Cross-linking the B7 Family Molecule B7-DC Directly Activates Immune Functions of Dendritic Cells

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Abstract

B7-DC molecules are known to function as ligands on antigen-presenting cells (APCs), enhancing T cell activation. In this study, cross-linking B7-DC with the monoclonal antibody sHIgM12 directly potentiates dendritic cell (DC) function by enhancing DC presentation of major histocompatibility complex–peptide complexes, promoting DC survival; and increasing secretion of interleukin (IL)-12p70, a key T helper cell type 1 promoting cytokine. Furthermore, ex vivo treatment of DCs or systemic treatment of mice with sHIgM12 increases the number of transplanted DCs that reach draining lymph nodes and increases the ability of lymph node APCs to activate naive T cells. Systemic administration of the antibody has an equivalent effect on DCs transferred at a distant site. These findings implicate B7-DC expressed on DCs in bidirectional communication; in addition to the established costimulatory and inhibitory functions associated with B7-DC, this molecule can also function as a conduit for extracellular signals to DCs, modifying DC functions.

Key words: dendritic cells • costimulation • B7 superfamily • B7-DC • IL-12

Introduction

B7-DC is a member of the extended B7 superfamily of co-stimulatory molecules that have been shown to play an important role in the regulation of T cell activation and differentiation (1, 2). Although B7-DC has less than 20% sequence identity at the amino acid level with classical B7.1 and B7.2, it shares an immunoglobulin fold and globular structure with other members of this family of costimulatory molecules. A homology search showed that B7-DC has the highest homology to B7-H1 (38% identity, 48% similarity; references 3 and 4). In addition to the high level of homology, B7-DC and B7-H1 have both been shown to bind PD-1, found on activated lymphocytes. B7-DC has been shown to have potent costimulatory properties for naive T cells in vitro (3). In these experiments, B7-DC fusion protein costimulated higher levels of T cell proliferation

and IFN-γ expression than B7.1 costimulation. In a separate study, however, B7-DC (PD-L2) was reported to inhibit cytokine production and cell cycle progression in previously activated T cells (5).

We had previously identified a naturally occurring human IgM antibody, sHIgM12, that specifically bound dendritic cells (DCs) and potentiated T cell activation and proliferation in vitro (unpublished data). Furthermore, B7-DC was identified as the ligand for sHIgM12 by DNA-mediated gene transfer, antibody blocking studies, and B7-DC knockout mice. The ability of IgM monomeric fragments to inhibit the intact pentamers from promoting T cell activation led us to investigate whether the antibody had direct effects on DC function. To study whether binding of sHIgM12 to B7-DC affects DC biology; DCs were treated in vitro with sHIgM12, polyclonal IgM control antibody, or LPS. DCs treated in vitro were analyzed for their ability to process and present a model antigen; and to secrete IL-12, a key immunomodulator, survive in culture in the absence of supporting cytokines, and to migrate to draining lymph nodes following adoptive transfer into syngeneic hosts. We find that an important aspect of B7-DC's

immune potentiating properties may be through the direct modulation of DC biology. Cross-linking of B7-DC on DCs increased antigen presentation and IL-12p70 production in vitro. Furthermore, anti–B7-DC treatment increased the survival of DCs in vitro and the migration of adoptively transferred DCs reaching draining lymph nodes in vivo.

Materials and Methods

Mice and Reagents. C57BL/6J and the green fluorescent (GFP) transgenic C57BL/6-TgN(ACTbEGFP)1Osb transgenic strains of mice were obtained from The Jackson Laboratory. B7-DC knockout and litter mate control bone marrow was acquired from Drew Pardoll, Johns Hopkins University. The knockout mice were generated by disruption of the second exon of the B7-DC gene on a 129/SvJ genetic background. The bone marrow was derived from animals of mixed genotype, as the knockout line is in the process of being backcrossed to C67BL/6. The B7-DC status of DCs derived from the bone-marrow cells was confirmed by flow cytometry. B7-DC–deficient DCs did not express epitopes recognized by rat anti-murine B7-DC-antibody (TY-25) nor by DC-reactive human antibody sHIgM12.

