The inhibitory receptor LILRB1 modulates the differentiation and regulatory potential of human dendritic cells

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Dendritic cells (DCs) link innate and adaptive immunity, initiating and regulating effector cell responses. They ubiquitously express members of the LILR (ILT, LIR, CD85) family of molecules, some of which recognize self-HLA molecules, but little is known of their possible functions in DC biology. We demonstrate that the inhibitory receptor LILRB1 (ILT2, LIR1, CD85j) is selectively up-regulated during DC differentiation from monocyte precursors in culture. Continuous ligation of LILRB1 modulated cellular differentiation, conferred a unique phenotype upon the resultant cells, induced a profound resistance to CD95-mediated cell death, and inhibited secretion of cytokines IL-10, IL-12p70, and TGF-β. These features remained stable even after exposure of the cells to bacterial LPS. Ligated DCs exhibited poor stimulatory activity for primary and memory T-cell proliferative responses, but this was substantially reversed by blockade of CD80 or its preferred ligand CTLA-4, or by depleting $CD4^+$ $CD25^+$ $CD127^{lo}$ regulatory T cells. Our findings suggest that ligation of LILRB1 on DCs by self-HLA molecules may play a key role in controlling the balance between the induction and suppression of adaptive immune responses. (Blood. 2008;111:3090-3096)

Introduction

Dendritic cells (DCs) play pivotal roles in the initiation, regulation, and maintenance of immune responses.[1] Activation of DCs through toll-like receptors (TLRs) for "infectious nonself" or other "danger" signals normally initiates a process of cellular differentiation resulting in "mature" DCs capable of stimulating T-cell and natural killer (NK)–cell responses. It has also been proposed that DCs are intimately involved in the prevention of inappropriate immune responses to "self"-antigens.[2] How this is achieved is not well known, but it is clear that DCs in a quiescent "immature" state can potentially control autoimmune attacks, through secretion of immunosuppressive cytokines such as IL-10 or TGF-β, for example, or by controlling the induction of $CD4^+$ $CD25^+$ regulatory T cells.

Recent studies have suggested a role for human leukocyte Ig-like receptors (LILRs, also known as ILT, LIR, or CD85) in regulating the function of myeloid cells, potentially implicating these molecules in the control of immune responses.[3-5] LILRs are encoded by a set of genes within the leukocyte receptor cluster on chromosome 19q13.4, adjacent to the killer Ig-like receptor (*KIR*) genes that are responsible for controlling NK- and CD8 T-cell survival and effector functions.[6] Similar to the KIR, the only known ligands for certain LILR molecules are "self"-HLA class I molecules. Ligation of these receptors results either in diminution of intracellular signaling by ITIM-associated phosphatase activity for inhibitory receptors with a long cytoplasmic tail or in ITAM-associated signaling through adaptor molecules recruited by activating receptors with a short cytoplasmic tail.

Unlike KIRs, which are expressed clonally to form a variegated repertoire by NK cells, LILR molecules appear to be almost ubiquitously expressed by myeloid cells including most types of DCs, with the exception of plasmacytoid DCs.[7] Typically, human DCs for experimental and/or therapeutic purposes are obtained by culturing monocytes in defined cytokines, followed by maturation with TLR agonists such as LPS and/or other stimuli.[8] Here we demonstrate that continuous ligation of the inhibitory receptor LILRB1 (ILT2, LIR1, CD85j)[9] by mimics of "self"-MHC molecules dramatically alters the cellular differentiation program and subsequent responses to the "infectious nonself" TLR agonist LPS, the capacity to induce and regulate T-cell responses, and susceptibility to cell death.

Methods

Generation of monocyte-derived DCs

DCs were derived from peripheral blood monocytes by culture for 6 days in RPMI 1640 containing penicillin (100 U/mL), streptomycin (100 μg/mL), and 1% autologous plasma, 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, London, United Kingdom), and 50 ng/mL recombinant human IL-4 (Peprotech). Half of the medium was replaced with fresh cytokines every 2 days until day 6 of culture, when bacterial LPS (Sigma, Poole, United Kingdom) was added to a concentration of 1 ng/mL for 24 hours as required.

To ligate cell surface receptors, purified anti-LILRB1 (clone HPF1) or HCMV-UL18-Fc was added to the cultures at 10 μg/mL in the presence of 2 μg/mL protein G (Sigma). Mouse IgG1 isotype MOPC21 or human IgG (Sigma) was used as control. HCMV-UL18-Fc fusion protein was produced by amplifying the UL18 gene from human cytomegalovirus (HCMV) strain AD169, cloning into sIgpIg vector (Sigma), transfecting 293T cells, and purifying UL18-Fc from culture supernatants by protein A chromatography.

