TREM-2 Mediated Signaling Induces Antigen Uptake and Retention in Mature Myeloid Dendritic Cells

Suresh Radhakrishnan, Laura N. Arneson, Jadee L. Upshaw, Charles L. Howe, Sara J. Felts, Marco Colonna, Paul J. Leibson, Moses Rodriguez and Larry R. Pease

*J Immunol* 2008; 181:7863-7872; doi: 10.4049/jimmunol.181.11.7863
http://www.jimmunol.org/content/181/11/7863

References

This article cites 42 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/181/11/7863.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Errata

An erratum has been published regarding this article. Please see next page or:
/content/184/11/6557.full.pdf
TREM-2 Mediated Signaling Induces Antigen Uptake and Retention in Mature Myeloid Dendritic Cells

Suresh Radhakrishnan,* Laura N. Arneson,* Jadee L. Upshaw,* Charles L. Howe,*† Sara J. Felts,* Marco Colonna,‡ Paul J. Leibson,* Moses Rodriguez,*† and Larry R. Pease2*

Myeloid dendritic cells (mDC) activated with a B7-DC-specific cross-linking IgM Ab (B7-DC XAb) take up and retain Ag and interact with T cell compartments to affect a number of biologic changes that together cause strong antitumor responses and blockade of inflammatory airway disease in animal models. The molecular events mediating the initial responses in mDC remain unclear. In this study we show that B7-DC XAb caused rapid phosphorylation of the adaptor protein DAP12 and intracellular kinases Syk and phospholipase C-γ1. Pretreatment of mDC with the Syk inhibitor mouseannol blocked B7-DC XAb-induced Ag uptake with a concomitant loss of tumor protection in mice. Vaccination with tumor lysate-pulsed wild-type B7-DC XAb-activated mDC, but not TREM-2 knockout XAb-activated mDC, protected mice from lethal melanoma challenge. Multimolecular caps appeared within minutes of B7-DC XAb binding to either human or mouse mDC, and FRET analysis showed that class II, CD80, CD86, and TREM-2 are recruited in tight association on the cell surface. When TREM-2 expression was reduced in wild-type mDC using short hairpin RNA or by using mDC from TREM-2 knockout mice, in vitro DC failed to take up Ag after B7-DC XAb stimulation. These results directly link TREM-2 signaling with one change in the mDC phenotype that occurs in response to this unique Ab. The parallel signaling events observed in both human and mouse mDC support the hypothesis that B7-DC cross-linking may be useful as a therapeutic immune modulator in human patients.

Dendritic cells (DC)3 are key targets in schemes to regulate immunity for the treatment of cancer, infectious diseases, transplant rejection, and autoimmune disease (1). Activation of DC through the TLR family initiates DC maturation resulting in down-regulation of Ag uptake and migration to regional lymph nodes, where they encounter and activate naive T cells (2, 3). As DC mature, the cell surface expression of a series of costimulatory molecules is up-regulated (4). These costimulatory molecules are critical for the activation of naive T cells into effector cells (4). The activated DC also produce inflammatory cytokines that influence the polarity of the ensuing immune response, determining the array of effector mechanisms capable to bear the infection (7, 8).

We have recently described a new approach for modulating the activity of DC that is distinct from previously defined mechanisms, yet results in potent immunomodulatory effects. B7-DC (or, PD-L2) is a B7 family member normally expressed on the cell surface of DC (9). The receptor for this protein is L, is expressed on activated T cells (10). Cross-linking B7-DC interactions in vivo with the human IgM B7-DC XAb alters a wide variety of important functions of mature DC (mDC), inducing 1) enhanced survival, 2) increased ability to process and present soluble Ag in the class I Ag-presenting pathway, 3) enhanced ability to activate naive T cells, 4) increased efficiency of seeding draining lymph nodes, and 5) up-regulation of the key immunomodulating cytokines IL-12 (11–12). However, mDC treated with B7-DC XAb do not display traditionally defined maturation phenotypes (12) such as up-regulation of the costimulatory markers CD80 or CD86 or a concomitant increase in cell surface expression of class II Ag-presenting molecules. Instead, treatment of immature mDC with B7-DC XAb results in increased Ag uptake and even restores the ability of TLR ligand-matured mDC to take up and retain Ag (13). The combination of TLR-9 ligand and B7-DC cross-linking results in a synergistic CTL response against peptide Ag (13). These differences in maturation lead to important biological distinctions between cells activated by traditional approaches compared with cells activated by cross-linking B7-DC. For example, mDC activated with B7-DC XAb are highly efficient modulators of the polarity of Th2 memory cells (14), effectively redirect T regulatory cells into effector cell phenotypes (15), and rapidly activate cytolytic T cell responses (16), whereas DC matured using TLR agonists are relatively inefficient inducers of these changes in T cell behavior.