The human monoclonal antibody sHIgM12 was isolated from the serum of a patient with Waldenstrom Macroglobulinemia. IgM antibody was purified from the serum by precipitation with water and size-exclusion column chromatography. The preparation of antibody used in these experiments was greater than 90% IgM by electrophoresis. A sharp light chain band was evident upon electrophoresis, indicating the presence of a predominant species of antibody, a finding consistent with our ability to obtain a single unambiguous amino acid sequence from the prepared antibody. Polyclonal human IgM antibody isolated in a similar fashion was used for nonspecific control treatments. Appropriate fluorophore-coupled anti-CD11c(HL3) and anti-K^b(AF6-88.5) were obtained from BD Biosciences. 25D1.16 (anti-K^b-SIINFEKL) antibody was kindly provided by Dr. Jonathan Yewdell, National Institutes of Health, Bethesda, MD. The TY-25 anti-B7-DC antibody is available from eBioscience. FITC-coupled goat anti–human IgM (Jackson ImmunoResearch Laboratories) and goat anti–mouse IgG secondary antibodies were obtained from Accurate Chemical and Scientific. Chicken ovalbumin was obtained from Sigma–Aldrich. The peptides were synthesized at the Mayo Protein Core Facility, Mayo Clinic.

Generation of Immature and Matured DCs In Vitro. DCs from the bone marrow were isolated using an established protocol (7). Briefly, bone marrow was isolated from the long bones of the hind legs. Erythrocytes were lysed by treatment with ACK at 37°C. The remaining cells were plated at the density of 10^6 per ml in 6 well plates (Becton Dickinson) in RPMI (Hyclone) supplemented with 10% FBS (Hyclone) and containing 10 ng/ml of murine GM-CSF and 1 ng/ml of murine IL-4 (PeproTech). The cells were incubated at 37°C with 5% CO2. After 2 d of culture the cells were gently washed and replaced with RPMI-10 containing the same concentration of GM-CSF and IL-4 for an additional 5 d.

Flow Cytometry. Cells were washed with FACS® buffer (0.5% BSA and 0.1% sodium azide in PBS) and centrifuged into a 96-well plate (Nunc). The indicated antibodies were added to the wells for a 30 min incubation on ice. Samples requiring an incubation with fluorochrome-conjugated secondary antibody were washed twice before an additional 20 min incubation on ice. After a subsequent three washes, cells were fixed with 1% parafor-

maldehyde and analyzed on a FACSCalibur™ (Becton Dickinson). Data was analyzed using CELLQuest™ Software (Becton Dickinson).

Cytokine ELISA. Day 7 bone marrow–derived DCs were treated with sHIgM12, polyclonal IgM control antibody or LPS at a final concentration of 10 μg/ml. The supernatants were collected 72 h after stimulation and an IL-12p70 ELISA (BD Biosciences) was performed according to manufacturer's instruction. The supernatant tested for each treatment groups was pooled from six separate wells per experimental group. The experimental groups were tested in triplicates and at numerous dilutions with each demonstrating a similar profile.

Cellular Metabolism Studies. DCs were plated on day 5 into 96-well plates at 2×10^4 cells/well. Cells were cultured with sHIgM12, A2B5 control antibody, or media to a final concentration of 10 μg/ml in RPMI-10 with GM-CSF and IL-4. In cytokine withdrawal assays, DCs were washed and cultured in RPMI-10 alone. After 1 h of culture, Alamar Blue (Biosource International) was added to a final concentration of 10% (vol/vol). Readings were taken at 24 h of culture on CytoFluor Multiplate Reader Series 4000 (Perseptive Biosystems). The fluorescence plate reader was set to an excitation wavelength of 520 nm and an emission reading of 590 nm. The data is representative of three separate plates with each data point done in triplicate.

Adoptively Transferred GFP DCs. Bone marrow from green fluorescent protein (GFP+) transgenic mice were used to derive cultured GFP(+) DCs. SIINFEKL peptide-pulsed DCs were treated with sHIgM12 or an isotype control antibody 16 h before subcutaneous injection into the footpad. In the variant systemic treatment experiment, untreated DCs were injected (day 0) into the footpad of mice that had received three intravenous injections (day −1, 0, +1) with either sHIgM12 or pHIgM (10 μg). DCs were isolated from the ipsilateral popliteal and inguinal lymph nodes 48 h after transfer. Contralateral lymph nodes from both treatment groups served as a control cohort. Samples were stained with PE-conjugated CD11c antibody and analyzed by flow cytometry. Isolated peptide-pulsed DCs were used as stimulators to induce proliferation of OT-1 TCR Tg splenocytes.

