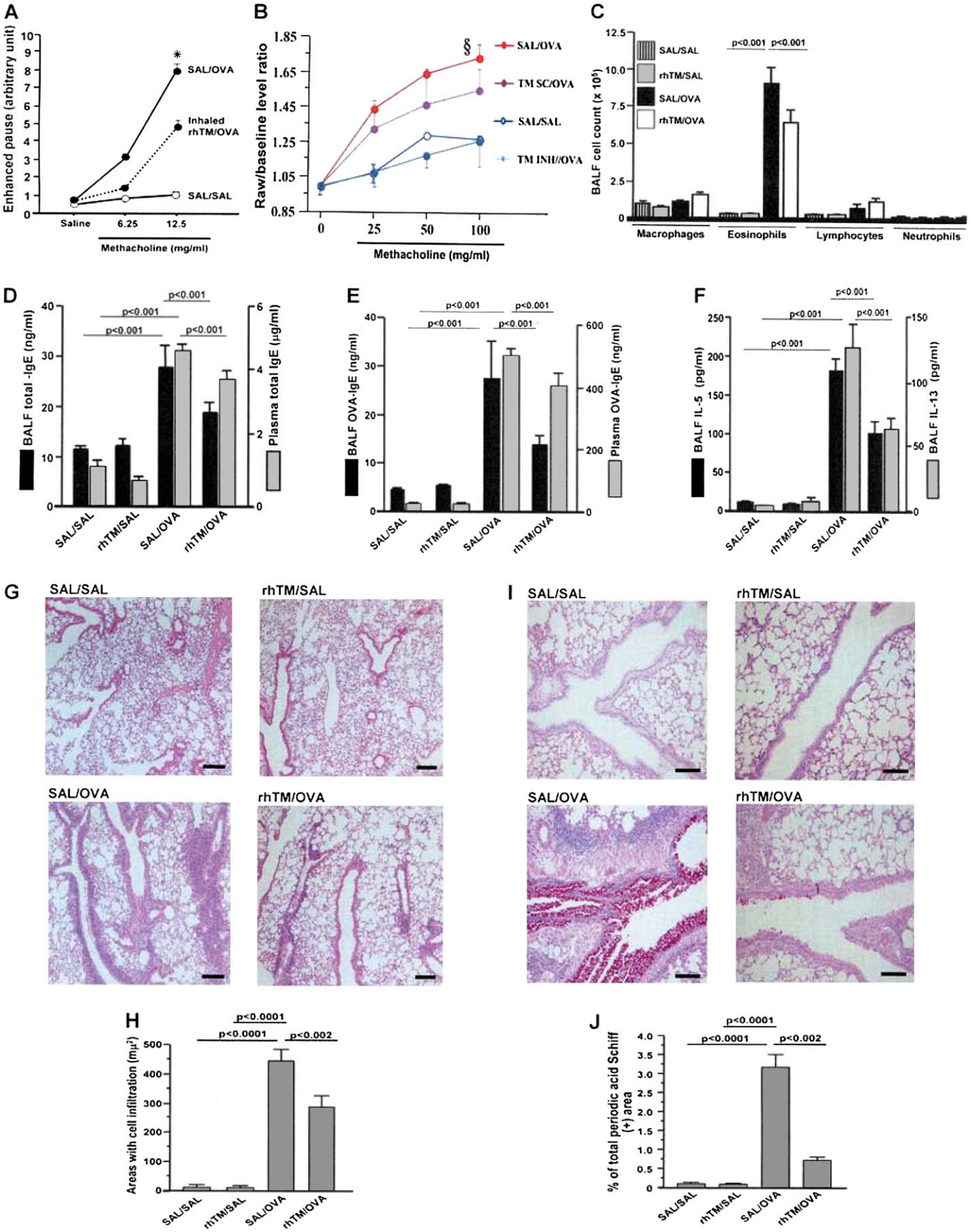

A
Enhanced pause (arbitrary unit)
SAL/OVA
Inhaled rhTM/OVA
SAL/SAL
Saline
6.25
12.5
Methacholine (mg/ml)
B
Raw/baseline level ratio
SAL/OVA
TM SC/OVA
SAL/SAL
TM INH//OVA
Methacholine (mg/ml)
C
BALF cell count (x 10⁵)
SAL/SAL
rhTM/SAL
SAL/OVA
rhTM/OVA
p<0.001
Macrophages
Eosinophils
Lymphocytes
Neutrophils
D
BALF total -IgE (ng/ml)
Plasma total IgE (µg/ml)
E
BALF OVA-IgE (ng/ml)
Plasma OVA-IgE (ng/ml)
F
BALF IL-5 (pg/ml)
BALF IL-13 (pg/ml)
G
SAL/SAL
rhTM/SAL
SAL/OVA
rhTM/OVA
I
SAL/SAL
rhTM/SAL
SAL/OVA
rhTM/OVA
H
Areas with cell infiltration (mµ²)
p<0.0001
p<0.002
J
% of total periodic acid Schiff (+) area
p<0.0001
p<0.002

Figure 1. Thrombomodulin (TM) treatment of mice protects against bronchial asthma. Mice were sensitized with ovalbumin + aluminum on Days 0 and 14, and challenged with ovalbumin by inhalation on Days 22, 23, 24, and 25. (*A*) Inhalation of 1 mg/kg of recombinant human (rh)TM administered on each day of the challenge inhibits airway hyperresponsiveness (AHR) measured as enhanced pause induced by increasing concentrations of methacholine. (*B*) Subcutaneous injection of 3 mg/kg rhTM administered weekly from the first day of sensitization also inhibits AHR measured invasively as airway resistance but with lower efficacy than inhaled rhTM. (*C*) The number of infiltrating eosinophils in bronchioalveolar lavage fluid (BALF) is reduced in mice treated with rhTM compared with groups receiving vehicle. (*D*, *E*) The concentration of total and ovalbumin-specific IgE is decreased in BALF and plasma from mice treated with rhTM compared with control mice. (*F*) The secretion of Th2 cytokines IL-5 and IL-13 in the lung is significantly suppressed by inhaled rhTM. (*G*, *H*) Hematoxylin and eosin staining shows airway remodeling along with enhanced infiltration of mononuclear cells in the peribronchiolar and perivascular areas in the lungs from mice with asthma, which is reduced in rhTM-treated mice. The scale bars indicate 200 μm. (*I*, *J*) Periodic acid-Schiff staining of lung sections from mice. n = 6 mice in each group. The scale bars indicate 100 μm. Error bars indicate SEM. Results shown here are representative of three separate experiments. SAL/SAL received saline by intraperitoneal injection and by inhalation; rhTM/SAL received saline by intraperitoneal injection and rhTM by inhalation; SAL/OVA received intraperitoneal injection of ovalbumin and inhaled saline; rhTM/OVA received intraperitoneal injection of ovalbumin and rhTM by inhalation. $^*P < 0.001$, compared with inhaled rhTM/OVA group. $\S P < 0.05$, compared with inhaled rhTM/OVA and subcutaneous rhTM/OVA groups. Raw = airway resistance.

involve thrombin (16). As aPC protects against bronchial asthma (17), we hypothesized that TM might also be protective in bronchial asthma. Some of the results of these studies have been previously reported in the form of an abstract (18).

METHODS

Additional detail on the materials and methods is provided in the online supplement.

Proteins

Plasmids that express TM-IgG2a Fc fusion proteins were derived from mouse TM cDNA (19) and mouse IgG2a cDNA (20). Proteins were expressed in COS cells and purified on anti-mouse IgG columns.

DC Generation and Maturation

Bone marrow–derived DCs were prepared as described (21). The cells were resuspended at 5×10^5 cells/ml in RPMI 1640 with 10% heat-inactivated fetal bovine serum containing 50 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and 3 ml/well in 6-well tissue culture plates was cultured. Recombinant human (rh)TM was added on Day 4 at a final concentration of 200 nM. CD141/TM$^+$ and CD141/TM$^-$ DCs were separated using anti-CD141 mAb and anti-rat IgG magnetic microbeads.

Experimental Bronchial Asthma

The Mie University Committee on animal investigation approved the experimental protocols, and the experiments were performed according to the guidelines for animal experiments of the National Institute of Health. Mice that lack the lectin domain (LeD mice) and receptor for advanced glycation end products (RAGE)-deficient mice have been previously characterized (22, 23). For induction of bronchial asthma, sensitized mice were exposed to aerosolized 2% ovalbumin in an exposure chamber on Days 22, 23, 24, and 25. AHR was measured on Day 26 and rechallenged with aerosolized 2% ovalbumin before killing on Day 27 (17).

Clinical Studies

Blood samples were drawn in tubes containing ethylenediaminetetraacetic acid from patients with stable chronic bronchial asthma (n = 8) (24). DCs were analyzed in whole blood after staining with anti-lineage-1–fluorescein isothiocyanate, anti-CD11c-PE-Cy5, and anti-human leukocyte antigen D-related (HLA-DR)-PE-Cy7. Informed consent was obtained from all participants before blood sampling. The study protocol was approved by the Mie University Hospital Institutional Review Board and the investigation was performed following the Helsinki Declaration.

Treatment with rhTM

Animals were treated with aerosolized 1 mg/kg rhTM for 20 minutes on Days 22, 23, 24, 25, and 26 immediately before each exposure to ovalbumin.

T Cell Stimulatory Activity of DCs

Before or after sorting into CD141/TM$^+$ and CD141/TM$^-$, DCs were stimulated with 100 μg/ml ovalbumin for 24 hours, cocultured with carboxyfluorescein succinimidyl ester–labeled CD4 T cells purified from OT-II mice, and proliferation of CD4 T cells was quantified (25).

Phagocytosis and Chemotaxis Assay

Phagocytosis (26) using fluorescein isothiocyanate–dextran was analyzed on a FACScan flow cytometer, and chemotaxis toward CCL3, CCL19, and CCL21 was analyzed by double-chamber assay (27).

Processing of DQ-ovalbumin by DCs

DCs were mixed with 0.1 mg/ml DQ-ovalbumin (DQ-OVA; Molecular Probes, Eugene, OR) for 15 minutes at 37°C and then washed with phosphate-buffered saline three times. Phagocytosis of DQ-OVA by DCs was defined as the percentage of DCs with positive fluorescent signals by flow cytometric analysis (28).

Statistics

All data are expressed as the mean ± SEM unless otherwise specified. The statistical difference between three or more variables was calculated by analysis of variance with *post hoc* analysis using Fisher predicted least significant difference test unless otherwise specified. The difference between the means of two variables was calculated by the Student *t* test or Mann-Whitney *U* test, depending on the distribution of the samples. Statistical analyses were performed using StatView 4.1 (Abacus Concepts, Piscataway, NJ) for the Macintosh. *P* less than 0.05 was considered as statistically significant.

RESULTS

Inhalation of Soluble TM Inhibits Allergic Bronchial Asthma

Balb/c mice were sensitized by intraperitoneal injection with ovalbumin, followed by aerosol challenge with ovalbumin. At the time of challenge, mice were treated by aerosol with rhTM (29). TM treatment reduced the severity of the disease, measured functionally either by enhanced pause or airway pressure (Figures 1A and 1B). TM was also efficacious when given subcutaneously, but to a lesser extent than when given by aerosol (Figure 1B). In bronchial asthma, bronchial alveolar lavage fluid (BALF) levels of IgE and total protein are increased, as are the number of cells, principally eosinophils (Figures 1C–1E). Treatment with TM reduced the total and ovalbumin-specific IgE, and the number of eosinophils in BALF. TM treatment also reduced the plasma levels of both total and specific IgE. As IL-5 and IL-13 are key mediators of allergic inflammation, their levels in BALF were determined. IL-5 and IL-13 levels were lower in animals treated with TM (Figure 1F). Lung histology of ovalbumin-challenged, untreated mice displayed airway remodeling with hyperplasia of mucin-