B7-DC interaction with PD-1 has been shown to result in either a positive response (9) or a negative response (10) by T cells. This discrepancy could be due either to the different model systems used or to the ability of B7-DC to interact with more than one of the receptors that differentially govern T cell responsiveness (17). The positive immune response observed when we treated animals with B7-DC XAb might also be attributable to a blockade of interactions between B7-DC and PD-1. However, adoptive transfer experiments using mDC activated in vitro under conditions in which B7-DC XAb did not physically block expression of accessible B7-DC on the cell surface demonstrated full immunomodulatory capabilities (18).

Received for publication June 3, 2008. Accepted for publication September 29, 2008.

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

1 This work was supported by Grants R01 CA104996-4 (to L.R.P.), R01 HL077296-3 (to L.R.P.), and R01 CA96859 (to L.R.P.) from the National Institutes of Health and by a grant from the Ralph Wilson Medical Research Foundation.
2 Address correspondence and reprint requests to Dr. Larry R. Pease, Department of Immunology, College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail address: pease.larry@mayo.edu
3 Abbreviations used in this paper: DC, dendritic cell; mDC, myeloid DC; poly(I:C), polyinosinic-polycytidylic acid; XAb, cross-linking Ab; PLC, phospholipase C; shRNA, short hairpin RNA.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00
How cross-linking B7-DC stimulates the immunomodulatory properties of mDC is not known. The plethora of immunomodulatory effects due to fundamental changes in mDC signaling is reflected in how mDC interact with T cell lineages. To understand the mechanisms involved, we previously showed that engagement of B7-DC by this IgM Ab protected mDC from cell death caused by cytokine withdrawal. This protection was mediated by intracellular signals involving PI3K, Akt, and NF-κB (11, 19). Thus, cross-linking of molecules on the surface of mDC by B7-DC XAb appears to elicit back-signals similar to events described following the ligation of B7-2 family members in studies using a CTLA-4-Ig fusion protein (20, 21). However, B7-DC XAb invoked responses remain poorly defined.

In this study we demonstrate that cross-linking of B7-DC molecules on mouse mDC derived from bone marrow precursors results in phosphorylation of the upstream adaptor protein DAP12 and the protein kinases Syk and phospholipase C (PLC)γ. Syk—PLCγ activation leads to the restoration of Ag uptake and retention in matured DC. Tumor lysate pulsed B7-DC XAb mDC pretreated with Syk inhibitor fail to take up and retain Ag or to protect mice against tumors upon adoptive transfer. Importantly, multimolecular complexes involving several cell surface molecules, including TREM-2 (Triggering receptor expressed by myeloid cells-2), form on B7-DC XAb-activated mDC. TREM-2 is an activating receptor of the Ig superfamily and regulates the development and function of DC, microglia, and osteoclasts (22). Our studies with TREM-2-deficient mDC show that TREM-2 initiates signaling through DAP12 and Syk and mediates activation and retention in matured DC upon cross-linking B7-DC XAb. These signals and changes in mDC function are necessary for B7-DC XAb to have its unique immunomodulatory properties.

Materials and Methods

Mice

C57BL/6 and B6.129J4-Cd80−/−Cd86−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 to 8-wk-old were obtained from The Jackson Laboratory. OT-II mice (H-2b) for ELISPOT assay were obtained from The Jackson Laboratory. Class II knockout mice (23) were a gift from C. David (Mayo Clinic, Rochester, MN). TREM-2 knockout mice (24) were bred in the mouse colony at Washington University School of Medicine (St. Louis, MO). All animals were maintained at the Mayo Clinic Animal Facility for at least 1 wk before use and protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Abs and other reagents