Results and Discussion

The central role that DCs play in the initiation of an effective immune response is due in part to the costimulation that is provided to naive T cells concurrent with antigen presentation. Among the cohort of costimulatory molecules known to be expressed by antigen-presenting cells, the role of B7-DC in priming or maintenance of an immune response is intriguing because of its relative DC specificity. There are contradictory observations, however, about whether B7-DC ligation to its cognate receptor(s) on T cell is stimulatory or inhibitory. Tseng et al. report that B7-DC strongly costimulates naive T cell activation and IFN-γ production (3). We likewise observed an increase in T cell stimulatory capacity of wild-type DCs upon cross-linking B7-DC (unpublished data). Latchman et al., however, report that B7-DC inhibits T cell activation and cytokine production (5). Furthermore, high levels of B7-DC also inhibited strong B7-CD28 costimulation. A possible interpretation of enhanced T cell responses after DC treatment with anti–B7-DC antibody is that the antibody blocks the T cell inhibitory interaction between B7-DC

and PD-1. However, when we have looked directly, the human IgM antibody sHIgM12 is not present on the surface of treated DCs after washings routinely performed before the use of DCs in our in vitro T cell proliferation assays (unpublished data). Furthermore, a corollary of the hypothesis that B7-DC mediates inhibition is that genetic disruption of this pathway should increase T cell proliferation or activation. T cell proliferation, however, was not enhanced upon induction with B7-DC deficient DCs. DCs from these knockout mice, however, are still proficient APCs after stimulation with anti-CD40 antibody. The reports regarding the stimulatory or inhibitory role of B7-DC may not be mutually exclusive as different experimental conditions, such as the use of naive (3) versus activated (5) T cells and the existence of cognate receptors on T cells other than PD-1, may explain the divergent findings (8–10). Also, our observations that sHIgM12 antibody does not remain bound to the surface of DCs in vitro does not rule out the possibility that the antibody may play some blocking role in vivo.

We have explored an alternative hypothesis. One possibility is that cross-linking of B7-DC may induce functional changes in DCs themselves. The implication of this view is that the B7-DC molecule may be involved in cross talk between DCs and their environment.

Cross-linking B7-DC Directly Induces Functional Changes in DCs. The uptake and presentation of antigen is a basic biological function of DCs. The effect of B7–DC cross-linking on DC presentation of antigen was tested after incubation with ovalbumin. DCs were concomitantly treated with either the human monoclonal antibody sHIgM12 or pHIgM control antibody. The processing and presentation of ovalbumin by the treated DCs was evaluated with an antibody (25D1.16) that specifically recognizes the immunodominant ovalbumin peptide (SIINFEKL) in the context of K^b MHC molecule (6). B7-DC cross-linking increases the level of MHC–peptide complexes (K^b–SIINFEKL) presented on the surface of wild-type DCs compared with unpulsed or pHIgM-treated DCs (Fig. 1 A, top panels). sHIgM12 treatment of B7-DC deficient DCs, however, did not affect presentation of ovalbumin peptide relative to pHIgM (Fig. 1 A, bottom panels). The difference in MHC peptide complexes presented on the surface of sHIgM12- versus pHIgM-treated DCs, was maximal at 24 h and decreased after 48 h of incubation (unpublished data). Levels of K^b and D^b MHC molecules, however, were not different between the treatment and control groups (unpublished data).