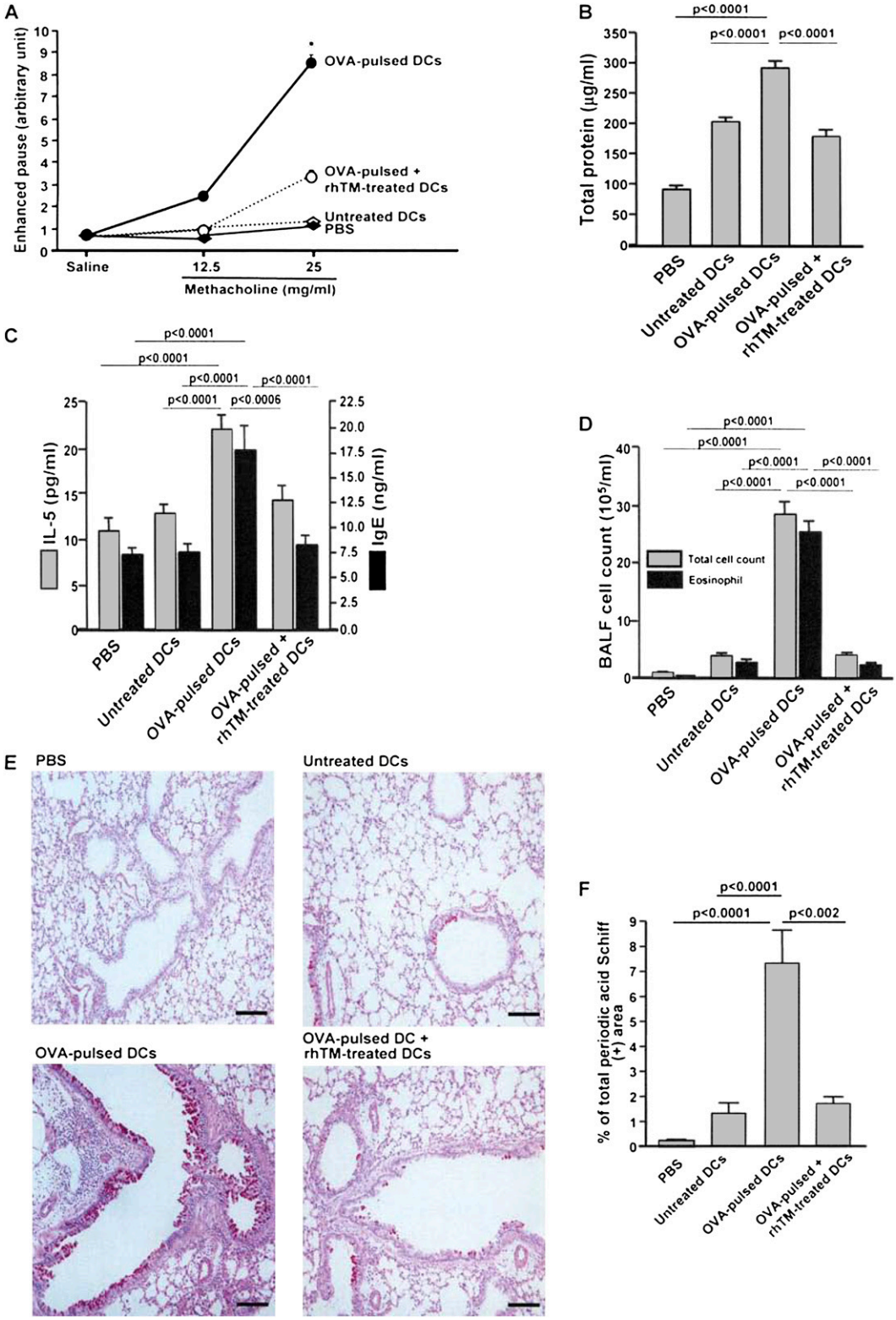


Figure 2. Adoptive transfer of thrombomodulin (TM)-treated dendritic cells (DCs) causes less bronchial asthma. (*A*) Ovalbumin (OVA)-pulsed DCs (10^6) treated with recombinant human (rh)TM *in vitro* were adoptively transferred to naive mice and airway hyperresponsiveness was measured. (*B–D*) The degree of lung inflammation measured by the concentration of total protein, IL-5, or IgE, and the number of inflammatory cells in bronchoalveolar lavage fluid (BALF). (*E, F*) Periodic acid-Schiff staining of lung sections from mice. The scale bars indicate 100 μm. n = 6 in each group. Error bars indicate SEM. Results shown here are representative of three separate experiments. Phosphate-buffered saline (PBS) group received PBS by intravenous injection by tail vein; untreated DCs group received intravenous control DCs; OVA-pulsed group received DCs pulsed with ovalbumin; OVA-pulsed + rhTM-treated DC group received DCs pulsed with ovalbumin and treated with rhTM. $^*P < 0.001$, compared with OVA-pulsed + rhTM-treated DCs.

secreting cells and inflammatory infiltrates (Figures 1G–1J). These findings were much reduced in TM-treated animals. Thus administration of TM during the challenge reduces asthma measured as lung function as well as its biochemical correlates, such as IgE. However, although the inhibitory activity of TM was statistically different, it was relatively weak at the dose of rhTM used in these experiments.

TM Treatment Alters DC Properties

DCs are professional antigen-presenting cells generated in the bone marrow before migrating through the blood to their target organs. On sensing a foreign body, DCs phagocytose it and then migrate to the draining lymph node where the processed antigen is presented to cognate T cells (6). Tolerogenic DCs can promote the development of regulatory T cells with

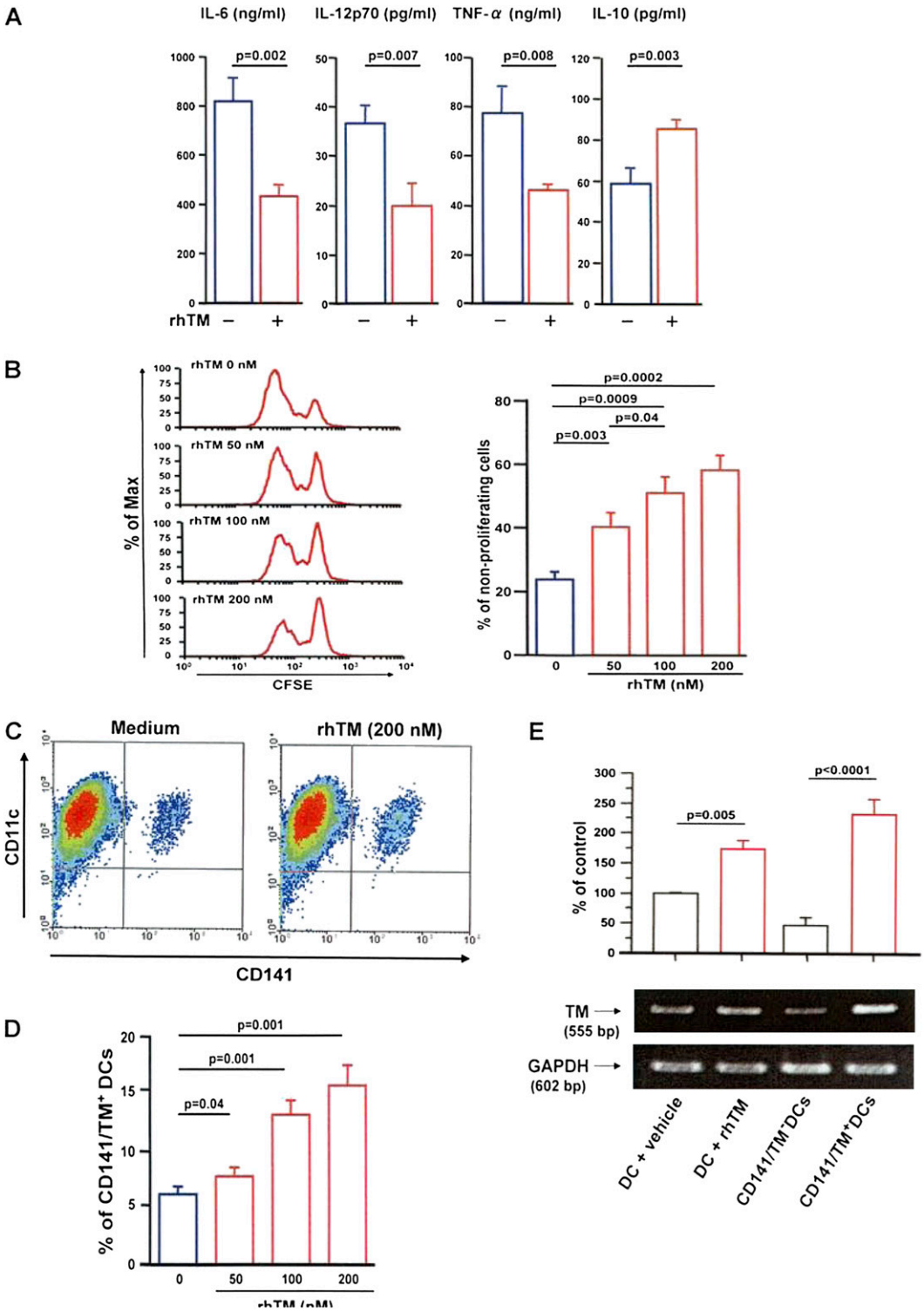


Figure 3. Thrombomodulin (TM) treatment modulates the properties of dendritic cells (DCs). (*A*) The concentration of cytokines was less in the supernatant of bone marrow–derived DCs (BMDCs) cultured for 24 hours in the presence of recombinant human (rh)TM. Bars represent the means of three independent experiments. (*B*) Effect of rhTM on T-cell stimulatory activity of BMDCs. BMDCs (C57BL/6) grown in the presence of the indicated concentrations of rhTM were cocultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4 T cells purified from OT-II mice. Proliferation of CD4 T cells was quantified by flow cytometry (*left*) and the percentage of live nonproliferating cells is shown as mean ± SD (*right*). Representative results from three independent experiments are shown. (*C*, *D*) Surface expression of CD141/TM in BMDCs is increased by rhTM treatment. Cell surface CD141/TM was analyzed by flow cytometry. The mean of three independent experiments is shown. Error bars indicate SD. (*E*) Expression of CD141/TM mRNA in BMDCs is increased by rhTM treatment. TM mRNA was evaluated by polymerase chain reaction PCR of RNA extracted from BMDCs treated with rhTM and in BMDCs sorted into CD141/TM$^-$ and CD141$^+$ DCs. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was analyzed as a control. Representative results from two separate experiments are shown. TNF = tumor necrosis factor.

suppressive activity. The number of regulatory T cells (CD4$^+$ CD25$^+$) in the lungs, mediastinal lymph, and spleen remained unaltered by treatment with inhaled TM, suggesting that the ability of TM to reduce asthma is not mediated by regulatory T cells (*see* Figures E1A–E1F in the online supplement).

Bronchial asthma can be induced in this model by treating naive DCs with a pulse of ovalbumin before adoptive transfer to naive host mice, which, on aerosol challenge with ovalbumin, then develop disease that is similar to that seen in animals sensitized intraperitoneally with ovalbumin. We tested whether TM could modulate mouse DCs generated *in vitro* from bone marrow cells by culturing in GM-CSF for 6 days and then matured by incubation with an ovalbumin pulse for 24 hours. During the differentiation phase, DCs were treated with TM *in vitro* for 2 days before the ovalbumin pulse, adoptive transfer, and challenge. DCs can be divided into two broad subsets in both mouse and human: mDC and pDC, which differ in their lineage markers and in the cytokines they secrete (9, 10). Differentiated bone marrow cells displayed the characteristic surface markers of mDCs and not pDCs (CD11b$^-$ B220$^+$ and CD11b$^-$ CD45RB$^+$ cells), irrespective of the presence of TM (Figure E1G).

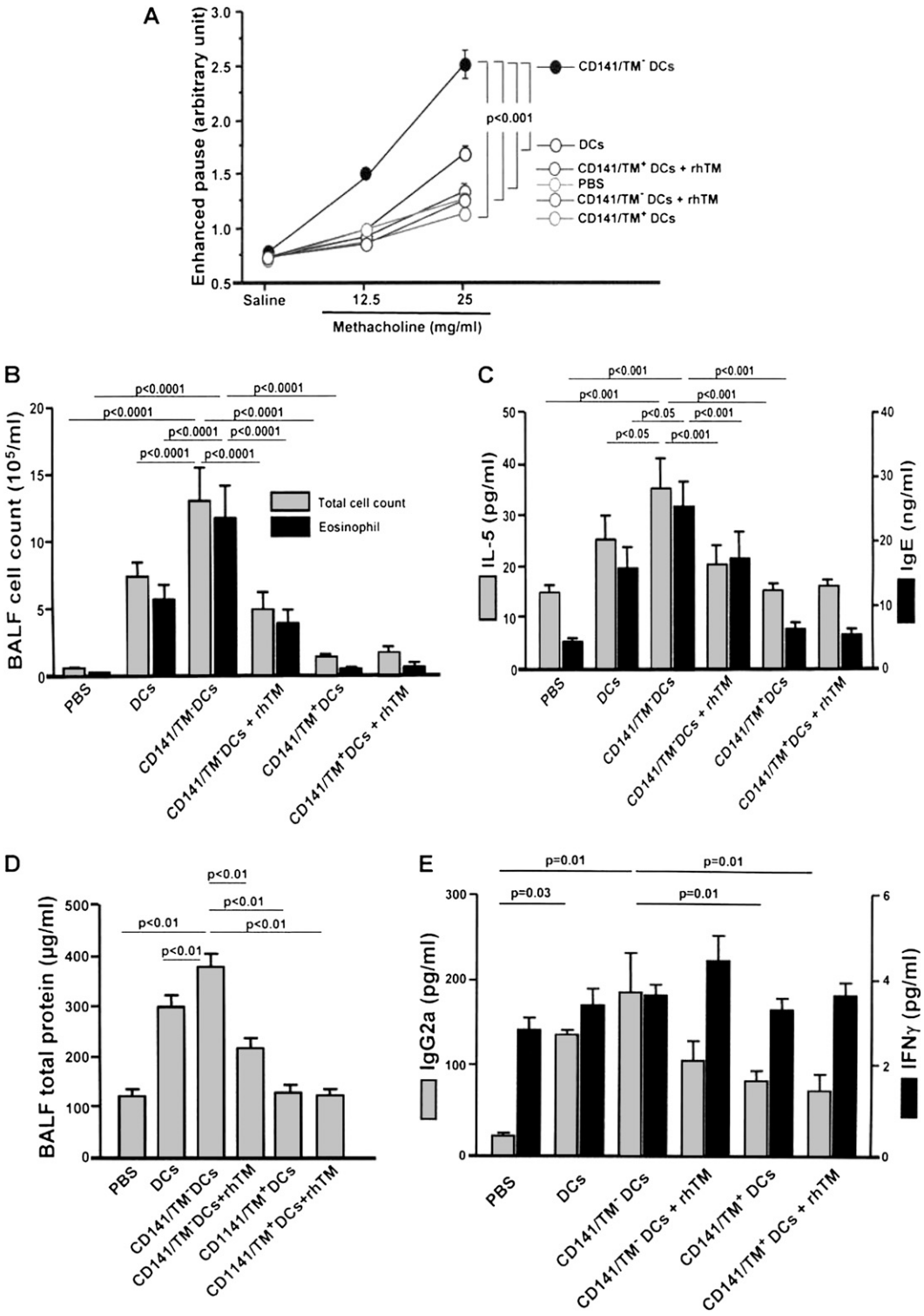
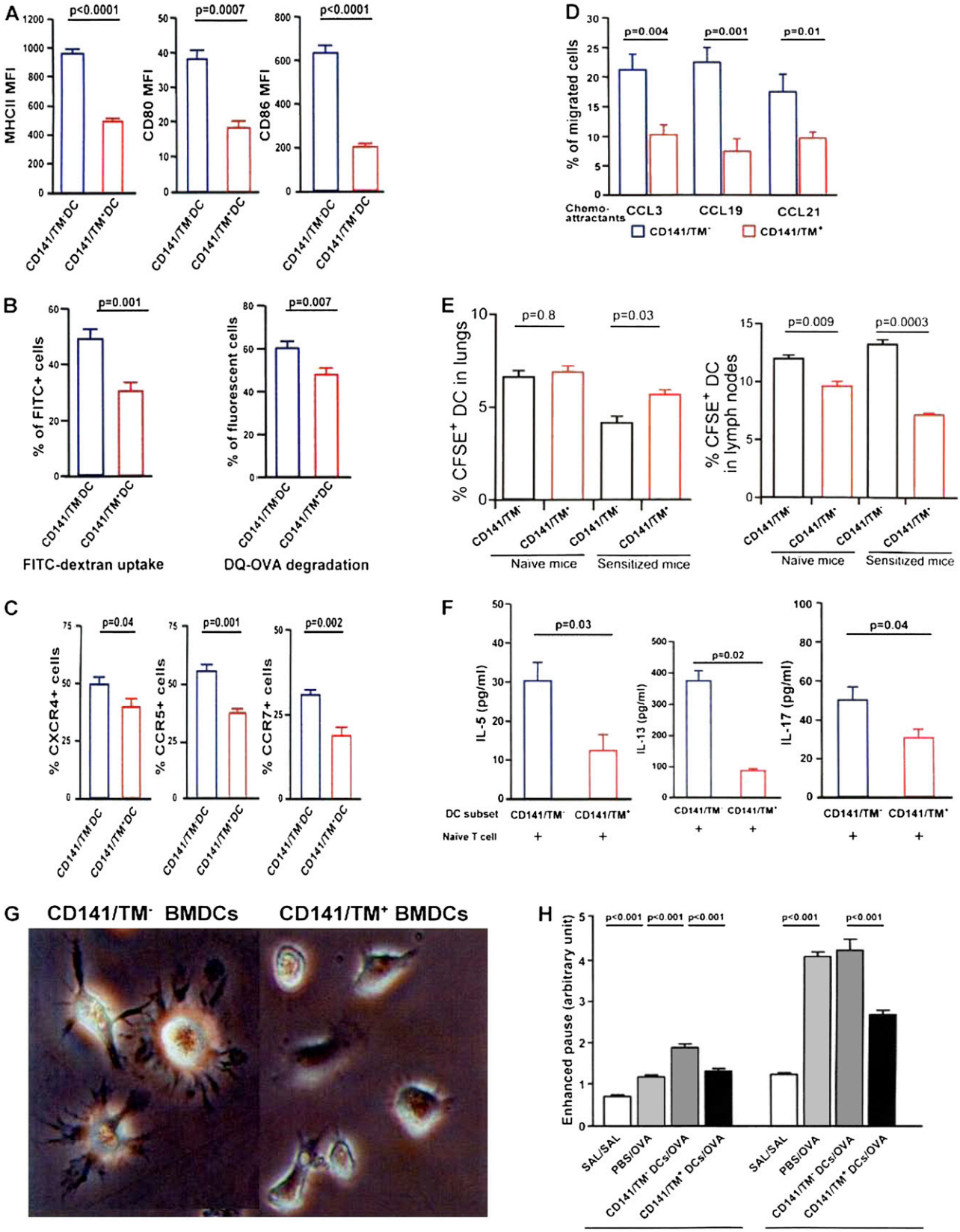


