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CUTTING EDGE

Cutting Edge: Expression of Functional CD137 Receptor by Dendritic Cells[1]

Ryan A. Wilcox,* Andrei I. Chapoval,* Kevin S. Gorski,† Mizuto Otsuji,† Tahiro Shin,† Dallas B. Flies,* Koji Tamada,* Robert S. Mittler,‡ Haruo Tsuchiya,† Drew M. Pardoll,† and Lieping Chen[2]*

Interaction between dendritic cells (DCs) and T cells is a prerequisite for the initiation of a T cell response. The molecular nature of this interaction remains to be fully characterized. We report in this work that freshly isolated mouse splenic DCs and bone marrow-derived DCs express CD137 on the cell surface and in soluble form. Triggering CD137 increased the secretion of IL-6 and IL-12 from DCs. More importantly, infusion of an agonistic mAb to CD137 into naive mice enhanced the ability of DCs to stimulate T cell proliferation in response to both alloantigens and a nominal Ag in vitro. This enhancement of DC function is not mediated through activation of T cells, because the effect was also observed in RAG-1 knockout mice that lack T cells. Our findings implicate CD137 as an important receptor involved in the modulation of DC function. ***The Journal of Immunology,*** **2002, 168: 4262–4267.**

Dendritic cells (DCs) are professional APCs that play a key role in the induction of a T cell response. Ample evidence exists indicating that immature DCs migrate from peripheral tissues, such as the skin and gut, to secondary lymphoid organs upon capturing Ags. When DCs migrate to and position themselves within the T cell zone of secondary lymphoid organs, they undergo maturation characterized by an increased capacity to process and present antigenic peptides and a simultaneous decline in their ability to phagocytose Ags (1). Following maturation, DCs up-regulate the expression of both MHC and costimulatory molecules within secondary lymphoid organs, where they may initiate a primary T cell response. CD40, a member of the TNFR superfamily, can induce DC maturation and thus improve the ability of the DC to stimulate a primary CTL response (2–5). In fact, CD40 cross-linking can trigger DC maturation in vivo and replace the need for CD4$^+$ T cell help (2–4, 6). However, other TNF superfamily molecules may also play a critical role in the regulation of DC function. Lu et al. (7) recently observed that CD4$^+$ T cells could stimulate DC maturation in a CD40-independent fashion. LIGHT, a member of the TNF superfamily expressed by activated T cells, has been shown to partially mature DCs upon binding its receptor, herpesvirus entry mediator (8). TNF-related activation-induced cytokine has been shown to both enhance the ability of DC to prime a T cell response and prolong DC survival (9–11). These observations thus highlight the importance of TNFR superfamily members in the regulation of DC maturation.

CD137 (4-1BB) is a TNFR superfamily member expressed by activated T lymphocytes (12, 13). The ligand for CD137 could be detected in DCs, activated macrophages, B cells, and activated T cells (12). In vitro studies have demonstrated that agonistic mAb to both CD137 and its ligand (CD137L) costimulate proliferation and cytokine secretion in both CD4$^+$ and CD8$^+$ T cells (14–18). However, studies performed in vivo suggest that CD137 plays a more prominent role in the generation of a CD8$^+$ CTL response than a Th cell response (17, 19–21). The systemic administration of mAbs against CD137 or gene transfer of CD137L into tumor cells induces potent cell-mediated immune responses against tumors (19, 22–25). Injection of anti-CD137 mAb in tumor-bearing mice leads to regression of well-established tumors in various mouse models (19) and prevents the death of T cells responding to superantigen stimulation (26). The studies using CD137 as well as CD137L-deficient mice revealed the importance of CD137 costimulation in graft-vs-host disease and antiviral CTL responses (27–30). Thus, the accumulating reports suggest a crucial role for CD137 costimulation in CD8$^+$ T cell responses.

We report in this work that mouse DCs express CD137. More importantly, engagement of DC-associated CD137 by agonistic mAb or CD137L delivers a stimulatory signal to DCs leading to secretion of cytokines and an improved ability to stimulate T cell responses.

Materials and Methods

Mice and cell lines

Female C57BL/6 (B6) and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Age-matched mice (6–10 wk old) were used for all experiments. Female C57BL/6 RAG-1 knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-1 mice, a TCR-transgenic mouse strain specific for an epitope derived from chicken OVA, were a gift from Dr. E. Celis (Mayo Clinic, Rochester, MN).