Binding of 25D1.16 antibody to class I–peptide complexes is strongly influenced by the cooperative binding with Fc receptors to stabilize the low affinity idiotypic recognition of Kb-SIINFEKL (unpublished data). The levels of Fc receptor (CD16/CD32) expressed on DCs, however, were not different in both treatment and control groups (unpublished data), indicating that the increased 25D1.16 binding truly reflects an increase in the limiting MHC–peptide complexes elaborated on the cell surface. The levels of accessory molecules involved in DC stimulation of an effec-
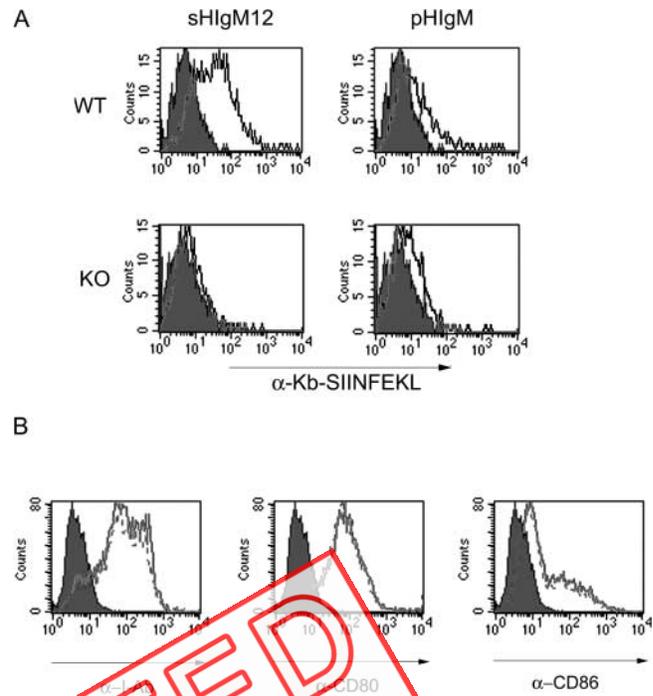
Figure 1. B7-DC cross-linking increases antigen presentation in vitro, but does not increase expression of cell surface accessory molecules. (A) Presentation of Kb-SIINFEKL complexes following treatment of ovalbumin protein–pulsed DCs with sHIgM12 (left panels) or pHIgM (right panels) antibodies. Bone marrow–derived DCs from wild-type (top panels) or B7-DC (bottom panels) mice were stained with 25-D1.16 antibody (dissolid) to detect specific MHC–peptide complexes. Shaded histogram represents impulsed DCs. (B) Immunostaining of ovalbumin-pulsed DCs for MHC class II, CD80, or CD86 molecules after sHIgM12 (solid line) and pHIgM (hashed line) antibody treatment. Shaded histogram represents staining isotype control antibody.

tive immune response were also tested. sHIgM12 cross-linking of B7-DC did not significantly increase surface expression of MHC class II or CD80 and CD86 costimulatory molecules (Fig. 1 B), or CD11c (unpublished data).

DC production of IL-12 was analyzed because this particular cytokine has a central role in stimulating the production of IFN-γ, a key factor in promoting Th1-type cellular immunity (11, 12). An ELISA was performed on culture supernatants to test for the presence of the active form of IL-12. As shown in Fig. 2 A, sHIgM12 treatment stimulated significantly higher levels of IL-12p70 released by DCs than did LPS, a strong danger signal. Polyclonal IgM treatment did not elicit detectable levels of IL-12p70. Interestingly, the sHIgM12-induced secretion of IL-12p70 was first detectable 24 h after it could be detected in LPS-activated DCs (unpublished data).

As cellular metabolism is an important parameter of DC viability and differentiation, we examined the effects of B7-DC cross-linking on DC biology by measuring DC metabolism of Alamar Blue under cytokine-deprived and –replete conditions. As shown in Fig. 2 B, GM-CSF/IL-4 withdrawal decreased metabolism in the cultures to 29% of the level found in DCs cultured in cytokine-supplemented media. Cross-linking B7-DC on DCs, however, resulted in