Figure 4. CD141/thrombomodulin (TM)$^+$ dendritic cells (DCs) protect from and CD141/TM$^-$ DCs exacerbate asthma. CD141/TM$^+$ and CD141/TM$^-$ DCs were purified by magnetic separation. DCs were stimulated with 100 μg/ml of ovalbumin for 24 hours and 10^6 cells were intravenously injected to BALB/c mice (n = 6); each group of mice was challenged with 2% ovalbumin for 20 minutes on 4 consecutive days. (*A*) Airway hyperresponsiveness was measured in response to methacholine. (*B–D*) The degree of lung inflammation was measured as the concentration of total protein, IL-5, or IgE, or the number of eosinophils or total cells in bronchoalveolar lavage fluid (BALF). (*E*) The Th1 cytokine IFN-γ and Th1 antibody IgG2a were measured and compared between groups. n = 6 in each group. Error bars indicate SEM. Results shown here are representative of three separate experiments. SAL/SAL received saline by intraperitoneal injection for sensitization and by intravenous injection; PBS/OVA group were sensitized with ovalbumin by intraperitoneal injection and treated intravenously with phosphate-buffered saline (PBS); CD141/TM$^-$ DCs/OVA were sensitized with ovalbumin and treated intravenously with CD141/TM$^-$ DCs; CD141/TM$^+$ DCs/OVA were sensitized with ovalbumin and treated intravenously with CD141/TM$^+$ DCs. After the magnetic separation a group of CD141/TM$^-$DCs (CD141/TM$^-$DCs + rhTM) and another group of CD141/TM$^+$DCs (CD141/TM$^+$DCs + rhTM) were further treated with recombinant human (rh)TM before adoptive transfer.

Adoptive transfer of TM-treated DCs reduced the severity of asthma measured by lung function and resulted in diminished BALF levels of total protein, IgE, IL-5, leukocytes, and eosinophils (Figures 2A–2D). Hyperplasia of mucin-secreting cells was also reduced in the lungs of mice that received a transfer of TM-treated DCs (Figures 2E and 2F). Thus, TM treatment of DCs *in vitro* interfered with their ability to induce asthma. The effect was independent of the role of TM in coagulation or complement, because PC activation and factor D were undetectable in the rhTM-containing culture media (data not shown). This raises the possibility that the lectin domain of TM might be involved (30). Overall, these *in vitro* findings may explain how *in vivo* administration of recombinant TM reduces asthma. We therefore investigated the effects of TM on the properties of DCs.

DCs treated with ovalbumin display increased expression of the surface markers of DC maturation, including major histocompatibility complex (MHC) class II, CD80, and CD86. When DCs were differentiated in the presence of TM, expression of these markers was dose-dependently reduced on the surface of



DCs (Figures E2A and E2B). Although the inhibitory activity of TM on expression of DC maturation markers is statistically different from control, it was relatively weak at the dose of rhTM used in these experiments. The phagocytosis and antigen-processing capacity of DCs was also reduced by TM treatment (Figure E2C). DCs migrate along chemokine gradients using chemokine receptors to sense chemoattractants. TM treatment of DCs reduced the expression of the chemokine receptors, CXCR4, CCR5, and CCR7 (Figure E2D). When migration toward the chemokines for these receptors (CCL3, CCL19, and

Figure 5. CD141/ thrombomodulin (TM)$^+$ dendritic cells (DCs) differ from CD141/TM$^-$ DCs. (*A–D*) CD141/TM$^-$ and CD141/TM$^+$ DCs were stimulated with ovalbumin. The surface expression of major histocompatibility complex (MHC) II, CD80, CD86, chemokine receptors (CXCR4, CCR5, CCR7), and the antigen uptake and processing capacity were analyzed by flow cytometry. Chemotaxis toward CCL3, CCL19, and CCL21 was analyzed by double-chamber assay. Representative results from three independent experiments are shown. (*E*) Mice were adoptively transferred with carboxyfluorescein succinimidyl ester (CFSE)-labeled CD141/TM$^+$ DCs and CD141/TM$^-$ DCs and the number of each type of DC homing to the lungs and regional lymph nodes was calculated. Error bars indicate SD. (*F*) CD141/TM$^-$ and CD141/TM$^+$ DCs (C57BL/6) were stimulated with ovalbumin and cocultured with naive CD4 T cells from OT-II mice. Supernatants were assayed for IL-5, IL-13, and IL-17. Representative results from two separate experiments are shown. Error bars indicate SD. (*G*) Phase contrast microscopy of CD141/TM$^+$ and CD141/TM$^-$ DCs. (*H*) Bone marrow cells were differentiated in the presence of recombinant human (rh)TM for Days 4 to 6. On Day 7, bone marrow–derived DCs (BMDCs) were sorted into CD141/TM$^+$ and CD141/TM$^-$ DCs and pulsed with ovalbumin for 24 hours before adoptive transfer to Balb/c mice (n = 4 in each group) sensitized with ovalbumin. After 7 days, the mice were challenged with aerosol ovalbumin and airway hyperresponsiveness was measured. Error bars indicate SD. Results shown here are representative of three separate experiments. SAL/SAL received saline for sensitization and treatment; PBS/OVA group were sensitized with ovalbumin and treated with phosphate-buffered saline (PBS); CD141/TM$^-$ DCs/OVA were sensitized with ovalbumin and treated with CD141/TM$^-$ DCs; CD141/TM$^+$ DCs/OVA were sensitized with ovalbumin and treated with CD141/TM$^+$ DCs. FITC = fluorescein isothiocyanate.

CCL21) was measured, TM-treated DCs migrated less, showing that the reduced levels of the chemokine receptors resulted in a lower response (Figure E2E). Lower amounts of proinflammatory cytokines are secreted by rhTM-treated DCs, whereas the antiinflammatory cytokine IL-10 is increased (Figure 3A). DCs interact with cognate T cells in lymph nodes, stimulating their clonal proliferation. DCs treated with TM suppress *in vitro* proliferation of cognate T cells purified from OT-II mice whose T-cell receptor is directed toward an ovalbumin peptide (Figure 3B). Interestingly, expression of TM itself (CD141) was induced on the surface of DCs by TM treatment of DCs, at least in part due to induction of TM mRNA (Figures 3C–3E, Figure E2F).

Treatment with GM-CSF induces incomplete maturation of DCs. We investigated whether induction of TM and inhibition of maturation by culturing with TM is due to the use of GM-CSF for differentiation into DCs or could be caused by TM during differentiation of DCs from bone marrow cells with Flt3-L instead of GM-CSF or with GM-CSF in the presence of lipopolysaccharide to cause further maturation. As with GM-CSF treatment, these other treatments also gave rise to less mature DCs that still were able to respond to culturing with TM by induction of CD141/TM expression and reductions in expression of the maturation markers, MHC class II, CD80, and CD86 (Figures E3A and E3B). Thus, irrespective of their state of maturation or agent used for induction of differentiation, TM treatment of DCs reduces expression of maturation markers and increases TM expression.

CD141/TM$^+$ DCs Are Tolerogenic; CD141/TM$^-$ DCs Are Immunogenic

We studied the functional differences between TM$^+$ and TM$^-$ DCs to see whether these subsets of DCs were responsible for the differences between TM-treated and control DCs. BMDCs were cultured in the presence of rhTM for 2 days before pulsing them with ovalbumin. We sorted the TM-treated DCs into CD141/TM$^+$ and CD141/TM$^-$ DCs before adoptive transfer into naive animals and challenging them. The purity of the sorted cells was greater than 93% (Figure E4). Mice that were adoptively transferred with CD141/TM$^-$ DCs had more disease than those transferred with control, unsorted DCs (Figure 4A). Animals adoptively transferred with CD141/TM$^+$ DCs were less hyperresponsive to ovalbumin, showing that DCs in which CD141/TM had been induced were tolerogenic. Adoptive transfer of CD141/TM$^-$ DCs exacerbated the increases in BALF levels of cells and eosinophils, IL-5, IgE, and total protein, as compared with control DCs (Figures 4B–4D). In contrast, adoptive transfer of CD141/TM$^+$ DCs caused a reduction in these levels. It is noteworthy that treatment with rhTM did not affect the expression of the Th1 cytokine interferon-γ but did significantly alter that of the Th1 antibody IgG2a (Figure 4E). Overall, these observations suggest that TM$^-$ DCs are immunogenic and the TM$^+$ DCs are tolerogenic.

To see whether CD141/TM$^+$ and CD141/TM$^-$ DCs could change their properties after sorting, CD141/TM$^+$ DCs were incubated during the ovalbumin pulse without TM and CD141/TM$^-$ DCs with TM. Although the properties of CD141/TM$^+$ DCs did not change, remaining tolerogenic, the immunogenic properties of CD141/TM$^-$ DCs were suppressed by 24 hours of TM treatment (Figures 4A–4E). Thus TM$^+$ DCs did not lose their TM expression or their tolerogenic properties by 24 hours in the absence of TM in the culture medium. In contrast, some TM$^-$ DCs had acquired a less immunogenic phenotype by treatment with TM.

As TM treatment of DCs during differentiation resulted in lower expression of maturation markers and changes in antigen presentation and T-cell stimulation, we investigated whether these properties were different in the CD141/TM$^+$ and CD141/TM$^-$ DC subsets. CD141/TM$^+$ DCs have lower levels of maturation markers (MHC class II, CD80, and CD86); exhibited less phagocytic capacity, reduced antigen processing, and less expression of the chemokine receptors CXCR4, CCR5, and CCR7; and migrated less toward the chemokines CCL3, CCL19, and CCL21 (Figures 5A–5D, Figure E5A–E5C). The decreased capacity for phagocytosis is probably due to downregulation of the mannose receptor, CD206, by TM (Figure E4D). Because CD141/TM$^+$ DCs migrated less *in vitro*, changes in migration of lung DCs in response to TM treatment were measured by challenging sensitized mice with fluorescent-labeled ovalbumin. In mice treated with TM there were fewer total DCs that had taken up fluorescent-labeled ovalbumin in the draining mediastinal lymph node and more in the lung than in untreated animals (Figures E6A and E6B). In addition, homing of CD141/TM$^+$ DCs to the lungs was significantly higher but lower to the mediastinal lymph nodes after adoptive transfer to sensitized mice. In naive mice, homing of CD141/TM$^+$ DCs to mediastinal lymph nodes was also significantly decreased after adoptive transfer (Figure 5E). CD141/TM$^+$ DCs secrete fewer proinflammatory cytokines but more antiinflammatory IL-10 compared with CD141/TM$^-$ DCs (Figure E7A). CD141/TM$^+$ DCs also stimulated cognate T cells to proliferate less (Figure E7B) and the production of Th2 and Th17 cytokines was reduced in the mixed cultures of DCs with T cells in comparison to CD141/TM$^-$ DCs (Figure 5F). CD141/TM$^+$ DCs are smaller and more spherical than CD141/TM$^-$ DCs and possess fewer dendrites (Figure 5G). CD141/TM$^+$ DCs from the lungs, spleens, and bone marrow of naive mice were characterized and found to be negative for lineage

TABLE 1. CHARACTERIZATION OF CD141/THROMBOMODULIN$^+$ FROM MOUSE LUNGS, SPLEEN, AND BONE MARROW

Marker	Spleen	Bone marrow	Lungs
CD11c	++	++	++
MHCII	+	+	+
CD86	+	+	±
CD80	+	+	±
CD4	−	−	−
CD8	−	−	−
B220	−	−	−
CD45RB	−	−	±
CD11b	++	++	+
CD205	−	−	−

CD11c-positive cells were isolated from the lungs and spleen of naive BALB/c mice using anti-CD11c–labeled beads, gated for CD141/thrombomodulin ™$^+$, and the presence of surface markers was assessed by flow cytometry as strongly positive (++), weakly positive (+), barely detectable (±), or negative (−) for the markers. Bone marrow–derived dendritic cells (BMDCs) derived by treatment with granulocyte-macrophage colony-stimulating factor were separated into CD141/TM$^+$ and similarly analyzed by flow cytometry.

markers as well as surface markers of plasmacytoid DCs (Table 1). In the lungs of naive animals, 10.73% of the DCs were CD141/TM$^+$ DCs (Figure E7C).