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[2] Address correspondence and reprint requests to Dr. Lieping Chen, Department of Immunology, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905. E-mail address: chen.lieping@mayo.edu

[3] Abbreviations used in this paper: DC, dendritic cell; sDC, spleen DC; BM, bone marrow; CD137L, CD137 ligand; SMART, switch mechanisms at the 5′ end of RNA transcript; rm, recombinant murine.

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P815 cells transfected to express mouse CD137L were previously described (31).

SMART cDNA preparation and virtual Northern blot analysis

Switch mechanisms at the 5′ end of RNA transcript (SMART) can be used to generate high yields of full-length double-stranded cDNA from as little as 50 ng total RNA. The method uses the addition of cytosines by reverse transcriptase at the end of its first strand synthesis run to extend the first strand. This extension primer together with oligo(dT) provides the opportunity to amplify cDNA using short rounds of PCR (Clontech Laboratories, Palo Alto, CA). For virtual Northern blot analysis, 4- to 6-wk-old female BALB/c mice were used for tissue RNA preparation. Total RNA were extracted with TRIzol (Life Technologies, Rockville, MD) and SMART cDNA (Clontech Laboratories) synthesis from tissues; sorted DCs and activated macrophages were performed according to the manufacturer's protocol. SMART PCR cDNAs were purified by PCR purification kit (Qiagen, Valencia, CA). A total of 0.5 mg/lane purified DNAs were run on a 1% agarose gel and transferred on a Nytran nylon membrane (Schleicher & Schuell, Keene, NH). Preparation of radioactive probes by PCR using primer sets (5′-GTAACGGCCGCCAGTGTGCTG-3′ and 5′-CGCCAGTGTGATG GATATCTGCA-3′), radiolabeling of probes, hybridization, washing, and autoradiography were done as previously described (32)

Abs and other reagents

2A is a rat IgG2a mAb specific for mouse CD137 (33). The mAb to mouse CD137L (clone 14B3) was generated in a similar fashion. Another mAb to mouse CD137 (clone 3H3) was described previously (18). The mAb was purified from the culture supernatant HiTrap Protein G-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) and was endotoxin-free, as determined by the *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, Falmouth, MA). The rat IgG control Ab was purchased from Sigma-Aldrich (St. Louis, MO). Both anti-CD3 and anti-CD28 Abs were purchased from BD PharMingen (San Diego, CA).

The PE-labeled CD137 mAb was purchased from eBioscience (San Diego, CA). FITC-labeled or biotinylated Abs against mouse B7-1, B7-2, CD40, I-A^b, OX40 ligand, and streptavidin-PE were purchased from BD PharMingen. Rat IgG and 2A were biotinylated using EZ-Link NHS-LC-LC Biotin according to the manufacturer's instructions (Pierce, Rockford, IL). FITC- and PE-labeled rat IgG and hamster IgG isotype control Abs were purchased from BD PharMingen.

The chicken $OVA_{257–264}$ peptide (SIINFEKL) representing the H-$2K^b$-restricted epitope recognized by OT-1 T cells was synthesized by the Mayo Clinic Molecular Biology Core Facility.

Generation of DC

The method to generate bone marrow (BM)-derived DC was previously described (34). Briefly, BM-DCs were cultured in complete medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) supplemented with 10 ng/ml murine GM-CSF obtained from J558L-GM-CSF supernatant (35) and 2 ng/ml recombinant murine (rm)IL-4 (R&D Systems, Minneapolis, MN). Nonadherent cells were removed and fresh medium was added on days 2 and 4. Nonadherent BM-DCs were harvested on day 6 for FACS analysis. Mature BM-DCs were generated by the inclusion of 10 μg/ml LPS (Sigma-Aldrich) for the last 48 h of culture.