Appropriate fluorophore-labeled Abs against murine Ab (25-9-17), murine class II specific IgM (26-9-3), allophycocyanin-labeled anti-mouse CD11c (HL3) FITC-labeled anti-human class II (3039), FITC-labeled anti-human CD28 (CD28.2), allophycocyanin-labeled anti-human CD28 (CD28.2), PE labeled anti-human CD8 (RPA-T4) and PE-labeled anti-human HLA-A, HLA-B, HLA-C (G46-2.6) were purchased from BD Pharmingen. Appropriate fluorophore labeled Abs against mouse class II (MS1/14.15.2), CD80 (16.10A1), CD86 (GL-1), CD11c (N418), allophycocyanin-labeled Ab against human DR (NL3), PE labeled anti-human CD80 (2D10.4), CD86 (IT2.2), murine B7-2 specific IgG Ab (TY25), and human B7-DC IgG Ab (MIH18) were all purchased from eBioscience. All secondary appropriately fluorophore-labeled F(ab)2 fragment Abs used in this study were obtained from Jackson ImmunoResearch Laboratories. An IgM Ab (28-13-3) specific for mouse class II H-2 K` was obtained from a hybridoma cell line from American Type Culture Collection (HB-41). Ab against the protein kinase Syk (4D10) was obtained from Santa Cruz Biotechnology. Anti-phosphotyrosine (4G10) and goat anti-mouse Abs were obtained from Upstate Cell Signaling Solutions. Anti-mouse TREM-2 Abs (239290) (for flow cytometry) and 237916 (for Western Blot) were purchased from R&D Systems. Rabbit Abs against PLC-γ1 (MC940) and DAP12 (MC547) were developed by Dr. P. Leibson (Mayo Clinic, Rochester, MN). OVA labeled with FITC or allophycocyanin was purchased from Molecular Probes. Protein A-Sepharose was purchased from Pierce.

DAPI (4′,6-diamidino-2-phenylindole), DNase, and LPS were obtained from Sigma-Aldrich. All inhibitors used in this study were obtained from Calbiochem unless otherwise indicated. Piceatannol was obtained from Sigma-Aldrich. Rac-1 inhibitor, N627366 was a gift from Dr. D. Billadeau (Mayo Clinic, Rochester, MN). CpG oligonucleotides as described (25) were synthesized in Mayo Research Center. The polynucleotide polyinosinic-polycytidylic acid poly(I:C) was purchased from Calbiochem. All human IgM Abs were purified as described (26) and used at 10 μg/ml.

Generation of mDC

The mDC from mouse bone marrow were generated as described (27). Bone marrow was isolated from the long bones of the hind legs. Erythrocytes were lysed by treatment with ammonium chloride/potassium bicarbonate/EDTA at 37°C. The remaining cells were plated 1 × 10^6 cells/ml in 6-well plates (BD Biosciences) in RPMI 1640 containing 10 ng/ml murine GM-CSF and 1 ng/ml murine IL-4 (PeproTech). The cells were incubated at 37°C with 5% CO2. After 7 d, the cells were washed and replated with RPMI 1640 containing the same concentration of GM-CSF and IL-4 for another 5 days. Human DC were derived from CD14+ mononuclear cells isolated from peripheral blood using magnetic bead sorting (Miltenyi Biotec). Buffy coat was obtained from a unit of blood donated by a normal human donor. The mDCs were isolated by centrifugation over Ficoll-Paque PLUS (Amersham Biosciences), and CD14+ cells were separated by positive selection with a mCD14 microbead (Miltenyi Biotec). The isolated cells were cultured in RPMI 1640 containing 10% FCS and 10% human AB serum (HP 10220, Valley BioMed) supplemented with 1% nonessential amino acids (Mediatech) (R&D Systems), 50 ng/ml murine GM-CSF (Berlex Laboratories) and 200 μg/ml of gentamicin. B7-DC XAb was added at concentrations of 10 μg/ml for 1 h, followed by addition of TLR 3 ligand polyinosinic-polycytidylic acid poly(I:C) for a period of 24 h before being used for the transduction procedure. The short hairpin control virus used to culture both bone marrow-derived mouse DC and peripheral blood-derived human DC are favorable toward myeloid cells. We refer to the DC in this study as mDC.

Production of short hairpin TREM-2 and short hairpin control virus

Short hairpin TREM-2 sequence 5′-TGAT CGCTTCTGGGTTCAAGAGCCAGAGATCTCCAGCATCTTTTTTC-3′ (28) containing a chloramphenicol resistance gene (generated and cloned into the pSUPER RNAi System, as provided by Dr. D. Billadeau) and a short hairpin Control sequence 5′-TGACCTGCTGAAAGTC TACAGCAGAGACCAAGCGACCTCAGCATTTTTTTT-3′ (28) containing the pgal promoter were cotransfected with VSV-G and gagpol plasmids, provided by Dr. R. Vile (Mayo Clinic, Rochester, MN) into 293T cells. Supernatant was collected at 48 and 72 h, pooled, filtered through a 0.45-micron filter and then harvested by centrifugation. For transducing mDC with the virus, the mDC culture medium was replaced with fresh medium containing 1 ml buffer containing 20 μM piceatannol containing the scrambled virus or virus encoding shRNA against TREM-2 and 2 ml of RPMI 1640 (multiplicity of infection ~30, see previous). Cytokines were added to a final concentration of 10 ng/ml murine GM-CSF and 1 ng/ml murine IL-4 at day 2 of mDC culture. Cells were maintained for another 3 days before using the mDC for Ag uptake assay as mentioned or for analysis of phosphorylation status of DAP12 and Syk proteins. Using this transient transduction procedure, intracellular staining showed that TREM-2 protein levels were reduced by ~60%.