CD141/TM$^+$ DCs Actively Suppress Asthma

The *in vivo* findings that CD141/TM$^+$ DCs caused less asthma could be due either to CD141/TM$^+$ DCs being passive or to CD141/TM$^+$ DCs actively suppressing the disease. Therefore, we determined whether CD141/TM$^+$ DCs could reduce the severity of disease after sensitization of the mice by administering ovalbumin intraperitoneally with a subsequent challenge of ovalbumin. In comparison to animals adoptively transferred with DCs matured with an irrelevant allergen, animals treated with CD141/TM$^+$ DCs were less hyperresponsive, whereas animals treated with CD141/TM$^-$ DCs had exacerbated asthma (Figure 5H). Thus CD141/TM$^+$ DCs have the ability to actively suppress the ongoing disease process.

TM Lectin Domain Interacts with DCs

TM is a transmembrane glycoprotein, ubiquitously expressed by endothelial cells (ECs) consisting of several domains with disparate functions, including binding to thrombin (epidermal growth factor [EGF] domains 5+6), activation of PC (EGF 4–6) and TAFI (EGF 3–6), and antagonism of high-mobility group protein B1 (HMGB1) by the lectin domain (15, 31, 32). Use of deletion fragments of mouse TM fused to the Fc domain of mouse IgG to stimulate DCs showed that only molecules containing the lectin domain were able to decrease maturation of DCs (Figure E8), suggesting that the lectin domain was responsible for altering the properties of DCs. To further clarify the biological relevance of the TM lectin domain, DCs from LeD mice (22) were shown to have lower CD141/TM expression than DCs from wild-type (WT) mice, suggesting that the lack of the lectin domain prevented expression of TM on DCs (Figure 6A). In contrast, the level of TM expression on ECs is not affected by loss of the lectin domain in these mice (22). DCs from LeD mice responded to the presence of TM during differentiation by increasing their CD141/TM expression, showing that cells lacking that domain still respond to exogenous TM containing the lectin domain (Figure 6A). To confirm the role of the TM lectin domain in bronchial asthma, bone marrow DCs from WT and LeD mice were prepared, pulsed with ovalbumin, and transferred intratracheally to WT mice (33). WT mice treated with ovalbumin-pulsed DCs from LeD mice had more AHR than WT mice treated with ovalbumin-pulsed DCs from WT mice (Figure 6B), suggesting that the lectin domain is probably critical for the inhibition of bronchial asthma.

Human Monocyte-Derived DCs Respond to TM

We tested whether full-length membrane-bound TM could also modulate DCs. By incubating human DCs with human lymphatic ECs, umbilical vein ECs, and alveolar epithelial cells, membrane-bound TM reduces the expression of maturation markers on human DCs (Figure 7A, Figure E9). This effect was reversed when we added polyclonal antibodies directed against

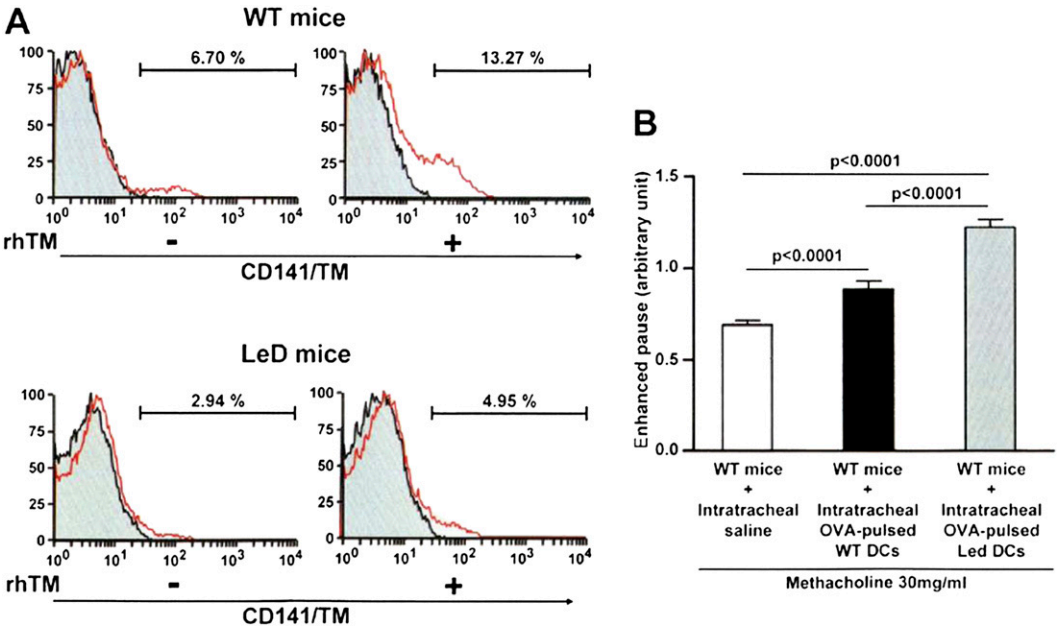


Figure 6. The lectin domain is critical for the protective effect of thrombomodulin (TM). (*A*) Mouse bone marrow–derived dendritic cells (BMDCs) from wild-type (WT) mice and mice lacking the lectin domain (LeD) were separated and cultured for 6 days in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant human (rh)TM was added from Day 4 to 6. On Day 7, BMDCs were cultured in the presence of ovalbumin (OVA) and rhTM for 24 hours and the expression of CD141/TM was evaluated by flow cytometry. Representative results from three independent experiments are shown. (*B*) Mice with BMDCs transferred from LeD mice have more hyperresponsiveness than mice with WT DCs. A total of 10^6 BMDCs prepared from WT and LeD mice were adoptively transferred to C57BL/6 mice (n = 4 in each group) and challenged with ovalbumin, and airway hyperresponsiveness was measured. Representative results from two independent experiments are shown. Error bars indicate SEM.

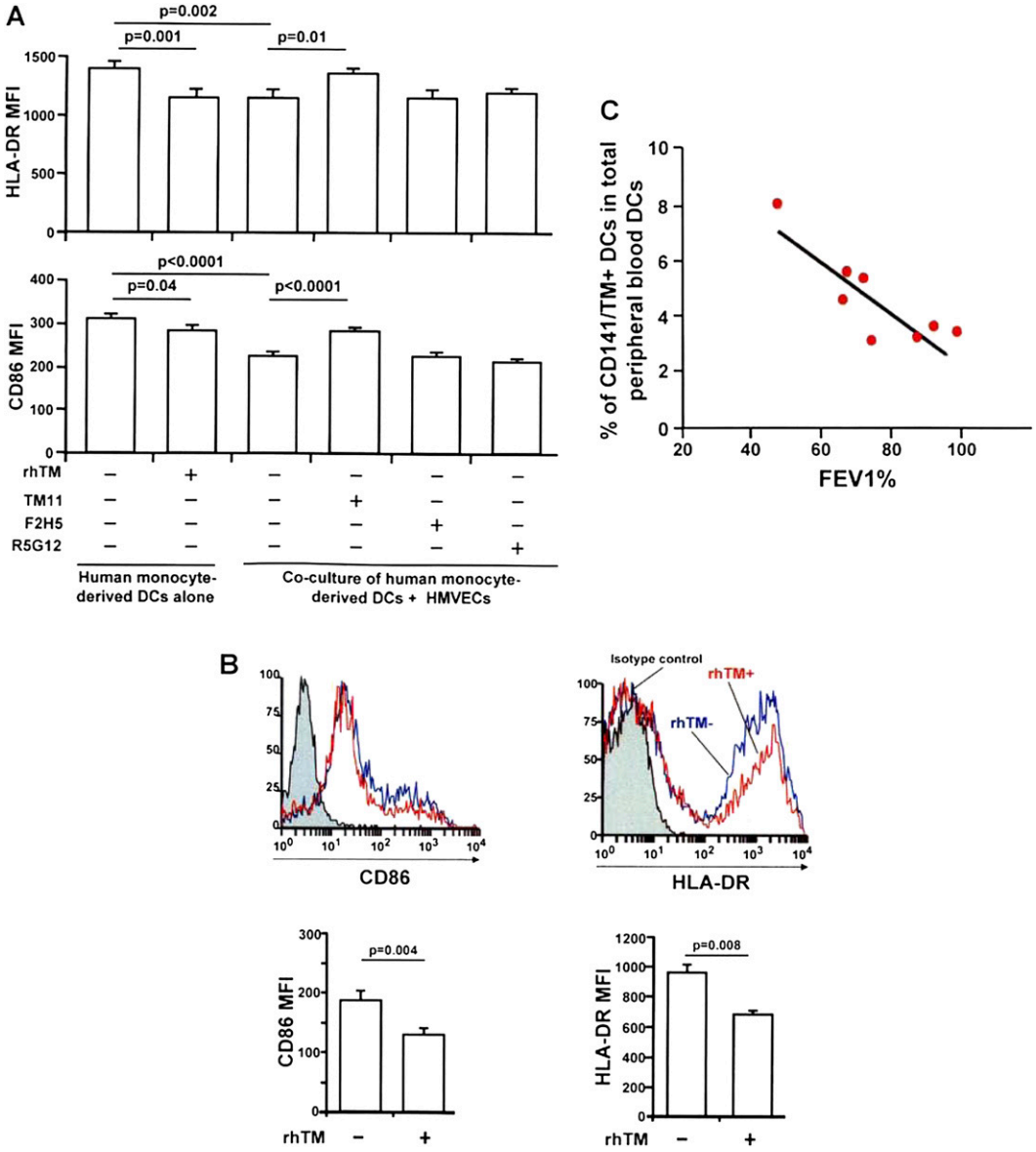


Figure 7. Thrombomodulin (TM) expressed on lymphatic endothelial cells reduces expression of maturation markers on human dendritic cells (DCs) and the percentage of TM$^+$ DCs is inversely correlated with lung function in patients with asthma. (*A*) Human monocytes were differentiated for 5 days with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 in the presence of recombinant human (rh)TM or a semiconfluent monolayer culture of normal human lymphatic microvascular endothelial cells (HMVECs). Polyclonal anti-TM (TM11) antibody and monoclonal anti-TM antibodies that inhibit thrombin binding (F2H5) and protein C or thrombin-activatable fibrinolysis inhibitor activation (R5G12) were added to the culture. Expression of MHC II and CD86 was analyzed by flow cytometry. Representative results from three independent experiments are shown. Statistics by analysis of variance with Dunn test as *post hoc* analysis. Error bars indicate SD. (*B*) Human monocytes were differentiated for 5 days in medium containing rhTM. Expression of maturation markers (MHC II and CD86) was analyzed by flow cytometry. Representative results from three independent experiments are shown. Error bars indicate SD. (*C*) Blood from patients with asthma (n = 8) was sampled and the expression of CD141/TM was evaluated on DCs gated using antibodies against human leukocyte antigen D–related (HLA-DR) and CD11c. A total of 120,000 events was analyzed two times by flow cytometry in each patient blood sample. Lung function was measured as FEV_1% in patients with bronchial asthma ($r = -0.7$; $P < 0.05$).

the whole TM molecule (TM11) but not when we added antibodies directed specifically against the thrombin-binding region (F2H5) or the PC and TAFI-activation domains (R5G12) (29). Thus these data confirm that the lectin domain of TM is responsible for regulating the expression of DC maturation markers and that membrane-bound TM was effective in modulation of DCs (Figure 7A, Figure E9). Similarly to mouse BMDCs, rhTM was also found to inhibit the increase in the expression of maturation markers (HLA-DR, CD86) and to increase the expression of TM (data not shown) on human monocyte–derived DCs (Figure 7B).

Levels of CD141/TM$^+$ DCs Inversely Correlate with Lung Function in Patients with Asthma

The number of CD141/TM$^+$ DCs in the peripheral blood of patients with asthma, determined by flow cytometry, correlated with airflow limitation. A significant and inverse correlation was found between the percentage of CD141/TM$^+$ DCs in total peripheral blood DCs and airflow limitation. Along with the preceding data, these findings are consistent with the hypothesis that CD141/TM$^+$ DCs ameliorate the disease (Figure 7C). Although there was an increase in CD 141/TM$^+$ DCs in patients with asthma as had been previously reported, the dose of steroids was not significantly associated with the number of peripheral DCs in patients with asthma (data not shown).

DISCUSSION

Originally we had hypothesized that TM would protect by activation of PC and/or TAFI, but instead found that the lectin domain was responsible for modulating the immunogenic properties of DCs. Surprisingly, TM treatment of DCs led to its own induction. That observation allowed us to sort the cells into CD141/TM$^+$ and CD141/TM$^-$ subsets, with the CD141/TM$^+$ DCs being tolerogenic and the CD141/TM$^-$ DCs being immunogenic. Thus administration of TM protects against

asthma because its lectin domain interacts with DCs, inducing CD141/TM on their surface and causing them to become tolerogenic.

The lectin domain of TM binds to Lewis Y (34), a blood group antigen, and is reported to interact directly with cells (22) and to antagonize HMGB1 (32). TM could interact with DCs via its own yet-to-be-identified receptor and/or by binding HMGB1, preventing its signaling through RAGE, which is known to stimulate maturation and activation of DCs. In the lung, when a foreign antigen is detected by DCs, the environment in which the DCs phagocytose the antigen and migrate to the draining lymph node will determine the subsequent behavior of that DC. In a scenario of ongoing inflammation, coagulation activation, and/or infection, TM can be oxidized, losing its ability to activate PC (35), be cleaved from the EC cell surface by proteases (36), and be down-regulated by inflammatory cytokines (37), and these conditions favor the activation of immunogenic DCs. Thrombin generated during asthmatic inflammation may exacerbate the immunogenic response through its receptors. Thus, the immunoregulatory effect of TM could also be explained by its ability to inhibit thrombin. Alternatively, in quiescent, noninflammatory conditions, the migrating DCs will be able to interact with intact TM on endothelial cells, causing inhibition of allergic responses (Figure E10).