80% maintenance of metabolism levels 24 h after GM-CSF/IL-4 withdrawal. Treatment of DCs with a control IgM antibody that binds DCs, A2B5 (a monoclonal antibody that binds c-series ganglioside; reference 13), did not significantly improve metabolism in the cultures compared with media alone (33 vs. 29%). The higher rate of Alamar Blue conversion after 24 h cytokine withdrawal in sHIgM12 treatment groups directly correlated with higher number of live trypan-blue and Annexin V–negative DCs in these cultures compared with control cultures (unpublished data). We do not rule out, however, the possibility that sHIgM12 antibody treatment may also influence the metabolic rates of individual living cells.
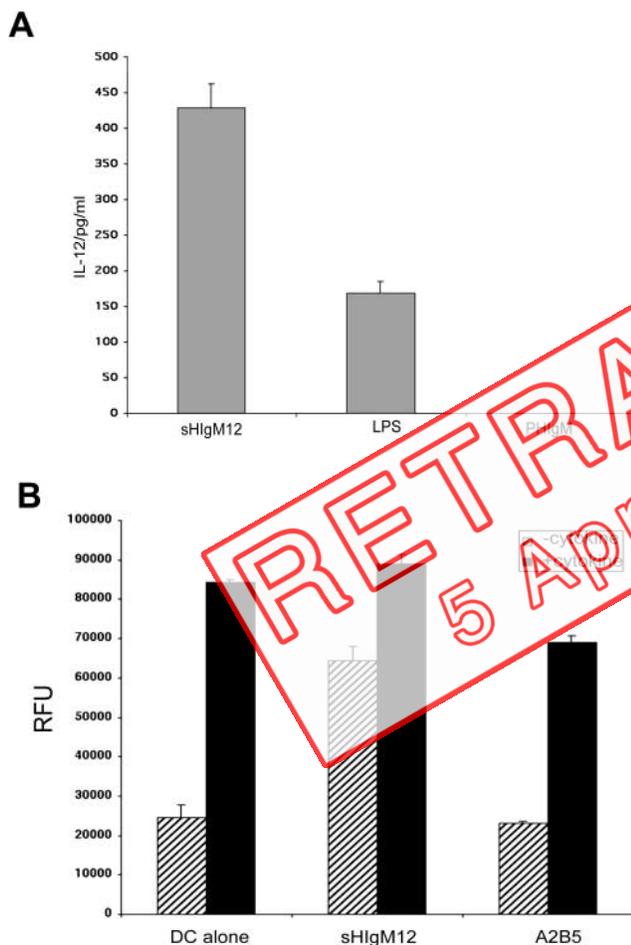

Figure 2. Murine bone marrow–derived DCs secrete high levels of IL-12p70 and exhibit prolonged survival in vitro following B7-DC cross-linking. (A) Day 7 bone marrow–derived DCs were treated with sHIgM12, polyclonal IgM control antibody or LPS at a final concentration of 10 μg/ml. The supernatants were collected 72 h after stimulation and an ELISA (BD Biosciences) was performed for the active fraction of IL-12 cytokine. The experimental groups were tested in triplicates and at numerous dilutions with each demonstrating a similar profile. (B) DC metabolism after treatment was accessed by reduction of Alamar Blue after a 24 h incubation period. DCs were plated on day 5 into 96-well plates at 2×10^4 cells/well. Cells were cultured with sHIgM12, A2B5 control antibody, or media with (solid) or without IL-4/GM-CSF (hashed) supplementation. The data is representative of three separate plates with each data point done in triplicate.

Antibody Enhances the Number of Adoptively Transferred DCs that Reach Draining Lymph Nodes. To further examine whether binding of sHIgM12 to B7-DC has a direct effect on DC viability, bone marrow–derived DCs from GFP transgenic mice were treated in vitro before adoptive-transfer into syngeneic, C57BL/6 mice. As shown in Fig. 3, A and B, fivefold more GFP+CD11c+ DCs were recovered from the draining popliteal and inguinal lymph nodes in mice receiving sHIgM12-treated DCs compared with those receiving pHIgM-treated DCs. The capacity of lymph node immigrants to potentiate an immune response was tested by concomitant treatment of the DCs with sHIgM12 and SI-INFEKL peptide before adoptive transfer. In vitro treatment of DCs with anti–B7-DC antibody not only increased the number of DCs recovered from the draining lymph nodes, but also increased by 10-fold the ability of these lymph node DCs to induce an antigen-specific T cell response (Fig. 2 C).

While the finding that of ex vivo antibody treatment influences the ability of adoptively transferred DCs to reach the draining lymph nodes has important therapeutic implications, we addressed the question of whether systemic administration of the antibody could have similar affects. Untreated GFP+ DCs were adoptively transferred into the footpad of naive animals to evaluate whether systemic administration of the antibody sHIgM12 would influence the number of DCs reaching draining lymph nodes. A fivefold increase in GFP+ DCs were recovered from draining lymph nodes in comparison to nodes recovered from mice receiving systemic control IgM antibody treatment (Fig. 4). This finding demonstrates that sIgM12 antibody administered intravenously can influence the ability of distant DCs to reach sites of T cell activation.