Immunoprecipitations and Western blots

Whole cell lysates were prepared from mouse or human DC stimulated with control Ab or B7-DC XAb. In experiments involving inhibition of Syk kinase, cells were preincubated with 10 μM piceatannol for 30 min. For suppression of TREM-2, mDC were transduced as described and were stimulated with control Ab or B7-DC XAb on day 6. Cells were lysed on ice for 10 min in 1 ml buffer containing 20 μM Tris-HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM Na3P04, 0.1% BSA, 1 mM Na2VO4, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100. Cellular debris was removed by centrifugation at 20,800 × g for 5 min at 4°C and the supernatant used for further analysis. For immunoprecipitation, Ab (10 μg) against mouse Syk (4D10) or PLC-γ1 (MC940) or
DAP12 (MC457) was bound to protein A-Sepharose beads at 4°C for 2 h under constant rotation. Supernatant from cell lysates were added to the Ab-coupled beads and incubated for 2 h at 4°C with constant rotation. Protein complexes were then eluted in 40 μl of SDS sample buffer, blocked by PAGE, and transferred to Immobilon-P membranes (Millipore). Tyrosine phosphorylated proteins were detected using anti-phosphotyrosine specific Ab, 4G10, followed by goat anti-mouse coupled to HRP (Santa Cruz Biotechnology) and the SuperSignal West Pico Chemiluminescence (Pierce). The total protein was visualized by staining the membrane with Ponceau staining solution (Pierce) for 30 s in case of analysis of whole cell lysate or in the case of immunoprecipitation assays, the membrane was stripped with 7M guanidine, blocked with BSA, probed with the Ab against the whole protein followed by protein A coupled to HRP (American Biosciences) and the SuperSignal West Pico Chemiluminescence (Pierce). Phosphorylated proteins were detected using the anti-phosphotyrosine specific 4G10, followed by goat anti-mouse coupled to HRP.

Live cell imaging for visualization of multicomponent complex

Mouse mDC were stained with anti-class II-FITC (MF/114.15.2), and either anti-CD80-PE (16.10A1) or anti-CD11c-PE (N418). Human mDC were stained with anti-class II-FITC (LN3) and anti-CD80-PE (2D10.4)/CD86-PE (IT2.2). DAPI was used to stain the nuclei. All incubations were conducted for 15 min at 37°C. The cells were subsequently stimulated with 10 μg/ml control Ab (shHgM39) or B7-DC XAb or B7-DC XAb and were observed every 5 min using time lapse confocal imaging at ×40 magnification with a LSM510 Laser scanning confocal microscope with a 37°C stage (Carl Zeiss).

Flow cytometry and fluorescence resonance energy transfer (FRET)

FRET occurs when certain fluorophores are in close enough proximity (<80 Å) such that when one has been excited (the donor), energy can be directly transferred to the other (the acceptor), causing it to fluoresce. A flow cytometry approach using fluorochrome-coupled Abs specific for cell surface molecules was used to study changes in cell surface interactions in response to cross-linking Ab treatment as previously described (29). Briefly, mouse mDC were stained with anti-class II allophycocyanin (M5/114.15.2) and anti-CD80-PE (16.10A1)/CD86-PE to focus on B7-DC XAb (237920). Human mDC were stained with allophycocyanin anti-class II (LN3) and anti-CD80-PE (2D10.4)/CD86-PE (IT2.2). All staining was for 15 min. In experiments involving blocking of B7-DC, both fluorophore-labeled Abs and purified anti-mouse B7-DC (TY-25) or purified anti-human B7-DC (MIH18) IgG mAb was added at 10 μg/ml for 15 min. Cells were stimulated with control Ab or B7-DC XAb or purified anti-class II IgM (25-9-3) and aliquots from different groups were taken at different time points. After 15 min of incubation, the cells were washed and fixed in 2% paraformaldehyde before analysis by FACS performed by the Mayo Flow Cytometry Core Facility using a FACSCalibur (BD Biosciences). Ab-induced generation of a FRET signal (upon excitation of PE at 488 nm and emission of allophycocyanin at 660 nm) was visualized in FL3 channel (650–670 nm LP). Data collected as log10 fluorescence were analyzed using CellQuest (BD Biosciences).