TM (CD141, BDCA-3) is currently used in panels of surface antigens to identify subsets of DCs in human peripheral blood (38). At least three DC subsets have been identified: BDCA-2$^+$/BDCA-4$^+$/CD123$^+$ pDC and two mDC subtypes, BDCA-1$^+$/CD11c$^+$/HLA-DR$^+$ (mDC1) and BDCA-3$^+$/CD32$^-$/HLA-DR$^+$ (mDC2). Increased numbers of the CD141/TM$^+$DC subset have been reported in peripheral blood from patients with infection, malignancy, autoimmune disease, and allergy (39–43), yet the relevance of these changes has not been delineated. Yerkovich and colleagues (41) showed that after allergen challenge the constitutive expression of TM on DCs was increased in atopic individuals compared with nonatopic subjects, and that CD141/TM$^+$ DCs are more prevalent in the peripheral blood of subjects with allergy and asthma compared with nonatopic subjects without asthma; the same group of investigators has also shown that TM$^+$ DCs are associated with Th2 polarized response, whereas TM$^-$ DCs are associated with a mixed Th1/Th2 response. In addition, McCarthy and colleagues reported that TM$^+$ DCs were more frequent in subjects with asthma than in healthy control subjects (44). The clinical significance of these findings, however, was an enigma because correlation of the peripheral number of CD141/TM$^+$DCs with lung functional parameters was not reported. In the present study, in addition to their high number in the peripheral blood of subjects with asthma compared with control subjects, the percentage of CD141/TM$^+$ DCs was significantly and inversely correlated with the percent forced expiratory volume in 1 second, a marker of airflow limitation in patients with asthma. These observations suggest that the increase in the number of TM$^+$ DCs in the peripheral blood is a compensatory response. However, because mouse and human TM may have different biological activities, the possibility of a detrimental effect of TM in humans cannot be completely ruled out.

In our *in vivo* studies we showed that inhalation of TM ameliorates bronchial asthma in mice and that modulation of the antigen-presenting properties and migratory ability of mDCs is critical for this inhibitory activity of TM. Taken together, these observations suggest that CD141/TM$^+$DCs have suppressive activity on allergic responses, and thus the reported increase in their circulating numbers in several diseases could be a compensatory, protective response. Human CD141/TM$^+$ DCs have reduced allostimulatory capacity *in vitro* and high expression of IRF-8, a transcription factor required for DC tolerogenic function, which is consistent with the protective activity of TM in bronchial asthma (45, 46). Alternatively, it has been proposed that BDCA3$^+$ DCs are the human equivalent of mouse CD8a$^+$ DCs that actively phagocytose necrotic cells (47). The results of this study show that TM is a modulator of DC immunostimulatory properties and a novel candidate drug for the prevention of bronchial asthma in atopic patients whose disease is quiescent.

Author Disclosure: T.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. O.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.B.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.G.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.N.D.-G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.M.C. holds a patent from the VIB, Belgium for the use of the lectin-like domain of thrombomodulin to treat inflammation. S.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.C.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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ONLINE DATA SUPPLEMENT

Inhibition of Allergic Bronchial Asthma by Thrombomodulin is mediated by Dendritic Cells

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Materials and Methods

Proteins. Soluble recombinant human (rh) TM (ART123; rhTM) was supplied by Asahi Kasei Corporation (Tokyo, Japan). The rhTM was clinical grade material approved for use in Japan and therefore does not contain LPS. The ovalbumin was purchased from Sigma-Aldrich and contains a low level of LPS. Plasmids that express TM-IgG2a Fc fusion proteins were derived from mouse TM cDNA (E1) and mouse IgG2a cDNA (E2). Proteins were expressed in COS cells and purified on anti-mouse IgG columns.

DC generation and maturation. Bone marrow-derived DCs were prepared as described (E3). The cells were resuspended at 5 x 10^5 cells/ml in RPMI1640 with 10% heat-inactivated fetal bovine serum (FBS) containing 50 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and 3 ml/well in 6-well tissue culture plates was cultured. Normal serum containing 19.2 ± 6.9 ng/ml high-mobility group box 1 protein (HMGB1) was passed over an anti-HMGB1 IgY column to prepare serum with low HMGB1 levels (2.2 ± 0.6 ng/ml). rhTM was added on day 4 at a final concentration of 200 nM. To induce maturation, cells were cultured for an additional 24 h in the presence of ovalbumin (200 μg/ml). CD141/TM^+ and CD141/TM^- DCs were separated using anti-CD141 mAb (R&D, Minneapolis, MN) and anti-rat IgG magnetic microbeads (Miltenyi Biotec, bergisch Gladbach, Germany). The purity of both the TM^+ and TM^- DC preparations was ⩾93%.

Experimental bronchial asthma. Mie University Committee on animal investigation approved the experimental protocols, and the experiments were performed according to the guidelines for animal experiments of the National Institute of Health. LeD mice from Dr E. Conway (University of Leuven) were previously characterized (E4). All experiments were repeated at least three times with similar results on each occasion. Female Balb/c mice were sensitized by an intraperitoneal injection of 10 μg of aluminium (Pierce Biotechnology Inc., Rockford, IL)-precipitated ovalbumin (Sigma, St. Louis, MO) on days 0 and 14. Control animals received an intraperitoneal injection of normal saline on days 0 and 14. For induction of bronchial asthma, sensitized mice were exposed to aerosolized 2% ovalbumin in an exposure chamber (MIPS, Osaka, Japan) on days 22, 23, 24 and 25; AHR was measured on day 26, rechallenged with aerosolized 2% ovalbumin before sacrifice by pentobarbital overdose on day 27 (E5).

Quantification of histological findings. The positive areas for periodic acid Schiff or nucleated cells were counted using an Olympus BX50 microscope combined with an Olympus DP70 digital camera (Tokyo, Japan) using the WinROOF image processing software (Mitani Corp., Fukui, Japan) for Windows. An average of 10 photos of high magnification areas were taken at random from all mice of each group and the total area with nucleated cells or the percentage of positive areas for periodic acid Schiff was calculated.

Clinical studies. Blood samples were drawn in tubes containing EDTA from patients with stable chronic bronchial asthma (n=8) (E6). DCs were analysed in whole blood after staining with anti-lineage-1-FITC (anti-CD3, -CD14, -CD16, -CD19, -CD20, -CD56), anti-CD11c-PE-Cy5, and anti-human leukocyte antigen D-related (HLA-DR)-PE-Cy7 (all from BD Biosciences, San Jose, CA). A total of 120,000 events was analysed by flow cytometry using CellQuest (Becton Dickinson, San Jose, CA). Expression of CD141/TM was analysed using PE-labelled rat anti-human TM (R&D, Minneapolis, MN). Informed consent was obtained from all participants before blood sampling, the study protocol was approved by

the Mie University Hospital Institutional Review Board and the investigation was carried out following the Helsinki Declaration.

Treatment with rhTM. Animals were treated with aerosolized 1 mg/kg rhTM for 20 min on days 22, 23, 24, 25 and 26 immediately before each exposure to ovalbumin. Aerosolized rhTM was administered using a multichamber pressurized nebulizer (PAR1, Turbo Boy M.I.P.S., Osaka, Japan). In one group of mice, 3mg/kg rhTM was administered by subcutaneous injection every week starting from the first day of sensitization.

Adoptive transfer of DCs. DCs from naïve mice were harvested on day 6 of culture and pulsed with ovalbumin for 24h in the presence or absence of rhTM. The cells were injected into the tail veins of BALB/c mice. Mice were challenged with aerosol ovalbumin on days 7, 8, 9 and 10 after adoptive transfer of DCs; pulmonary function was (AHR) was measured on day 11 and sacrificed on day 12 after adoptive transfer of DCs.

Homing of dendritic cells to the lungs and lymph nodes. DCs were generated from BALB/c bone marrow cells in the presence of rhTM (200 nM). On day 6, $CD141^{-}$DCs and $CD141^{+}$DCs were purified by magnetic separation. Cells were cultured for 24 hours in the presence of ovalbumin (200 μg/ml) and labeled with 1 μM CFSE (CFDA-SE: Invitrogen). 1 x 10^{6} DCs were i.p. injected to naïve or OVA-sensitized mice. After OVA-challenge (daily, 3 times), single cell suspensions were prepared from either digested lung tissue or mediastinal lymph nodes by dispersing the tissue through a 70-μm nylon tissue strainer (BD Falcon). The resultant suspension was treated with ACK buffer to remove any residual red blood cells, washed twice, and stained with PE/Cy5-labeled anti-CD11c and PE-F4/80. SSC(low), CD11c(high), F4/80(negative) cells were defined as dendritic cells. The percentages of CFSE-positive cells in dendritic cell population were calculated.

Measurement of AHR. AHR to increasing concentrations of aerosolized methacholine was measured non-invasively as enhanced pause (Penh), a dimensionless index that reflects changes in amplitude of pressure waveform and expiratory time, using a whole body

plethysmograph system (Buxco, Sharon, CT). For invasive measurement of AHR, mice were anesthetized with 70 mg/kg pentobarbital given intraperitoneally (Dainippon Pharmaceutical Co., Osaka, Japan) after the final ovalbumin or saline challenge, intubated with a stainless cannula and connected to a ventilator (Buxco Electronics, Elan Series, Osaka, Japan). After baseline determination of airway resistance, mice were challenged with increasing doses of methacholine nebulized directly into the ventilatory circuit. The collected data was analyzed off-line using Excel (Microsoft Corporation, Redmond, WA).

T cell stimulatory activity of DCs. DCs before or after sorting into CD141/TM$^+$ and CD141/TM$^-$ DCs, were stimulated with 100 μg/ml ovalbumin for 24h, co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-labelled CD4 T cells purified from OT-II mice (K. Takahashi, Yokohama City University Graduate School of Medicine) and proliferation of CD4 T cells was quantified (E7). Supernatants were assayed for the expression of IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17 and Tumor Necrosis Factor-α (TNF-α) by ELISA.

Phagocytosis and chemotaxis assay. 10^5 DCs were suspended in 100 μl PBS containing 1% BSA and incubated with 0.1 mg/m FITC-dextran (Sigma Chemical, St. Louis, MO) at 37°C for 15 min. Incubations were stopped by adding 2 ml ice-cold PBS containing 1% BSA (E8). The cells were washed three times with cold PBS and analyzed on a FACScan flow cytometer. Chemotaxis toward CCL3, CCL19, and CCL21 was analyzed by double chamber assay (E9).

Processing of DQ-OVA by DCs. DCs were mixed with 0.1 mg/ml DQ-OVA (Molecular Probes, Eugene, OR) for 15 min at 37ºC and then washed with PBS three times. Cells were cultured for a further 4h at 37ºC in RPMI1640 with 10% heat-inactivated FBS. Phagocytosis of DQ-OVA by DCs was defined as the percentage of DCs with positive fluorescent signals by flow cytometric analysis (E10).

Biochemical analysis

The level of total protein in bronchial alveolar lavage fluid (BALF) was measured using a dye-binding assay (BCA™ protein assay kit, Pierce, Rockford, IL, USA) following the

manufacturer's instructions. The total cell count in BALF was measured using a nucleocounter from ChemoMetec (Allerød, Denmark). For differential cell counting BALF was centrifuged using a cytospin and the cells were stained with May-Grunwald-Giemsa (Merck, Darmstadt, Germany). The BALF concentrations of cytokines were measured using commercial immunoassay kits specific for mouse cytokines. The immunoassay kits for measuring interleukin(IL)-5, interferon-γ (IFN-γ), IL-6, IL-12p70, IL-10, IL-4, tumor necrosis factor-α (TNF-α), IgE and IgG2a were purchased from BD Biosciences Pharmingen (San Diego, CA). IL-17 (R&D, MN) and IL-13 (R&D, MN) was measured by using commercial EIA kits. All cytokines were measured following the manufacturer's instructions. The detection range of the commercial EIA assay for IFN-γ was 3.1-200 pg/ml, for IL-5, IL-6 and TNF-α was 15.6-1000 pg/ml, for IL-10 31.3-2000 pg/ml, for IL-12p70 62.5-1000 pg/ml, for IgE 1.6-100 ng/ml, for IgG2a 3.1-200 ng/ml, for IL-13 4-500 pg/ml and for IL-17 was 4-500 pg/ml.

Statistics. All data are expressed as the mean ± standard error (s.e.m.) unless otherwise specified. The statistical difference between three or more variables was calculated by ANOVA with post hoc analysis using Fisher's predicted least significant difference test. The difference between the means of two variables was calculated by the Student t-test or Mann-Whitney U test depending on the distribution of the samples. Statistical analyses were carried out using StatView 4.1 (Abacus Concepts) for the Macintosh. $p < 0.05$ was considered as statistically significant.

Figures Legends

Figure E1. The population of regulatory T cells is unaffected in mice treated with rhTM and *in vitro*-differentiated bone marrow cells are not plasmacytoid DCs. (**A, B, C, D**) Mice were sensitized with ovalbumin + aluminum on days 0 and 14, treated with inhaled rhTM and 1h after challenged with ovalbumin by inhalation on days 22, 23, 24 and 25. Flow cytometry analysis of T cells isolated from the lungs (**A**, **B**), mediastinal lymph nodes (**C**, **D**) and spleen (**E**, **F**) showed no differences in the CD4 population positive for CD25 between SAL/OVA and rhTM/OVA groups. Statistical analysis: one-way ANOVA. Data are expressed as mean ± s.e.m. SAL/SAL was treated with saline by ip injection and by inhalation; SAL/OVA received ip

injection of ovalbumin + aluminum and inhaled saline; rhTM/OVA received ip injection of ovalbumin + aluminum and rhTM by inhalation. Representative results from three experiments are shown. (**G**) Bone marrow cells were isolated and treated with GM-CSF from day 0 and then from days 4 to 6 with 200nM rhTM. By flow cytometry analysis all cells were positive for CD11b but negative for the plasmacytoid DC markers B220 and CD45RB. Representative results from three experiments are shown.