The method to generate DCs from spleens (sDC) of B6 or B6-RAG-$1^{-/-}$ mice was previously described (36). Briefly, spleens were dissected into small pieces (~1 mm^3) and incubated at 37°C in complete RPMI 1640 supplemented with 2 mg/ml collagenase (Sigma-Aldrich), 100 mg/ml DNase (Sigma-Aldrich), and 10 μg/ml polymyxin B (Sigma-Aldrich) for 30–45 min. Cell suspension was obtained by vigorous pipetting and was passed through a nylon mesh filter and washed with complete RPMI 1640. After lysis of RBCs with ACK lysis buffer, $CD11c^+$ sDC were isolated using CD11c microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The cells isolated were 90% $CD11c^+CD11b^+$ and 10% $CD11c^+CD11b^-$.

T cell stimulation

Twenty-four-well plates were coated overnight with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 mAb. Both $CD4^+$ and $CD8^+$ T cells were purified from the spleens and lymph nodes of B6 mice with CD4 or CD8 microbeads, according to the manufacturer's instructions (Miltenyi Biotec). Freshly isolated T cells, as well as T cells that had been activated with anti-CD3/anti-CD28 for 48 h, were stained for CD137 expression.

FACS analysis

DCs were isolated and stained at 4°C for 30 min with 1 μg of the Abs indicated and 10 μg of anti-CD16 and anti-CD32 (American Type Culture Collection, Manassas, VA) in 50 ml PBS supplemented with 3% FBS and 0.02% azide. DCs were washed and stained an additional 30 min at 4°C with 1 μg of the appropriate secondary Ab before washing and FACS analysis. For CD137 staining, either biotinylated mAb 2A and streptavidin-PE or PE-conjugated anti-CD137 (eBioscience) was used. Fluorescence was analyzed by a FACScan (BD Biosciences, Mountain View, CA).

In vitro proliferation assays

sDC were isolated from B6 or B6-RAG-$1^{-/-}$ mice that had received 100 μg mAb 2A or rat IgG i.p 24 and 72 h previously. For the MLR, sDC were cocultured with 4×10^5 BALB/c lymph node cells in a flat-bottom 96-well plate at the responder:stimulator ratios indicated. For OT-1 stimulation experiments, $CD8^+$ T cells were purified from OT-1 mice using CD8 microbeads according to the manufacturer's instructions (Miltenyi Biotec). A total of 1×10^4 purified OT-1 cells were cocultured with 4×10^4 sDC in a 96-well, U-bottom plate in the presence (1 ng/ml) or absence of OVA peptide. T cell proliferation was assessed by the addition of 1 μCi/well [^{3}H]TdR during the last 15 h of a 2- or 3-day culture. [^{3}H]TdR incorporation was measured in a MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland).

ELISA

For detection of soluble CD137, supernatants were collected from BM-DCs cultured for 48 h in the presence or absence of LPS. Similarly, sDC were cultured in complete RPMI 1640 supplemented with rmGM-CSF and rmIL-4, and supernatants were collected 24 and 48 h later. A 96-well ELISA plate (Dynatech, Chantilly, VA) was coated with 50 μl of the capture mAb 3H3 (1 μg/ml) at 4°C overnight. Biotinylated mAb 2A was used to detect soluble CD137 in DC supernatants and CD137Ig fusion protein in a standard curve. IL-6 and IL-12 were detected in cell-free supernatants by sandwich ELISA according to the manufacturer's instructions (BD PharMingen).

Results and Discussion

In an effort to determine the gene expression pattern of DCs, we screened a subtractive cDNA library between BM-derived GM-CSF-cultured DCs and activated macrophages. After RNA extraction, cDNAs were synthesized and amplified by PCR. CD137 message was found in abundance in DCs. Virtual Northern blotting using CD137 cDNA as a probe showed that CD137 mRNA could be detected in BM-DC, sDC, and a few monocyte/macrophage-derived tumor cell lines including RAW 264-7 and PU 5-18. Expression of CD137 was not found in normal tissues, including brain, lung, thymus, heart, liver, spleen, kidney, muscle, lymph node, and BM, as well as activated macrophages derived from BM or the peritoneal cavity. This expression pattern appears to be different from B7-1 (restricted to DC) and I-A^d α (broad expression) (Fig. 1*A*). In addition to a dominant 2.1- to 2.5-kb cDNA, which may encode full-length CD137, there was an additional ~1-kb band found in some cell lines. However, it is unknown whether this additional species of cDNA represents the alternative splice form of CD137 (see Fig. 1*D*). The data thus suggest that DCs express CD137. To confirm this finding, we examined the surface expression of CD137 by BM-DC and sDC using CD137-specific mAb 2A. BM-DCs were generated in vitro in the presence of GM-CSF and IL-4. These BM-DCs had a typical DC morphology and expressed DC markers, including CD11c, CD11b, and high levels of I-A^b (data not shown). Both immature and mature BM-DCs that had been cultured in the presence of LPS for 48 h were stained for CD137. As shown in Fig. 1*B*, both the immature and mature BM-DC expressed CD137. Like B7-1 and B7-2, CD137 was upregulated on LPS-matured DC. However, it should be noted that the level of expression of CD137 on the surface of immature BM-DC was variable among different DC preparations, albeit detectable (R. A. Wilcox and L. Chen, unpublished data). As shown