Together these data provide strong evidence that B7-DC can function as a conduit for the communication of signals directly to DCs from ligands in the environment. We have demonstrated that cross-linking of B7-DC induces changes in antigen processing, cell survival, and lymphokine production in DC populations grown in the absence of T cells. Direct modification of DC function is observed in cells bearing genetic deficiencies in TLR-4 receptors, indicating that LPS is not a contributing factor in these studies (unpublished data). Furthermore, we have demonstrated that the ability of DCs to reach draining lymph nodes after adoptive transfer into immunocompetent hosts is enhanced by treatment with these antibodies. One possible explanation of this finding is that antiapoptotic effects of antibody treatment effectively raises the number of surviving cells thereby increasing the number of cells that make it to the lymph nodes. Another intriguing possibility is that B7-DC interacts with cellular ligands that provide signals that influence migration.

An important question is whether systemic administration of anti–B7-DC cross-linking antibody can potentiate the response of resident DCs. The possibility that this is the case is suggested by our recent findings that systemic treatment of mice with sHIgM12 antibody enhances resistance to transplantable B16 melanoma and potentiates T cell responses to systemically administered soluble protein (un-

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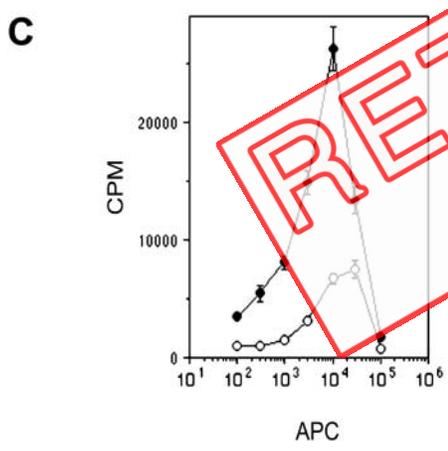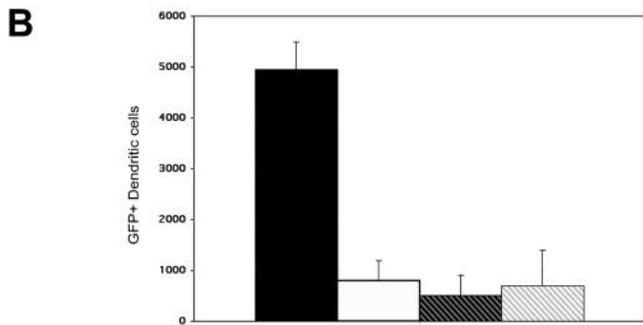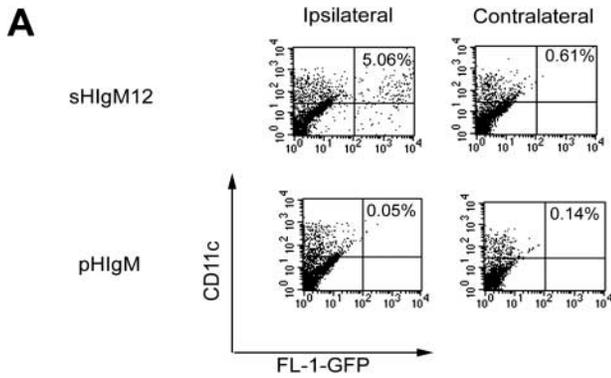
Figure 3. Anti-B7.DC treatment enhances potency and number of adoptively-transferred DCs recovered from draining lymph node. (A) Flow cytometric analysis, and (B) enumeration of CD11c⁺; GFP⁺ cells in draining lymph nodes of treated mice. Treatment with sHIgM12 (solid); or pIgM (hashed). Isolation of cells from ipsilateral (dark); or contralateral (light) lymph nodes. (C) Proliferation of OT-I TCR Tg splenocytes after incubation with isolated DCs. Ovalbumin-pulsed DCs were treated before adoptive transfer with either sHIgM12 (filled) or pIgM (open) control. Each group was performed in triplicate. The data is representative of three independent experiments.