Ag uptake and retention assay

Experiments to assess the accumulation of Ag (reflecting both Ag uptake and retention) were conducted as previously described (13). Day 5 mDC were matured with TLR ligand CpG oligonucleotides (10 μg/ml) for mouse mDC (11) or poly(I:C) (10 μg/ml) for human mDC. The matured cells were incubated with OVA (1 mg/ml) labeled with FITC or allophycocyanin and control Ab or B7-DC XAb for 2 h, washed, and analyzed by FACScalibur (BD Biosciences). In studies involving inhibitors, cells were pretreated 30 min as indicated in the experiments or at 10 μM concentration before the addition of control Ab or B7-DC XAb.

T cell stimulation by mDC

To ensure that inhibition of Ag uptake by various signaling inhibitors did not compromise all DC functions, mDC were tested for their ability to stimulate T cell proliferation. Naive mouse splenocytes were harvested from the OT-II mice, plated in triplicate (3 × 10^5), and stimulated in vitro for 3 days with titrated doses of mDC that were pretreated with different inhibitors (10 μg/ml), pulsed with 10 μM OT-II peptide (ISQAVHAA-HAEINEAGR), and treated with control Ab or B7-DC XAb overnight. The cells were pulsed with [3H]thymidine for 18 h before harvest. Tritium incorporation was measured by liquid scintillation.
Tumor vaccine experiments

All in vivo tumor experiments were conducted as previously described (30). Briefly, all the groups of mice were injected with 0.5 × 10^6 B16 melanoma cells in the right flank. In addition, some mice were vaccinated with B16 melanoma lysate-pulsed wild-type or TREM-2 knockout mDC (2 × 10^6, i.p.) treated with control Ab or with B7-DC XAb. In some experiments, mice received mDC that were pretreated for 15 min with 10 μg/ml the Syk inhibitor piceatannol before the addition of lysate and B7-DC XAb. After 7 days, draining lymph node cells (from two mice in each group) were harvested, pooled, and used as effectors against the 51Cr-labeled B16 melanoma target cells. The remaining mice were monitored for the tumor growth and mice bearing tumors of size 17 × 17 mm were euthanized as per the Institutional Animal Care and Use Committee recommendations.

Results

DAP12-Syk-PLCγ mediates B7-DC XAb-induced Ag uptake and retention in mature human mDC

We have demonstrated earlier that cross-linking B7-DC on mature mDC from mice leads to restoration of the ability of the mDC to take up and retain protein Ag (13). Matured human mDC also responded to B7-DC XAb treatment by regaining the ability to take up and retain Ag (Fig. 1B). Initial studies into the mechanisms regulating this process showed that global protein phosphorylation was induced as early as one minute after treatment of mDC with B7-DC XAb (data not shown). To determine what kinases were activated and what signaling intermediates mediated the Ag uptake response, we tested whether Src, Syk, and PLC-γ inhibitors blocked steps in the activation pathway. Blockade of Src kinases with PP2 resulted in the inhibition of Ag accumulation by matured mDC upon B7-DC cross-linking (Fig. 1B). Similarly, pharmacologic agents that block calcium-dependent protein kinase C activity, P3K, and the Rho family GTPase RAC1 were also inhibitory (Fig. 1C). B7-DC XAb treatment of mDC resulted in the activation of ERK, but not p38 (data not shown). However, inhibitors of Rho-A, MEK, and p38 pathways did not influence B7-DC XAb-induced Ag accumulation (Fig. 1C). Assessment of cell viability using Alamar Blue or Annexin V binding showed that 94–98% of the mDC treated with B7-DC XAb were viable and remained so in the presence of the various inhibitors (data not shown).

As no signaling elements in B7-DC have been identified, it seemed likely that an association with adapter molecules containing signaling domains would be required to mediate an mDC response. DAP12 is an adaptor molecule that can couple receptor molecules like TREM-2 that lack innate signaling capability to downstream activation pathways (31–33). DAP12 was rapidly phosphorylated on tyrosine upon cross-linking B7-DC XAb (Fig. 2A). Further analysis showed that B7-DC XAb stimulation of human mDC also caused the tyrosine phosphorylation of p72 Syk (Fig. 2B), shown in other systems to be downstream of a Src kinase (34) and associated with Fc receptor-mediated Ag uptake by B cells, macrophages, and DC (35). PLC-γ1, a downstream substrate for Syk (36) was also phosphorylated (Fig. 2C). Vav1, a RhoGEF activated by Syk and required for cytoskeletal rearrangements

![FIGURE 2.](http://www.jimmunol.org/)

The DAP12-Syk-PLCγ is activated by B7-DC XAb and is required for regained Ag uptake in matured mDC. Matured human mDC were treated with the B7-DC XAb for varying times. Immunoprecipitates were prepared using Abs specific for DAP12 (A), Syk (B), and PLC-γ (C) and analyzed for activation by blotting for phosphorylation (pTyr). After stripping, the membranes were rebotted with the specific Abs used for immunoprecipitations. D and E. Cells were pretreated with the indicated concentrations or with 10 μM Syk inhibitor piceatannol. F and G. Cells were pretreated with piceatannol or the active or inactive forms of PLC-γ1 inhibitor (U73122/U73343) and Ag uptake was assessed by pulsing DC with FITC-conjugated OVA and measuring intracellular fluorescence 16 h later in CD11c+ cells by flow cytometry. Data are representative of three or more experiments.
during Ag uptake in B cells (37) also was phosphorylated in DC following activation with B7-DC XAb (data not shown).