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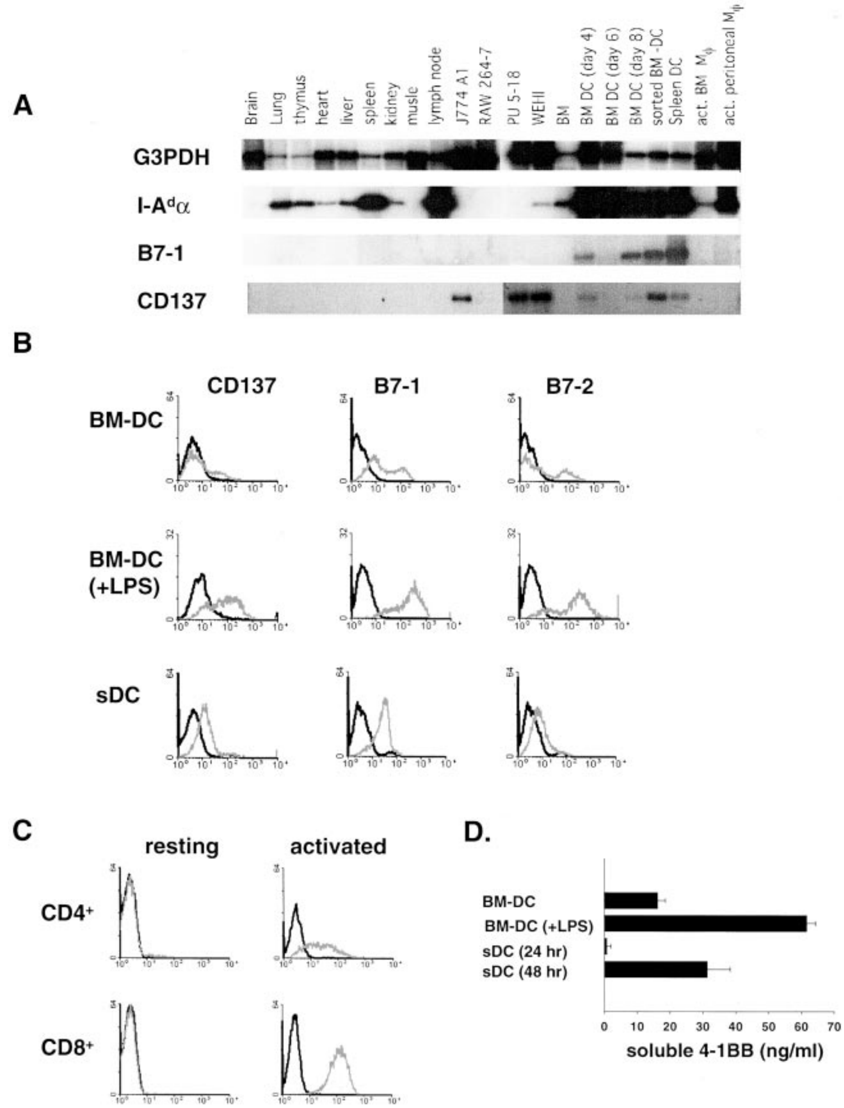

