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

Suresh Radhakrishnan,* Laura N. Arneson,* Jaehee L. Upshaw,* Charles L. Howe,*** 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 piceatannol 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.

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 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--PLC-γ 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 Ag uptake and retention that occurs in matured DC upon cross-linking of the 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/6j and B6.129j-CD80−/−CD86−/− (CD80/CD86−/−) mice aged 6 to 8 wk old were obtained from The Jackson Laboratory. OT-2 mice were purchased from the National Institutes of Health (NIH) and bred in the laboratory. Class II knockout mice (23) were a gift from Dr. D. Billadeau (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 in the Mayo Clinic Institutional Animal Care and Use Committee-approved animal facility.

**Abs and other reagents**

Appropriate fluorophore-labeled Abs against murine IgGα (25-9-17), murine class II specific IgM (25-9-3), allophycocyanin-labeled anti-mouse CD11c (HL3) FITC-labeled anti-human class II (T209), FITC-labeled anti-human CD28 (CD28.2), allophycocyanin-labeled anti-human CD28 (CD28.2), PE labeled anti-human CD8, TRP-2 (T24) and PE-labeled anti-human HLA-A, HLA-B, HLA-C (IW-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 (LN13), PE-labeled anti-human CD80 (2D10.4), CD86 (IT2.2), murine B7-DC 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 Kk 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 Bio-technology. Anti-phosphotyrosine (4G10) and goat anti-mouse Abs were obtained from Upstate Cell Signaling Solutions. Anti-mouse TREM-2 Abs were purchased from R&D Systems. Rabbit Abs against PLC-γ1 (MC490) and DAP12 (MC457) 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, NVC23766 was a gift from Dr. D. Bil-ladeau (Mayo Clinic, Rochester, MN). CpG oligonucleotides as described (25) were synthesized in Mayo Core Facility. The polynucleotide polyinosinic-polycytidylic acid poly(IC) 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 × 10⁷ 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 for 4 days, 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 mDC were isolated by centrifugation over Ficoll-Paque PLUS (Amersham Biosciences), and CD14+ cells were separated by positive magnetic cell sorting. The isolated cells in RPMI 1640 containing 10 ng/ml human AB serum (HP 102-20, Valley Bio-medical) with 1% nonessential amino acids (Mediatech) and 1% rR(D&D) 10% and 50 ng/ml GM-CSF (Berlex Laboratories) were plated at 1 × 10⁶ cells/ml in 6-well plates for 6–8 days. Medium was changed with fresh RPMI 1640 with 1% FCS. Maturation of DC was achieved by addition of TLR ligands and poly(I·C), 100 μg/ml for a period of 24 h before being used for Ag uptake assays. All nonhuman cell lines used to culture bone marrow-derived mouse DC and peripheral blood-derived human DC are favorable toward myeloid differentiation; we refer to the DC in this study as mDC.

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

We isolated the hairpin containing the short hairpin TREM-2 sequence 5’-TGAT GACCTGGGTCAAGAGCCAGAGTCTCAGCATCAGCCGATCATGCACTGAC 3’ (28) from plasmid and cloned into the pSUPER RNAi System, as provided by Dr. D. Billadeau (Mayo Clinic, Rochester, MN). The shRNA sequences for TREM-2 were employed. 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 × 10⁶ 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 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 mM Tris-HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM NaBP₄, 0.1% BSA, 1 mM NaVO₃, 1 mM PMSF, 5 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 (MC490) or
FIGURE 1. Kinase and cytoskeleton reorganization inhibitors affect OVA-FITC uptake by matured human mDC. A. Human mDC were left untreated (filled histogram) or were matured overnight with poly(I:C). On day 6 the mDC cultures were activated with the isotype control Ab (gray-lined histogram) or with B7-DC XAb (open histogram) and pulsed with OVA-FITC. Uptake of OVA-FITC was assessed 24 h later by flow cytometry. B and C. Day 6 matured mDC were preincubated without or with the Src kinase inhibitor PP2 (0.3, 1, 3, or 10 μM), the PKC inhibitor Bim (50 nM), Rho A inhibitor Y-27632 (25 μM), PI3K inhibitor LY294002 (10 μM), PLC-γ inhibitor U73122 (10 μM), MEK inhibitor PD98059 (10 μM), p38 MAPK inhibitor SB203580 (1 μM), or Rac1 inhibitor NSC23766 (50 μM) before activation with isotype control Ab or B7-DC XAb. All cells were pulsed with OVA-FITC at the time of treatment and analyzed for OVA-FITC uptake 24 h later by flow cytometry. Data are representative of three or more experiments.

DAP12 (MC457) was bound to protein A-Sepharose beads and incubated 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, boiled, resolved by SDS-PAGE, and transferred to Immobilon-P membrane (Millipore). Tyrosine-phosphorylated proteins were detected using anti-phosphotyrosine antibody, Ab, 4G10, followed by goat anti-mouse HRP and reacted with HRP-labeled Chemiluminescence (Amersham Biotechnology) and the SuperSignal West Pico kit. Autoradiographs of the bound 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, TREP-2 was detected by blot using mouse Ab (237920) and 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)/anti-CD11c-PE (N418). Human mDC were stained with anti-class II-FITC (LN3) and anti-CD86-PE (2D10.4)/anti-CD80-PE (16.10A1)/CD86-PE (GL-1) or anti-CD11c-PE (N418). Human mDC were left untreated (open histogram) or were pulsed with OVA (1 mg/ml) for 15 min. Cells were stimulated with control Ab or B7-DC XAb or purified anti-mouse class II IgM (25-9-3) or allogeneic MHC molecules 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 log$_{10}$, 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) or mouse mDC (11) or poly(I:C) (10 μg/ml) for 3 days with titrated doses of mDC that were pretreated with different inhibitors (10 μM) before activation 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 μM), pulsed with 10 μM OT-II peptide (IQAYVHA-AHAEINEAGR), 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 \( \times 10^6 \) 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 \( \times 10^6 \), 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 \( \mu \)g/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 x 17 were euthanized as per the Institutional Animal Care and Use Committee recommendations.