Pretreatment of mDC with piceatannol inhibited phosphorylation of Syk and PLC-γ1 (Fig. 2, D and E) and inhibited accumulation of tagged proteins by matured mDC in a dose responsive manner (Fig. 2F). Inhibition of PLC-γ using U73122, but not with the inactive analog U73343 (Fig. 2G) also blocked the accumulation of Ag. Thus, the ability of mDC to regain the ability to take up and retain Ag in response to B7-DC XAb is mediated by the activation of multiple kinases, especially Syk and PLC-γ, which are tied to B7-DC by DAP12.

Intact Syk pathway in mDC is necessary for the protection of mice against tumor

We next tested whether the mDC signaling intermediates we identified in vitro were important for the immunomodulatory affects observed using B7-DC XAb. We showed previously that administration of B7-DC XAb into mice leads to the generation of a potent CTL response and tumor clearance (30). Mice immunized with B16 tumor lysate and receiving B7-DC XAb treatment are protected against B16 melanoma tumor (38). Because Syk was the upstream kinase involved in inducing Ag uptake in mDC, we asked whether inhibition of Syk affected B7-DC XAb-induced tumor immunity in a B16 melanoma model. Myeloid DC were treated with piceatannol before being pulsed with the tumor cell lysate and treated with B7-DC XAb or control Ab. Adoptive transfer of the mDC pulsed with the B16 melanoma tumor cell lysate and activated with the B7-DC XAb induced a potent CTL response (Fig. 3A) and protected the mice against a lethal challenge with B16 tumor (Fig. 3C). However, mice that received piceatannol-treated mDC did not mount a cytotoxic response and failed to clear the B16 tumor (Fig. 3, B and C). Peptide-pulsed mDC treated with piceatannol were still able to stimulate T cell proliferation in vitro (Fig. 3D). Taken together, these findings indicate that the B7-DC XAb-activation of the Syk pathway in mDC is required for the processing of Ags from tumor lysates and the subsequent induction of tumor immunity.

B7-DC XAb leads to multimolecular cap on the surface of the mDC

Because B7-DC has a short cytoplasmic tail and is thereby lacking inherent signaling capability, we hypothesized that B7-DC XAb may be able to cause topological changes or clustering of membrane molecules that could recruit and activate DAP12/Syk pathways in mDC. To test this ability, we looked for changes in membrane localization of key molecules involved in the mDC stimulation of naive T cells. We found that class II, CD80, and CD86 molecules were reorganized into an indistinct cap-like cluster on the cell membranes of both mouse and human mDC within 15 min after Ab treatment (Fig. 4, A and B). The relationship between co-capped molecules and the kinetics of cap formation were investigated further using FRET. Within 10 min of B7-DC XAb treatment, class II, CD80, and CD86 molecules moved into close juxtaposition (<80 Å) as judged by the induction of a strong FRET signal (Fig. 5, A and B). However, no FRET signal could be detected when mDC were incubated with a different IgM Ab that binds to mouse class II molecules (Fig. 5C). Moreover, if the acceptor fluorophore was quenched as a result of this photobleaching, emission of donor fluorophore increased, further confirming FRET and the implied proximity of the rearranged class II and CD80/CD86 molecules on the cell surface (data not shown). The ability of B7-DC XAb to form capped structures on the surface of human and mouse mDC was abolished by blockade of B7-DC XAb with the B7-DC-specific IgG Ab MIH18 on human mDC (Fig. 5D, top row) and by the IgG Ab TY-25 on mouse mDC (Fig. 5D, bottom row). These observations are consistent with our previous findings that the ability of B7-DC XAb to induce functional changes in mDC is dependent on direct binding to B7-DC (11, 12). To test whether the complex was internalized, cells were stripped of the bound fluorophore-labeled Abs using Hanks buffer (pH 2.5) and subsequently analyzed for FRET signal. Signal was lost when cells were injected with B16 live tumor (n = 5 mice per group). Mice were monitored for tumor growth (presented as the average size in millimeters plus or minus deviation from the mean). Mice with tumor size of 17 × 17 mm were euthanized. D, mDC or mDC pretreated with piceatannol were pulsed with OT-II-specific peptide (ISQAVHAAHAEINEAGR) and control Ab or B7-DC XAb (○) before adoptive transfer into mice at the same time they were injected with B16 live tumor (n = 5 mice per group). Mice were analyzed for the ability to kill the B16 targets in a chromium release assay (performed in triplicate using spleens pooled from three mice per group). C, mDC were pulsed with the tumor cell lysate and treated with control Ab (○), B7-DC XAb (●), or B7-DC XAb plus piceatannol (□) before adoptive transfer into mice and day 7 after transfer, splenocytes from mice were analyzed for the ability to kill the B16 targets in a chromium release assay (performed in triplicate using spleens pooled from three mice per group).
Following treatment with B7-DC XAb, co-capping (FRET) of CD11c with class II still occurred in CD80/CD86/mDC and co-capping of CD80 with CD86 still occurred in class II/mDC (Fig. 5E), indicating that B7-DC XAb can induce clustering of these particular molecules independently of one another.