FIGURE 1. BM-DC and sDC express CD137. *A*, SMART cDNAs at 0.5 mg/lane derived from various tissues, monocyte/macrophage cell lines, BM-DC and sDC cultures, and activated (act.) peritoneal and BM-derived macrophages were electrophoresed. Southern blots were performed using the probes indicated. *B*, Both immature and LPS (10 μg/ml)-matured BM-DC or freshly isolated sDC were stained with anti-CD137, anti-B7-1, and B7-2 (gray histograms) or isotype control mAb (bold histograms). *C*, Both $CD4^+$ and $CD8^+$ T cells were stained with anti-CD137 (gray histogram) or isotype control mAb (bold histogram) before and after activation (48 h) with anti-CD3 and anti-CD28. *D*, A total of 1×10^5 BM-DCs or sDC were cultured in triplicate in a flat-bottom 96-well plate. Soluble CD137 was detected in cell-free supernatants by sandwich ELISA 24 or 48 h later.

in Fig. 1*C*, both $CD4^+$ and $CD8^+$ T cells express CD137 following activation by anti-CD3 mAb, although expression levels were greater for $CD8^+$ T cells. The levels of CD137 expression observed in the mature BM-DC were comparable to those observed on $CD4^+$ T cells.

We have detected a soluble form of CD137 in the culture supernatant by sandwich ELISA using two mAb against different epitopes of mouse CD137. As was observed for CD137 expressed on the cell surface, LPS-treated BM-DCs release higher levels of soluble CD137 than untreated DCs (Fig. 1*D*). Soluble CD137 likely represents an alternative splice form, as multiple RNA transcripts representing both cell-associated and soluble CD137 have been identified in T cells (37). However, the possibility that CD137 is cleaved by proteases from the cell surface of the DCs cannot be excluded. The significance of soluble CD137 is not yet clear, but it is possible that soluble CD137 plays a role in the down-regulation of a T cell response by blocking its endogenous ligand (data not shown).

We next examined the expression of CD137 on the surface of freshly isolated sDCs. The freshly isolated sDCs were largely $CD11c^+CD11b^+$, although ~10% of them were $CD11c^+CD11b^-$ and may represent the recently characterized murine equivalent of the human plasmacytoid DC (38). Importantly, the purified sDCs did not contain any $CD14^+$ cells, suggesting that contamination with monocytes or macrophages was negligible in our preparation. Furthermore, these sDCs expressed high levels of I-A^b (data not shown) and both B7-1 and B7-2 (Fig. 1*B*). As was observed with the BM-DC, freshly isolated sDCs also express CD137, albeit to a greater extent than immature BM-DC (Fig. 1*B*), suggesting that immature DCs may constitutively express CD137 in vivo. Although CD137 transcripts could not be detected in the spleen or lymph node by Northern blot analysis (Fig. 1*A*), this technique may not be sufficiently sensitive to detect CD137 transcripts in DCs and activated T cells present in secondary lymphoid organs. No significant change in CD137 expression was observed upon stimulation with LPS, although the in vitro culture of sDC in medium increased expression levels of B7-1, B7-2, and CD137, compared with freshly isolated sDC (33). Similarly, we also detected low levels ($<$5 ng/ml) of soluble CD137 in sDC supernatants during the first 24 h of culture and extended in vitro incubation for an additional 24 h increased the levels of soluble CD137 significantly (Fig. 1*D*).

To determine whether ligation of DC-associated CD137 stimulates DCs, we cocultured BM-DC with CD137L/P815 cells. As shown in Fig. 2*A*, BM-DCs stimulated with irradiated CD137L/P815 secreted high levels of IL-6. IL-12 was also detected in the

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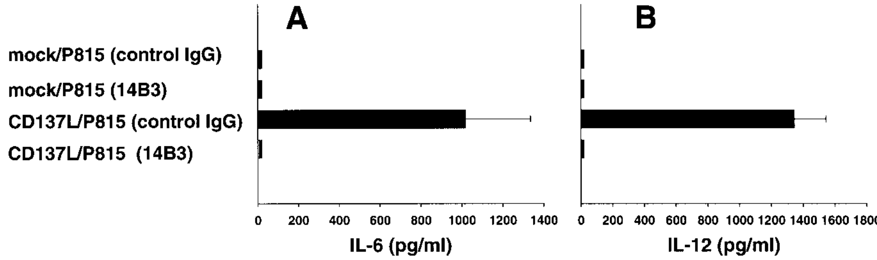

FIGURE 2. CD137L stimulates BM-DC to secrete cytokines in vitro. A total of 1×10^5 BM-DCs were cocultured with 2×10^5 irradiated mock/P815 or CD137L/P815 in triplicate in a 96-well plate with GM-CSF (10 ng/ml) and IL-4 (1 ng/ml). A control rat IgG or anti-CD137L mAb (14B3) was added to each well (10 μg/ml), and the supernatants were collected 48 h later for IL-6 (*A*) or IL-12 (*B*) ELISA. The cocultures used for the detection of IL-12 were supplemented with 1 μg/ml LPS to increase the sensitivity of the assays.