Results

DAP12-Syk-PLC\(\gamma\) 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. 1A). 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-\(\gamma\) 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 (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-\(\gamma\), a downstream substrate for Syk (36) was also phosphorylated (Fig. 2C). Vav1, a RhoGEF activated by Syk and required for cytoskeletal rearrangements
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 the B16 tumor (Fig. 3C). However, mice that received piceatannol-treated mDC did not mount a cytotoxic response and failed to clear the 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 clustering on the surface of the mDC

Because B7-DC has a short cytoplasmic tail and is therefore 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 distinct 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 stripped at 15 min, though a weak signal was detected if the cells were stripped at 30 min, suggesting internalization of some of the complex (data not shown). The independent recruitment of CD80, CD86, and class II molecules into the cap was investigated using mDC derived from CD80/CD86 double knockout or MHC class II knockout mice.
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 of 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 communoprecipitation of TREM-2 with the class II molecule I-A^b 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, we assessed the activation of Ag uptake and retention by mature TREM-2-deficient mDC using a knockdown strategy. Transduction of a retrovirus containing shDNA for TREM-2 into mouse mDC substantially reduced the expression of TREM-2 on the cell surface (Fig. 6C). This reduction was associated with the absence of the phosphorylation of DAP12 and Syk (Fig. 6, D and E). Furthermore, using mature mDC, the shDNA-transduced cells accumulated very little OVA when treated with B7-DC XAb (Fig. 6F, right). When mDC were transduced with virus containing a scrambled shDNA sequence, expression of TREM-2 (Fig. 6C), phosphorylation of DAP12, and Syk (Fig. 6, D and E), and accumulation of OVA (Fig. 6F, left) were similar to that observed for nontransduced mDC (13).

The important contribution 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, p38 MAPKs, and STAT1, leading to the secretion of IFN-γ and the suppression of T cell proliferation (20). 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.

FIGURE 5. Reorganization of B7-DC XAb-activated DC cell surface results in close membrane clustering between class II-CD80-CD86. The distribution of class II (labeled with allophycocyanin-conjugated Ab) and CD80/CD86 molecules (labeled with PE-conjugated Abs) was visualized over time by FRET following treatment of human XAb (open histogram) or with isotype control Ab (filled histogram). C. Lack of induction of FRET in CD80-allophycocyanin and CD80-PE in mouse mDC by the I-A b-specific IgM Ab 25-9-3 (open histogram) and isotype control Ab-treated samples (filled histogram). Binding of the class II-specific IgM Ab to the mouse mDC analyzed is also shown (right panel). D. Inhibition of B7-DC XAb-induced FRET by B7-DC-specific IgG (green line histogram) compared with no IgG as control (red line histogram). Absence of FRET induced by isotype control Ab is also shown (filled histogram). E. Class II-allophycocyanin/CD11c-PE FRET in mDC from CD80+/−/CD86+/− mice (top). FRET signal at zero minutes (left) after treatment with control Ab (filled histogram) at mean fluorescence intensity (MFI) 80 or B7-DC XAb (open histogram) (MFI 98). FRET signal 15 min (right) after stimulation with isotype-matched control Ab (filled histogram) (MFI 190). CD80-allophycocyanin/CD86-PE FRET signal from class II+/− mDC (bottom). FRET signal at 0 min (left) after stimulation with control Ab (filled histogram (MFI 13) or B7-DC XAb (open histogram) (MFI 10). FRET signal 15 min (right) after stimulation with isotype-matched control Ab (filled histogram) (MFI 12.5) or B7-DC XAb (open histogram) (MFI 115). Data are representative of three or more experiments.
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
unlikely that cross-linking B7-DC in isolation does not mean for intercellular communication. However, it is possible that reorganization of the DC membrane during cross-linking with the B7-DC molecule induces intracellular signaling, therefore B7-DC. What is interesting is that cross-linking B7-DC (PD-L2) is a stimulus that has distinct physiological consequences that might be exploited in the treatment of human diseases. The potential importance of these novel DC activation pathways is illustrated by our findings that B7-DC XAb binding to human immune regulatory cells in vitro and in vivo results in in vitro and in vivo. Thus, with this report, we have new understanding of the molecular mechanisms linking 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). Moreover, changes in gene expression induced by B7-DC cross-linking of resident DC alters the polarity of the immune response against alligator Ag introduced into the airways in a mouse model of allergic asthma (26). The observations documented in this study demonstrate that human IgM activates signaling pathways in human DC that closely mirror activated pathways in the mouse, providing a basis for our hypothesis that B7-DC XAb will be a potent modulator of human immune responses and the rationale for developing this reagent to modulate DC function to treat human disease.

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Disclosures

The authors have no financial conflict of interest.

References

7. Jankovic, D. M., H. Hauschka, K. Finberg, S. Hiery, P. Caspar, C. M. Collazo, and A. Sher. 2002. In the absence of TLR2, CD4+ T cell responses to intracellular pathogens fail to activate Th1, Th2, Th17, and are host protective in vivo. IL-12 setting.


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.

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