TREM-2 mediates activation through a DAP12-Syk signaling pathway and is required for Ag accumulation by mature mDC treated with B7-DC XAb

TREM-2, a recently described pattern recognition receptor expressed on monocytes and cultured DC, is known to signal through DAP12 (39, 40). As visualized by FRET, an association of TREM-2 with class II molecules was observed within 5 min and was increased in 15 min after treatment of mDC with B7-DC XAb (Fig. 6A). In contrast, TREM-2 was not associated with class II molecules on mDC following treatment with isotype control Ab. The association of TREM-2 with class II molecules was confirmed by coimmunoprecipitation of TREM-2 with the class II molecule I-Ab in lysates isolated from mDC 5 min after B7-DC XAb treatment (Fig. 6B).

To evaluate the functional importance of TREM-2 in the transduction of B7-DC XAb-induced signals was further confirmed using mDC derived from TREM-2 knockout mice. Although phosphorylation of DAP12 and Syk was readily induced in wild-type mDC activated with the MTAb B7-DC XAb, phosphorylation of these same signaling intermediates was not observed in TREM-2 knockout mDC (Fig. 7, A and B), and the matured knockout mDC did not regain the ability to accumulate OVA after Ab treatment (Fig. 7C). Furthermore, whereas wild-type mDC pulsed with B16 tumor lysate and B7-DC XAb functioned efficiently as an antitumor vaccine (fully protecting 5/5 animals from B16 melanoma challenge), animals receiving a TREM-2 knockout mDC vaccine succumbed to the melanoma (5/5 animals; p < 0.05).

Discussion

The ability of B7-DC XAb to induce a spectrum of biologic changes in mDC is consistent with the hypothesis that B7-DC may function as a signaling receptor on DC. Others have provided evidence for reverse signaling through a number of B7 family members, including CD80 and CD86 in response to CTLA-4-Ig or CD28-Ig ligands and B7-H1 in response to autoantibodies (20, 21, 41). Ligation of CD80 and CD86 by CTLA-4-Ig induces activation of NF-κB and MAPK pathways and the production of IL-6 and IFN-γ by DC, supporting potent T cell responses (20, 21). In contrast, CD28-Ig has been shown to activate NF-κB and MAPK pathways and the production of IL-6 and IFN-γ by DC, supporting potent T cell responses (21). In this study, we have used both human and mouse mDC to show that cross-linking B7-DC with the IgM Ab B7-DC XAb results in a reorganization of cell surface molecules, the activation of intracellular kinases, and the induction of Ag uptake and retention by mature mDC. These signaling events...
require TREM-2 and are mediated by the activation of Syk. Furthermore, both TREM-2 and Syk are required for B7-DC XAb-induced tumor immunity in mice. Although our data show that CD80 and CD86 are present in the molecular caps formed by B7-DC cross-linking, their role in Syk activation is unknown. Our previous studies showed that CD80 and CD86 are not required for Th1-polarization of recall responses induced by B7-DC XAb (12). We have also documented the expression of an array of immune mediators resulting from cross-linking B7-DC on mDC (26, 30) with a role in B7-DC XAb action are not yet clear. A key conclusion from all these experiments is that engagement of B7 family members with different ligands can induce a spectrum of immunomodulatory signals that can either up-regulate or down-regulate the immune response.

Engagement of B7-DC with B7-DC XAb results in the formation of multimolecular clusters including cell surface molecules involved in the generation of a productive T cell response (CD80, CD86, class II, and TREM-2). The ability of B7-DC XAb to induce these structures on the surface of cells is in contrast to the inability of an anti-class II IgM to induce comparable molecular...
clusters. The fact that bivalent Abs specific for B7-DC block membrane rearrangements induced by B7-DC XAb on both human and mouse mDC demonstrates that the induction of molecular complexes is mediated through B7-DC in both species.