CD137L/P815-stimulated BM-DCs that were cultured with a low dose of LPS (Fig. 2*B*). These cytokines were not detectable in the mock/P815 cocultures. In addition, the stimulatory effect of CD137L/P815 could be neutralized completely by anti-CD137L mAb. Our results indicate that CD137 ligation delivers an activation signal to DCs.

Given the importance of DCs in the induction of a T cell response, we sought to determine whether signaling through DC-associated CD137 in vivo could enhance their T cell stimulatory function. We reported previously that administration of CD137 agonistic mAb 2A induced the activation of $CD8^+$ T cells in vivo and led to regression of established tumors (19). We adopted this system to examine whether 2A could activate DCs in vivo. To do so, B6 mice were infused with 2A mAb and sDCs were subsequently isolated from the mice. Purified DCs were tested immediately without further manipulation for their ability to stimulate allogeneic T cells. As shown, sDCs isolated from the mice given 2A were better stimulators in the allogeneic MLR than those from the mice given the control rat IgG (Fig. 3*A*), as demonstrated by the >30% increase in thymidine incorporation. Similarly, OVA peptide-pulsed sDCs from 2A-treated mice were better stimulators of OT-1 transgenic T cells than those sDCs isolated from control IgG-treated mice (Fig. 3*B*).

While our results suggest that triggering of CD137 by 2A improved the ability of these sDCs to stimulate T cells, it is not yet clear whether this is mediated by a direct ligation of CD137 on DC or by an indirect effect through ligation of CD137 on T cells. Although the mice used in our experiments for the preparation of sDCs are naive and CD137 is undetectable by FACS analysis on T cells from these mice (data not shown), the possibility remains that CD137 is expressed in very low levels or is selectively expressed in a small subset of T cells. To exclude this possibility, we decided to