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Figure E3. TM suppresses expression of DC maturation markers and increased cell surface CD141/TM expression on mature DCs. (**A**) DCs cultured in the presence or absence of rhTM (200nM, from day 4 to 6) were assessed by flow cytometry for relative expression of MHC II, CD80, CD86 and CD141 after overnight maturation with LPS (200 ng/ml). Left panels are the flow cytometry traces and right panels the Mean Fluorescent Intensity (MFI), Data are the mean ± s.d. (**B**) BM cells were cultured in the present of recombinant Flt3-L for 6 days to derive DCs. rhTM was added to the culture medium from days 4 to 6. On day 6, DCs were stimulated with 100 μg/ml of ovalbumin overnight and expression of MHC II, CD86, CD80, and CD141 was assessed by flow cytometry. Left panels are the flow cytometry traces

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Figure E5. CD141/TM+ DCs are immature and have lower mannose receptor (CD206) expression. (**A, B** and **C**) CD141/TM- and CD141/TM+ DCs were stimulated with ovalbumin in triplicate cultures. Expression of maturation markers (MHC II, CD80, and CD86), phagocytosis and antigen processing, chemokine receptors (CXCR4, CCR5, and CCR7) were analyzed by flow cytometry (left panels) and the percentage of cells determined (right panels: data are expressed as mean ± s.d.). (**D**) BMDCs cultured in the presence or absence of 200nM rhTM from day 4 to 6 were assessed for relative expression of CD206 after overnight incubation with 100 μg/ml ovalbumin. CD141/TM+ and CD141/TM- DCs were purified before overnight treatment with 100 μg/ml ovalbumin. The percentage of cells that express the mannose receptor, assessed by flow cytometry, was reduced in BMDCs treated with rhTM and in CD141/TM+ BMDCs. Data are expressed as mean ± s.d. Each panel shows representative results from three independent experiments. Statistics by Student's t test.

Figure E6. rhTM inhalation increases DCs in the lungs and decreases their migration to lymph nodes. (A, B) Mice were sensitized with ovalbumin and challenged on days 22, 23, 24, and 25 with FITC-labelled ovalbumin. FITC labeled DCs were counted in the lungs and mediastinal lymph nodes. Representative results from two independent experiments are shown. Error bars indicate s.d. SAL/OVA was sensitized with ovalbumin and treated with saline; rhTM/OVA was sensitized with ovalbumin and treated with rhTM. Statistics by Student's t test.

Figure E7. Secretion of cytokines from CD141/TM+ and CD141/TM- DCs and decreased T-cell proliferative activity of CD141/TM+ DCs. (**A**) 106 DCs were stimulated with 100 ng/ml of LPS in triplicate cultures for 24h with or without TM and culture supernatants were analyzed for the expression of IL-6, IL-12p70, TNF-α, and IL-10 by ELISA. Error bars

indicate mean ± s.d. Statistical analysis by ANOVA. (**B**) Effect on T cell stimulatory activity of DCs. DCs grown in the presence of the indicated concentrations of rhTM were co-cultured with CFSE-labelled CD4 T cells purified from OT-II mice. Proliferation of CD4 T cells was quantified by flow cytometry and the percentage of non-proliferating cells is shown as mean ± s.d. Error bars indicate mean ± s.d. Each panel shows representative results from three independent experiments. Statistical analysis by Student's t test. (**C**) The percentage of CD141/TM+ cells was measured by flow cytometry in the lung of naïve mice by gating for CD11c and CD141 positive cells.

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Figure E9. Lectin domain from membrane-bound TM of vascular endothelial cells and alveolar epithelial cells is critical for inhibition of the maturation of DCs. Human monocytes were incubated for 5 days with 50 ng/ml GM-CSF and 50 ng/ml IL-4 in the presence of rhTM or with (**A**) a semiconfluent monolayer culture of normal human umbilical vein endothelial cells (HUVECs) or (**B**) A549 alveolar epithelial cells. Cells were cultured in HMGB1-depleted medium. Polyclonal anti-TM (TM11) antibody and monoclonal anti-TM antibodies that inhibit thrombin binding (F2H5) and PC or TAFI activation (R5G12) were added to the culture. Expression of maturation markers (MHC II and CD86) was analyzed by flow cytometry. The upper panels show the MFI for MHC II and the lower panel for CD86. Data are expressed as mean ± s.d. Each panel shows representative results from three independent experiments. Statistical analysis by ANOVA.

Figure E10. Schematic overview of the responses of dendritic cells (DCs) to thrombomodulin (TM) and TM inhibition of the migration of lung DCs to lymph nodes. CD141/TM DCs mature by capture of allergic antigens leading to migration from the lungs to lymph nodes and stimulation of cognate T-cells. Presence of TM inhibits maturation, antigen capture and migration as well as causing expression of TM on the DCs. CD141/TM^{+} DCs inhibit the immunogenic activities of CD141/TM^{-} DCs.

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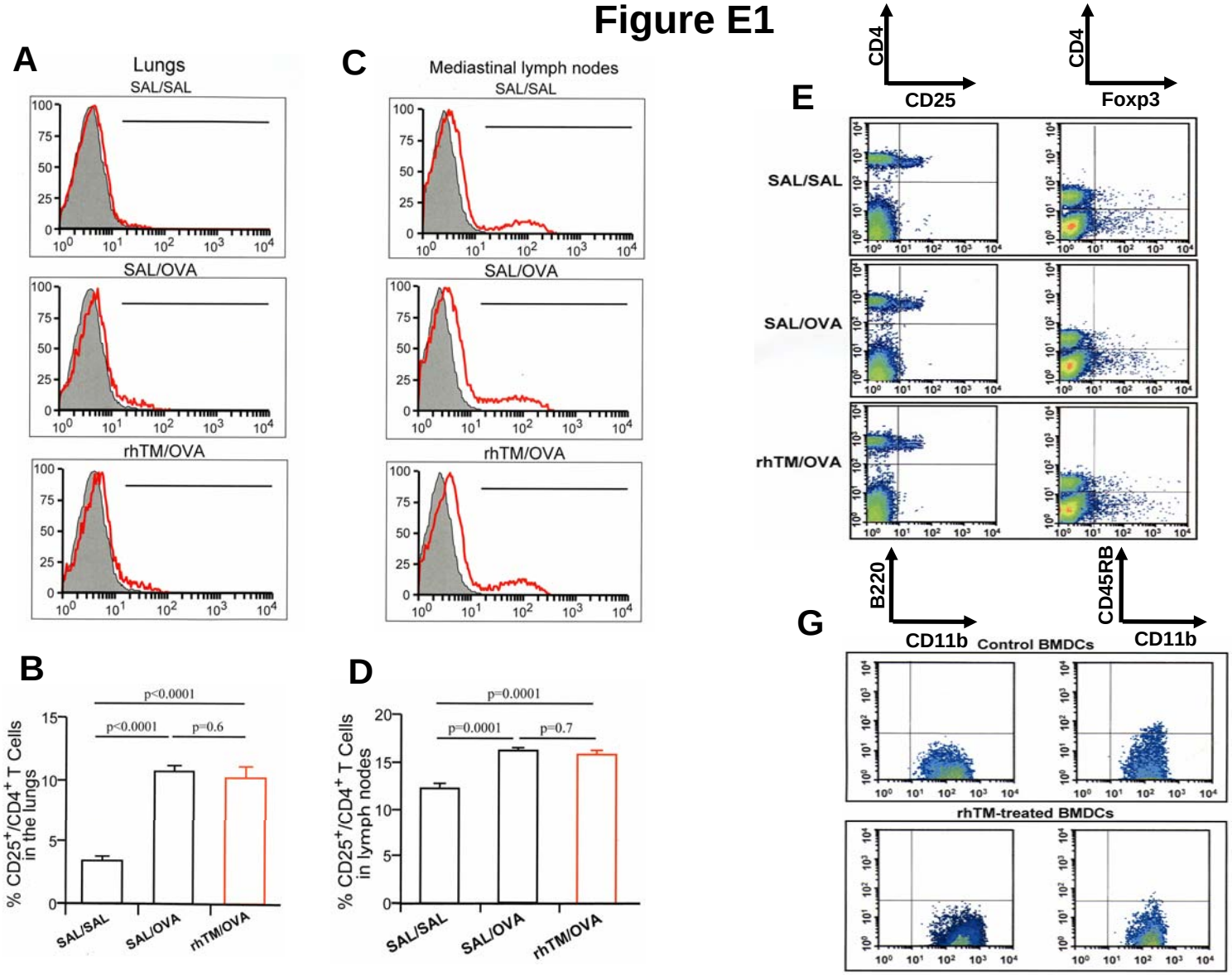
Figure E1



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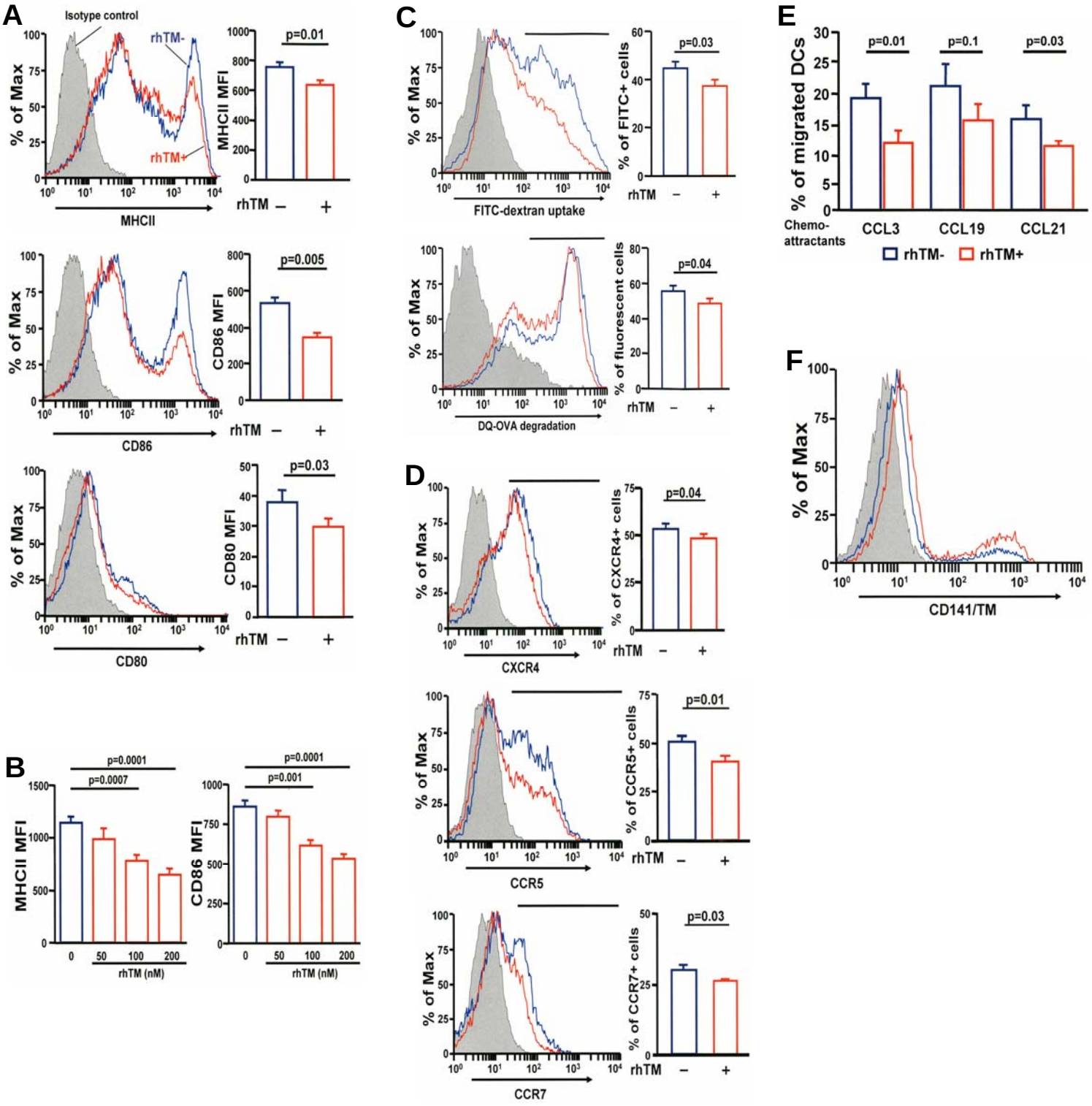

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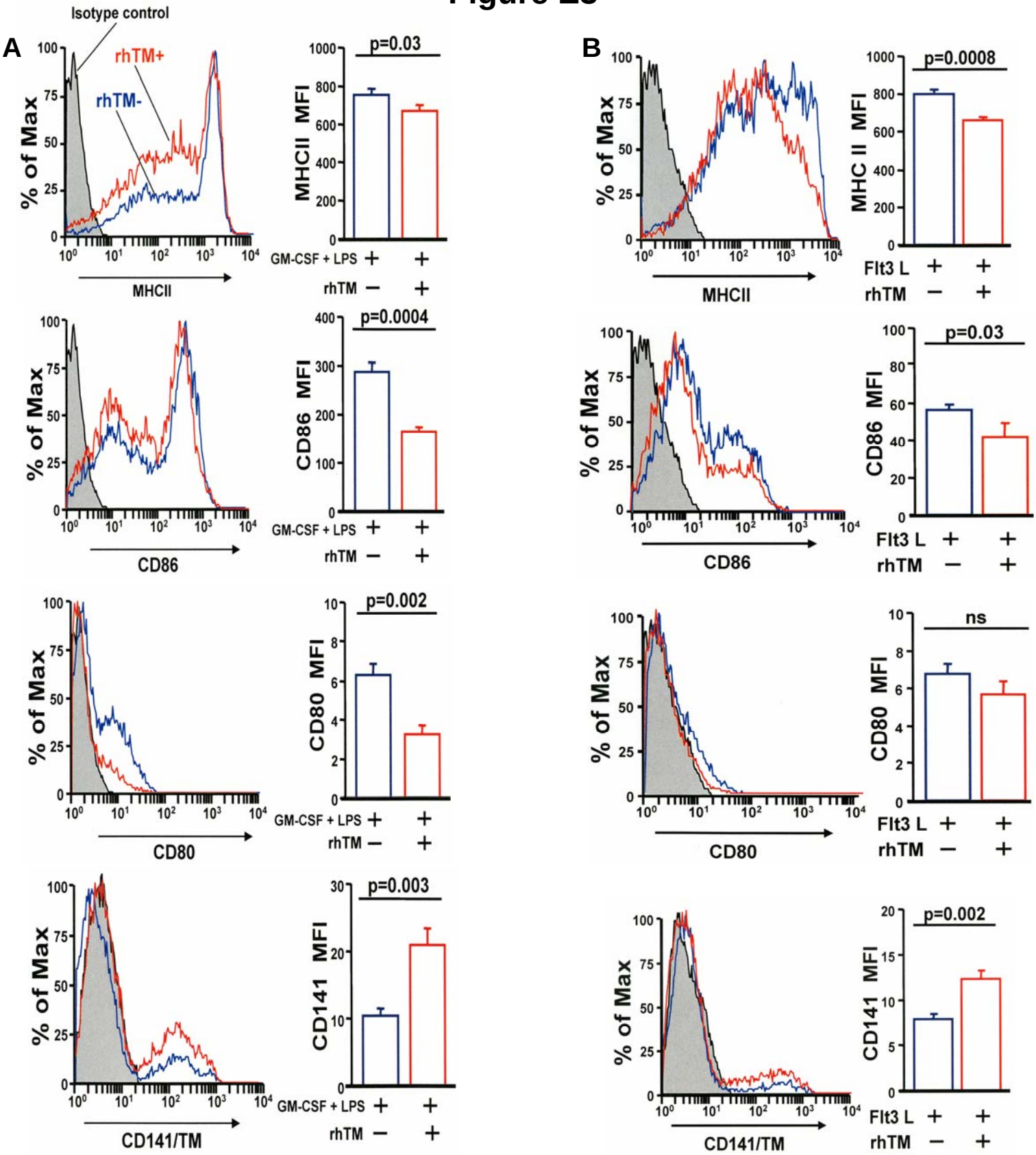