The structures of human and mouse B7-DC differ with respect to the size of their cytoplasmic tails (9, 10). Although human B7-DC has a tail of 30 aa that could be a potential site for interactions with intracellular signaling molecules, mouse B7-DC is missing most of this structure, having only a few amino acids on the cytoplasmic side of the cell membrane. The B7-DC transmembrane domain is unremarkable in both species and contains no charged residues. Similar to all members of the B7 family, the extracellular domains of B7-DC are predicted to fold into two Ig-like domains. Molecules with these folds have a tendency to form heterodimers: CD8, Igs, TCR, and more recently B7 family members being notable examples (42). Whether the formation of multimolecular structures featuring the close juxtaposition of molecules such as class II, CD80, CD86, CD11c with B7-DC is critical for the generation of intracellular signals or is the consequence of signals generated by other molecular interactions remains to be tested. Nonetheless, the formation of molecular clusters containing TREM-2 in response to B7-DC XAb treatment demonstrates the principle that molecules other than B7-DC with intrinsic signaling capability may be recruited in this process.

One unanswered question is whether activation of mDC with B7-DC XAb represents a normal physiological process. It seems
immunoprecipitated from mDC derived from wild-type (WT) or TREM-2 DC function to treat human disease. That B7-DC XAb will be a potent modulator of human immune pathways in the mouse, providing a basis for our hypothesis that B7-DC XAb has potent immunomodulation of the DC membrane during interactions with the T cell and that this has distinctive physiological consequences that might be exploited in the treatment of allergic asthma (26). The observations documented in this study demonstrate that this human IgM activated signaling pathways in human DC that closely mirror acti-
vated signaling pathways in a mouse model of allergic asthma (26).

FIGURE 7. TREM-2 is required for B7-DC XAb-induced activation of Ag uptake in mature mDC. Tyrosine phosphorylation of DAP12 (A) or Syk (B) immunoprecipitated from mDC derived from wild-type (WT) or TREM-2 KO mice after incubation with B7-DC XAb or isotype control. C. OVA FITC uptake induced by the control Ab (filled histogram) or B7-DC XAb (open histogram) in matured mDC from wild-type (left) or TREM-2 KO mice. Data are representative of three or more experiments.

unlikely that cross-linking B7-DC in isolation is the means for intercellular communication. However, it is possible that reorgani-
zation of the DC membrane during cross-linking with the extracellular surface induces intracellular signaling pathways also involving B7-DC. What is interesting is that a cross-linking-induced PKCθ is a stimulus that has distinct physiological consequences that might be exploited in the treatment of melanoma challenge (26, 30, 38).

Thus, with this report, we have new understanding of the mole-
cular mechanisms limiting B7-DC XAb binding to mDC, the recruitment of TREM-2 into membrane clusters, the activation of Syk to an enhanced uptake and retention of Ag in mature mDC, and the induction of tumor immunity in mice. Our interpretation is that DC residing in tumors or draining lymph nodes are activated by cross-linking B7-DC and that the induced changes enhance their ability to capture tumor Ags and mobilize a protective cellular immune response against developing melanoma nodules (see also 11, 14, 30, 38).

Acknowledgments
We thank Bogoljub Ciric, Virginia P. Van Keulen, Dianne Khurana, and Kristina Bruns for technical support and help from Timothy Kottke and Richard Vile in establishing viral titers.

Disclosures
The authors have no financial conflict of interest.

References


Letter of Retraction


In the course of investigating suspicious patterns of experimental results in the laboratory, a systematic and in-depth study of key findings in this article was carried out using blinded protocols. In these repeat studies, no evidence was found to support our original conclusions that B7-DC XAb modulates dendritic cell functions. We do not believe our failure to reproduce our earlier findings is the result of a technical problem. A member of the B7-DC XAb investigative team, Dr. Suresh Radhakrishnan, who was involved in or had access to all the work on this subject, was found in a formal investigation to have engaged in scientific misconduct in unpublished experiments involving the B7-DC XAb reagent. This finding of misconduct and our inability to reproduce key findings using blinded protocols has undermined our confidence in our published reports. We seek, therefore, to retract this body of work.

Laura N. Arneson
University of Minnesota Law School
Minneapolis, MN

Jadee L. Upshaw
Charles L. Howe
Sara J. Felts
Moses Rodriguez
Larry R. Pease
Mayo Clinic
Rochester, MN

Marco Colonna
Washington University
St. Louis, MO