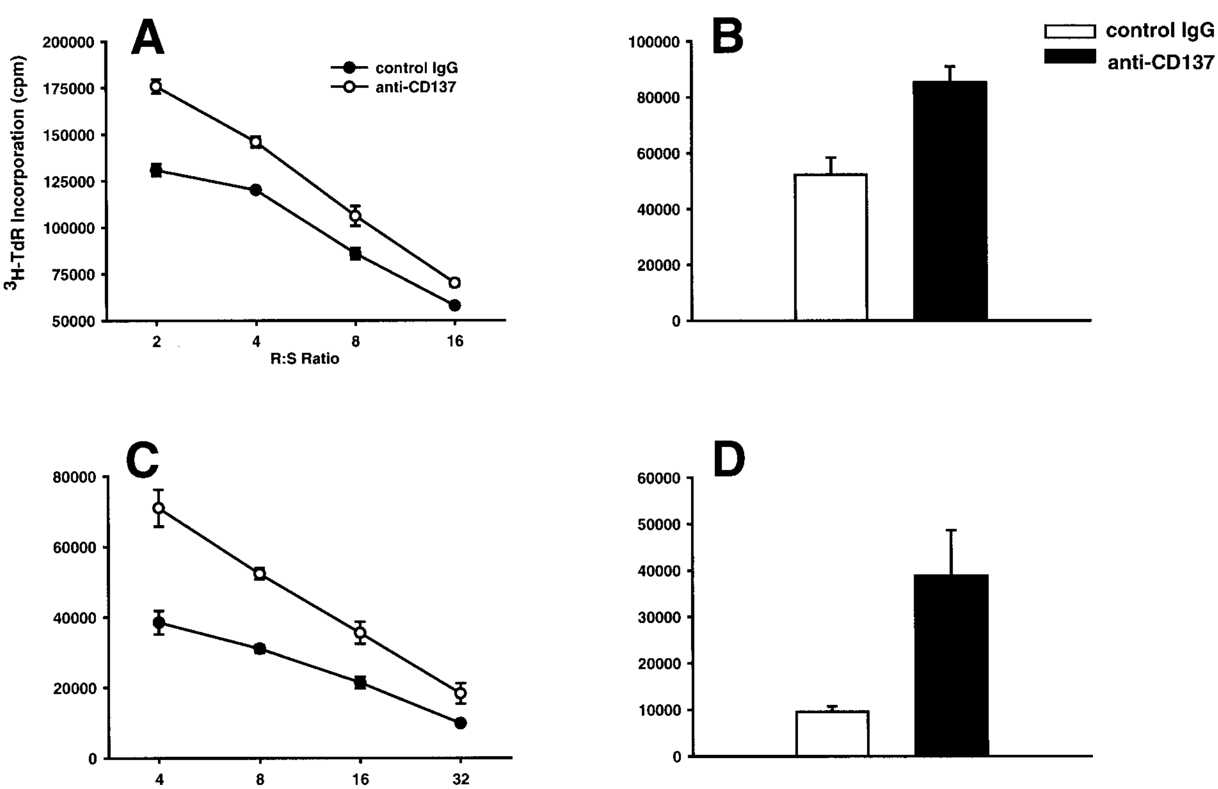

FIGURE 3. Administration of anti-CD137 mAb promotes the ability of sDC to stimulate T cell proliferation. Wild-type (*A* and *B*) or RAG-1-deficient (*C* and *D*) C57BL/6 mice were given control rat IgG or 2A at 100 μg/mouse 72 and 24 h before isolating sDC. Purified sDC were either used as stimulators in an allogeneic MLR with T cells from BALB/c mice at the R:S ratios indicated (*A* and *C*) or were pulsed with 1 ng/ml OVA peptide and used to stimulate naive OT-1 T cells (*B* and *D*).

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examine the effect of 2A on DC activation in RAG-1 knockout mice (B6 background) that lack T cells and B cells. In a similar fashion, administration of 2A greatly improved the ability of sDCs isolated from these mice to stimulate proliferation of both allogeneic T cells (Fig. 3*C*) and OVA-specific OT-1 cells (Fig. 3*D*). Our results support the notion that anti-CD137 mAb directly stimulates DC-associated CD137, resulting in a significant enhancement of their capacity to stimulate naive T cells.

The mechanism involved in the regulation of DC function upon CD137 ligation is unknown. It was reported that binding of CD137 on T cells by its ligand recruited TNFR-associated factor-2 and led to activation of p38 MAPK, apoptosis signal-regulating kinase-1, and c-Jun N-terminal/stress-activated protein kinases (39–41). These signaling events presumably increase secretion of cytokines and expression of additional cell surface molecules. Our results suggest that cytokines such as IL-6, and particularly IL-12, may play a critical role in this process. However, we were unable to find any significant changes upon CD137 ligation in the expression of CD80, CD86, 4-1BBL, OX40 ligand, CD40, B7-H1, B7-H2, B7-H3, and B7-DC on the cell surface (data not shown). Therefore, CD137 signaling may enhance DC function through a yet unknown mechanism.

We reported previously that the systemic administration of mAb against CD137 could eradicate established tumors in mice by the vigorous amplification of tumor-specific $CD8^+$ CTL activity (19). A combination of anti-CD137 mAb and IL-12 (22) or a peptide vaccine (33) can further increase immunity against tumors that are resistant to anti-CD137 mAb. Our findings in this study suggest that, in addition to directly triggering CD137 on primed tumor-specific T cells, administration of agonistic CD137 mAb may also stimulate DCs directly and enhance their ability to stimulate the vigorous T cell response observed in tumor-bearing mice treated with anti-CD137 mAb. CD137 signaling on the DC may thus explain, at least in part, the potent effect of anti-CD137 mAb in the activation of tumor-specific CTL. In addition, CD137 is also expressed on activated mouse NK cells (31) and human monocytes (42). Interestingly, CD137 signaling on human monocytes induced cell contact-dependent apoptosis of B cells (42). Taken together with the observation that CD137L is also found on activated T cells, B cells, macrophages, and even some tumor cells (43), our results suggest that CD137 and its ligand may play a role in the cross-talk among these cells during the generation of an immune response. The work presented here may have profound implications for both our understanding of DC immunobiology and our mechanistic understanding of CD137-based immunotherapy.

Acknowledgments

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