published data). Understanding how systemically administered antibody mediates these important biological affects may provide insights into how DC biology might be manipulated to gain advantage in a number of disease states that intersect with the immune system.
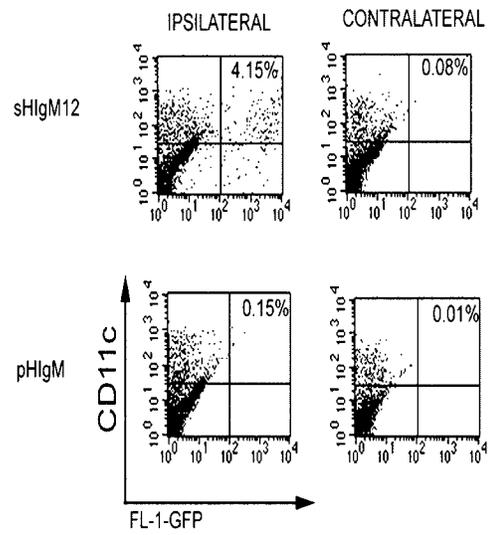
Figure 4. Systemic sHIgM12 treatment increases the number of adoptively transferred DCs recovered from the draining lymph node. Untreated GFP⁺ DCs were injected (day 0) into the footpad of mice that had received intravenous injections of sHIgM12 or pHIgM on days −1, 0, +1. Ipsilateral and contralateral draining lymph nodes were recovered 48 h later. Staining and flow cytometric analysis was performed as described in Materials and Methods.

The observed consequences of sHIgM12 binding to B7-DC on DC biology is akin to recent data on TNF-related activation-induced cytokine (TRANCE)-treated DCs. Josien and colleagues observed that TRANCE-treated DCs were better inducers of primary and secondary T cell responses in vivo (14). Furthermore, TRANCE-receptor activator of NF-κB (RANK) interaction on DCs increased the in vivo longevity and subsequent migration of DCs to the draining lymph node by 5–10 fold (14). Although signaling through TNFR superfamily members, like CD40 and RANK, is known to modulate DC biology, the downstream consequences of B7 superfamily binding has not been established. There is data to suggest a regulatory role of B7.1 and B7.2 molecules expressed on B cells and lymphomas. Signaling through these costimulatory molecules was reported to regulate B cell proliferation, antibody production, and Bcl-X(L) expression in these cells (15, 16).

Our data suggests that ligation of B7-DC on DCs has similar regulatory properties. To our knowledge, the role of B7 superfamily members in regulating DC biology has not been previously reported. In contrast to classical B7.1 and B7.2, murine B7-DC has a very short cytoplasmic domain (4 amino acids) that would not be expected to associate with signaling intermediates. A possible mechanism for B7-DC–mediated signaling in DCs is through the interaction with a coreceptor that contains intracellular signaling motifs. The presence of two charged amino acids within the transmembrane domain of murine B7-DC suggests a potential binding partner (3). The association of receptor subunits via charged amino acids within the transmembrane region is seen in a number of immune receptor complexes (i.e., TCR-CD3, BCR-Ig α/β, and Ly49-DAP12) on lymphocytes.

Recently, DCs have also been shown to express activating surface receptors that associate with signaling coreceptors components. FcγR and TREM-2 are immunoglobulin superfamily members, that require the association of FcγR–associated γ chain and DAP12, respectively, to activate DCs (17, 18). Interestingly, TREM-2/DAP12 promotes a partial activation of DCs through nuclear factor (NF)-κB–independent up-regulation of classical maturation markers (MHC class II, CD80, and CD86) without concurrent secretion of IL-12 (18). In contrast, cross-linking of B7-DC mobilizes IL-12 secretion without increasing surface maturation marker. It is interesting to speculate whether DCs may express a series of surface receptors that have distinct, perhaps complementary, function depending upon the immunological context.

In conclusion, the key role that DCs play in regulating the immune response has made them a focal point for therapeutic intervention in a number of clinical settings (19–23). Our observations that B7-DC cross-linking in vitro has a profound effect on DC viability, migration, and IL-12 production provide a rational scheme for interventions that require ex vivo DC manipulation. Furthermore, it will be interesting to see whether other signaling pathways that alter DC biology, such as CD40 and RANK, will have additive or synergistic effects when targeted in conjunction with B7-DC.

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