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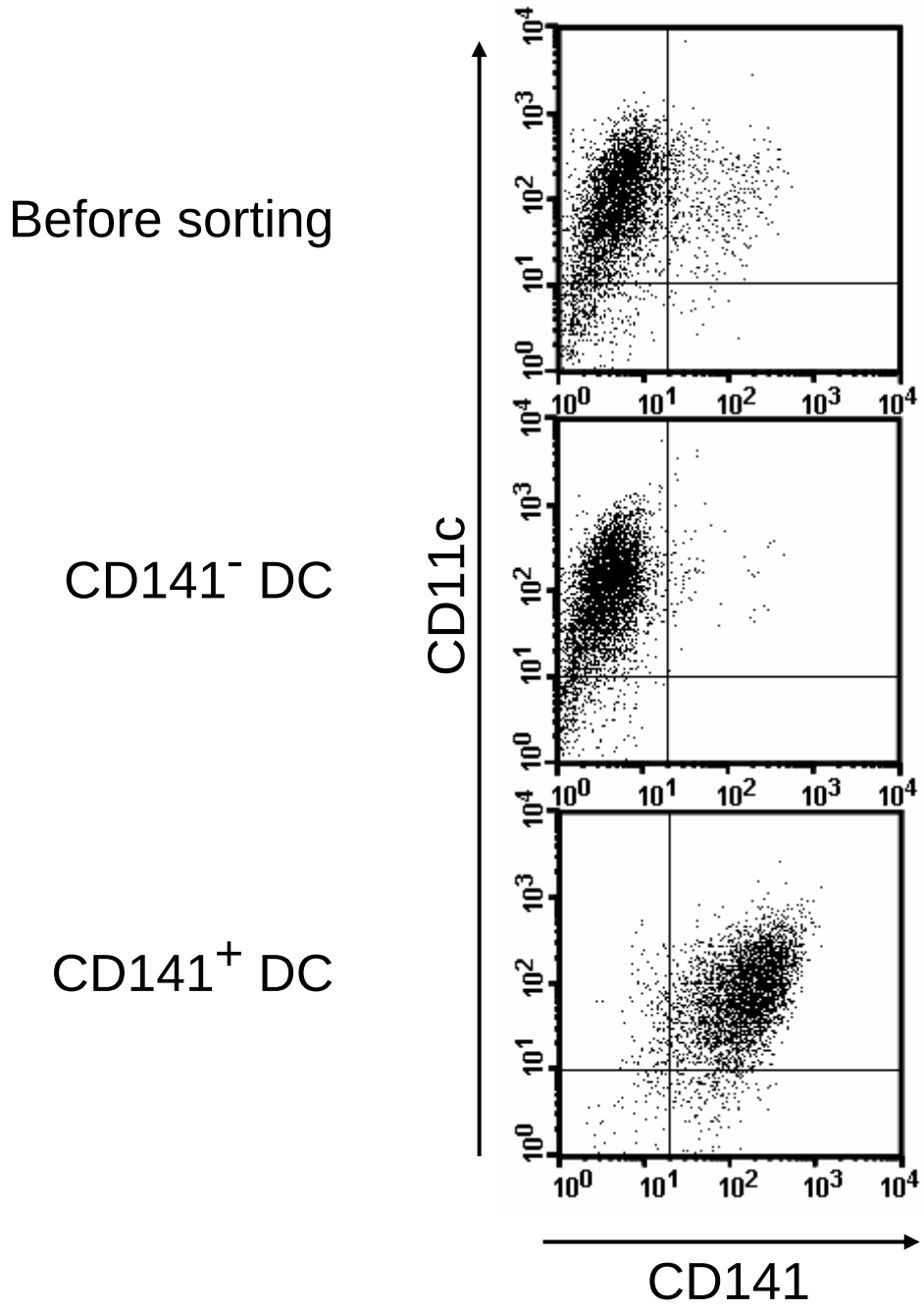

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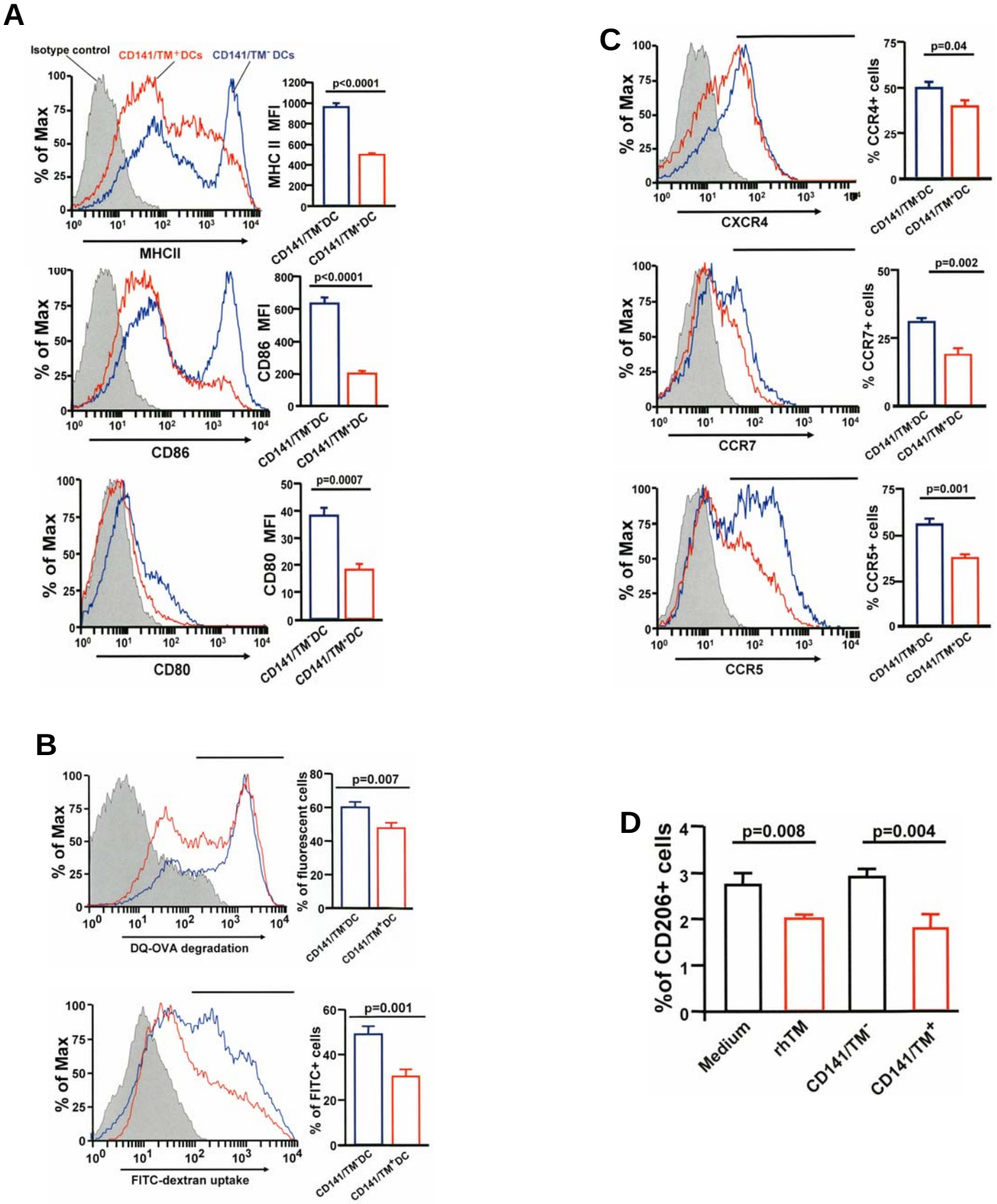

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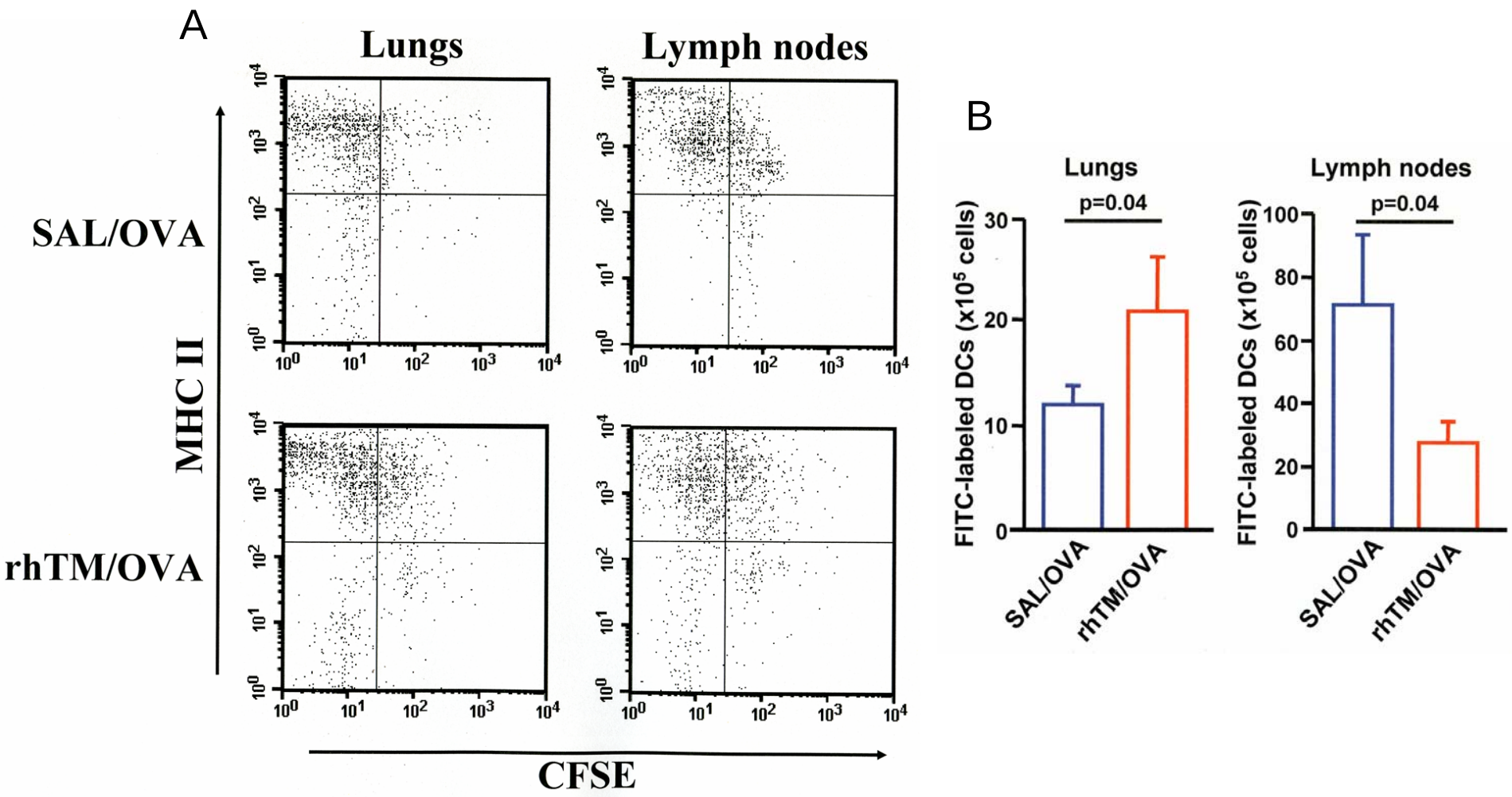
Figure E6



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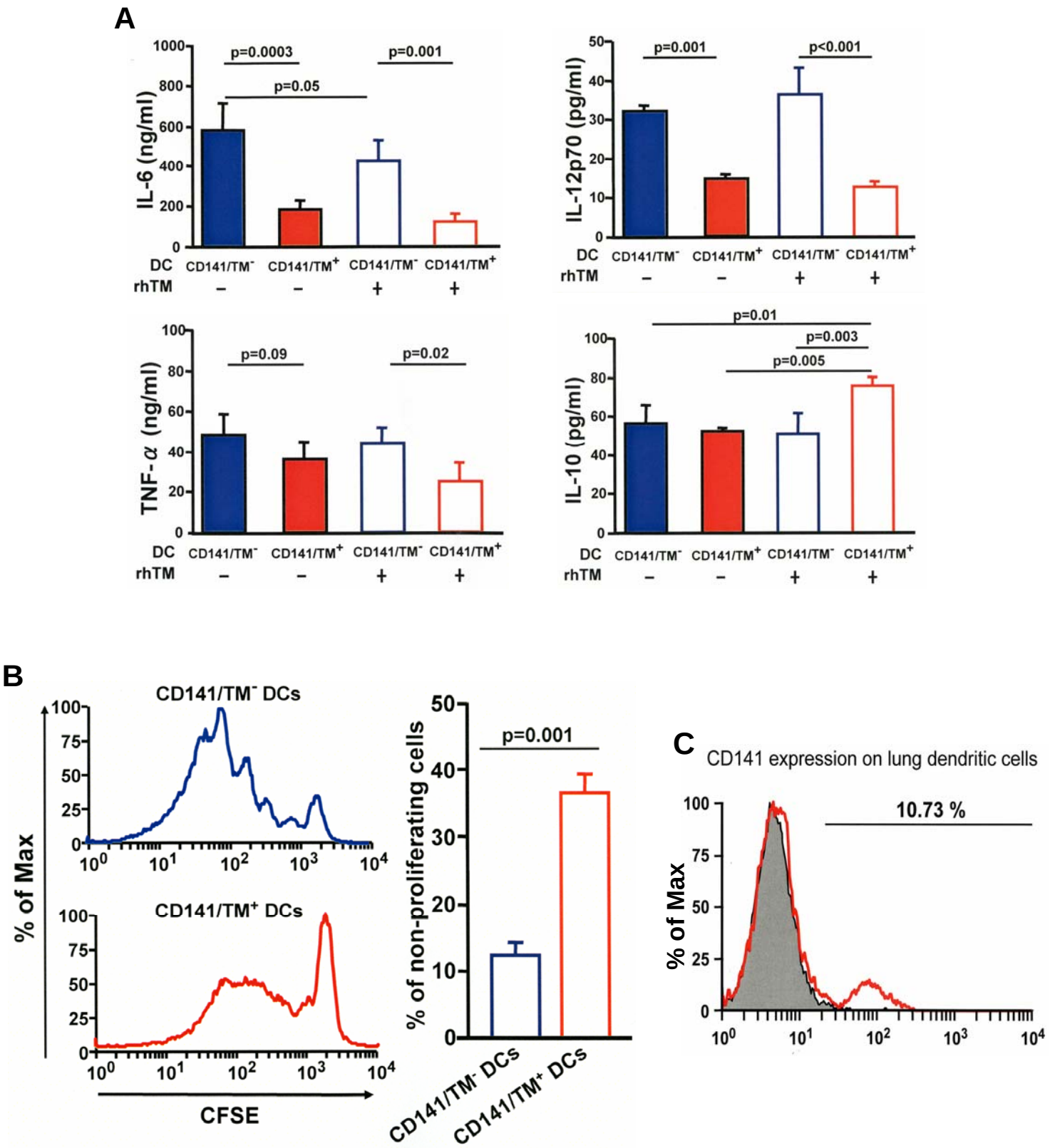

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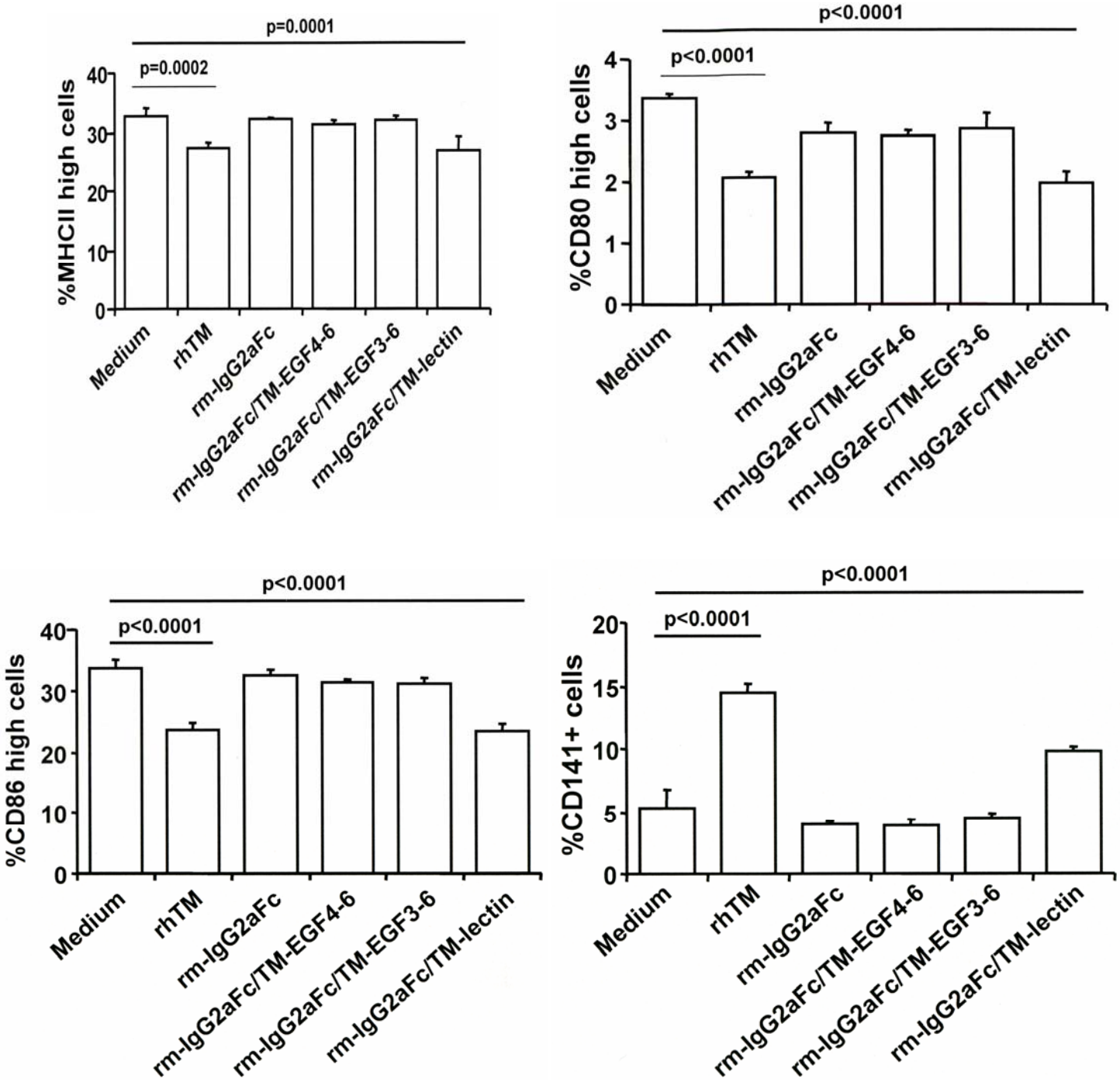

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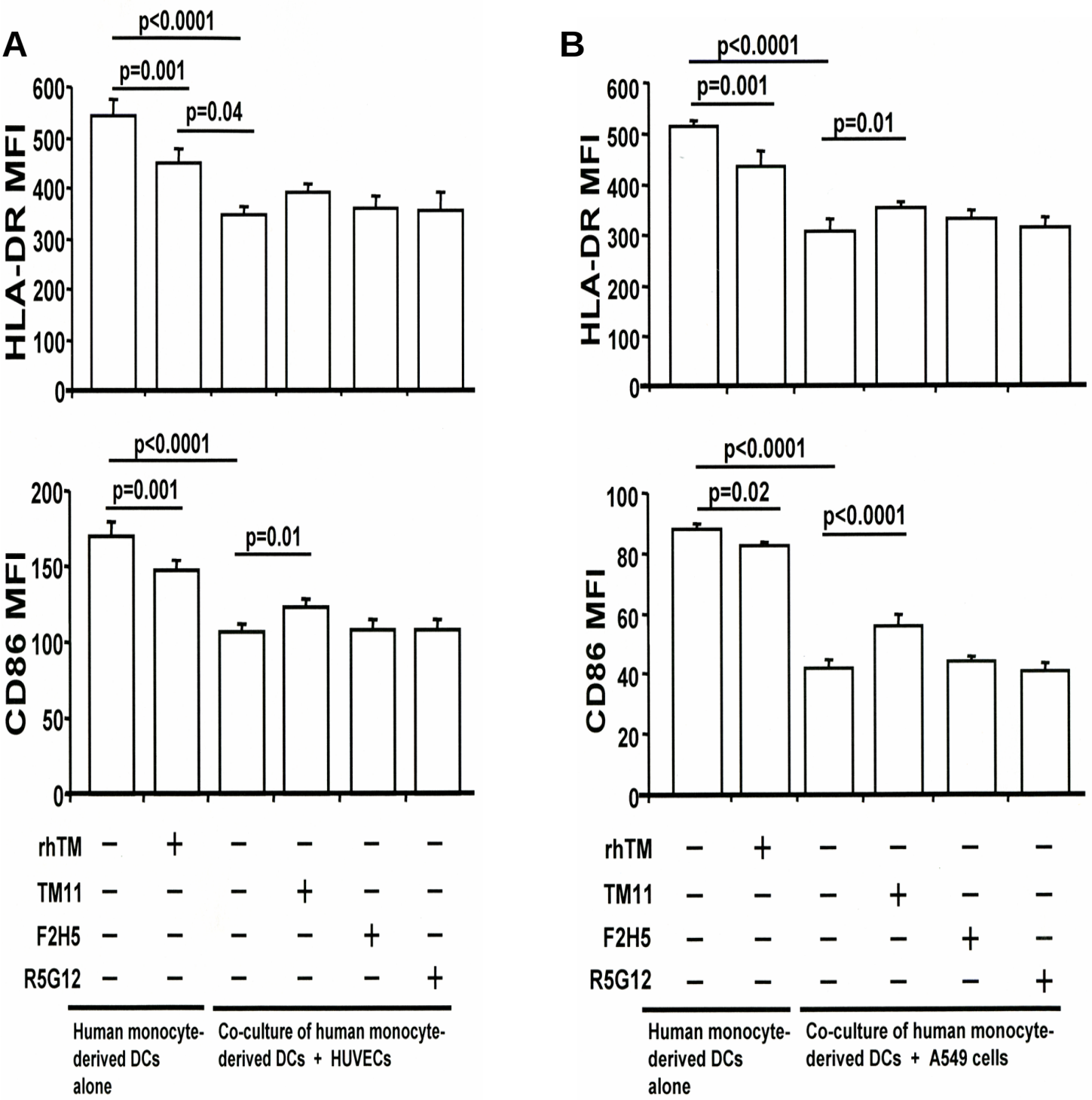

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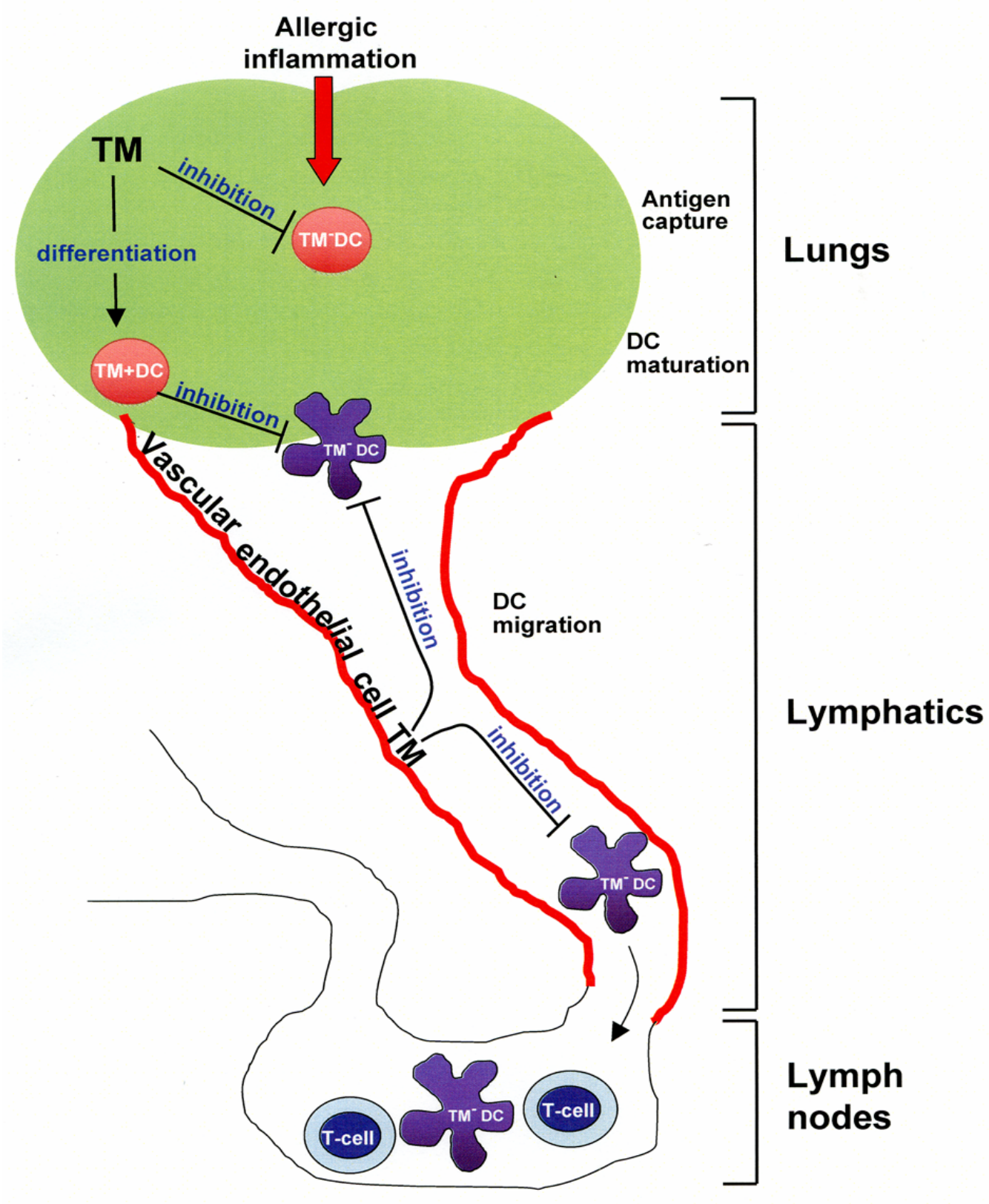
Figure E10



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