Submitted May 9, 2007; accepted November 26, 2007. Prepublished online as *Blood* First Edition paper, December 19, 2007; DOI 10.1182/blood-2007-05-089771.

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Flow cytometry

DC populations were washed and incubated with the following labeled antibodies (BD Biosciences, Little Chalfont, United Kingdom): anti–CD83-FITC, anti–CD1a-FITC, anti–CD14-FITC, anti–CD38-FITC, anti–CD63-FITC, anti–CD80-PE, anti–CD40-PE, anti–CD68-PE, anti–CCR7-PE, anti–CD86-Cychrome, anti–HLA-DR-Cychrome, or labeled mouse isotype controls. After incubation at 4°C for 1 hour, cells were washed and analyzed on a FACSCAN flow cytometer (BD Biosciences) using Cellquest software (BD Biosciences). DCs were identified on the basis of forward and side scatter characteristics, and a minimum of 20 000 gated events was collected. For endocytic assays, cells (5×10^5) were incubated at 37°C or 4°C with 1 mg/mL FITC-labeled 40K-Dextran (Invitrogen, Paisley, United Kingdom) for 1 hour. Cells were washed extensively and analyzed by flow cytometry as above.

T-cell proliferation assays

LILRB1- or IgG-treated cell preparations, subsequently cultured in the presence or absence of LPS, were washed, irradiated with 50 Gy, and added in graded numbers to 96-well round-bottomed microplates. For MLRs, allogeneic responder lymphocytes were labeled with CFSE (Invitrogen) and 5×10^4 cells were added to triplicate wells containing DC stimulators. Culture medium was RPMI 1640 with antibiotics, L-glutamine, and 5% human AB serum (Sigma). Plates were incubated at 37°C 5% CO_2 for either 7 or 10 days, washed, and stained with anti–CD3-CyChrome (BD Biosciences), and CFSE fluorescence of gated $CD3^+$ lymphocytes was analyzed by flow cytometry. MLRs were also performed in the presence of 10 μg/mL purified anti-CD80 (clone L307.4) or anti–CTLA-4 (clone BNI3) mAbs (BD Biosciences) with irradiated stimulators. In addition, MLRs were performed using $CD4^+$ $CD45RO^+$ T cells and $CD4^+$ $CD45RO^+$ $CD25^-$ T cells purified using magnetic microbeads (Dynal, Bromborough, United Kingdom) or $CD4^+$ $CD25^+$ $CD127^{lo}$ T cells purified by flow cytometric cell sorting as responders. For memory responses, cell populations were incubated at 37°C 5% CO_2 overnight with graded doses of tetanus toxoid (Sigma). After washing, the cells were cultured in the presence of 1 ng/mL LPS, irradiated, and used to stimulate 5×10^4 autologous CFSE-labeled lymphocytes in 96-well microplates for 7 days before flow cytometric analysis as above.

Quantitation of DC cytokine production

Culture supernatants from LILRB1-ligated or IgG-treated DC preparations were collected prior to (day 6) and after (day 7) exposure to LPS and stored at − 80°C. Levels of IL-10, IL-12p70, and TGFβ were assayed using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Oxfordshire, United Kingdom).

Apoptosis analysis

Apoptosis in cell populations was assessed by staining with annexin V–FITC or intracellular anti–active caspase-3–PE (BD Biosciences). To assess the effect of CD95, cell preparations (2×10^5) were washed and incubated overnight in RPMI 1640 culture medium with antibiotics, L-glutamine, 5% human AB serum, 5 μg/mL purified anti-CD95 monoclonal antibody (clone DX2; BD Biosciences), or isotype control IgG and 2 μg/mL protein G. Cells were washed and stained with annexin V–FITC before flow cytometric analysis as above.

Phosphotyrosine analysis

The tyrosine phosphorylation status of LILRB receptors was analyzed in purified monocytes and monocyte-derived DCs using a commercial antibody array (R&D Systems) according to the manufacturer's instructions.

Results

We first examined expression of LILRB receptors during the normal differentiation of monocytes into immature DCs in culture using available mAbs and flow cytometry. There was pronounced up-regulation of LILRB1, whereas expression of LILRB2 and LILRB4 was increased to a much lesser extent (Figure 1A). Levels of LILRB1 remained high after exposure of the cells to LPS to induce maturation. LILRB1, LILRB2, and to a lesser extent LILRB4 were constitutively phosphorylated in ex vivo–purified monocytes (Figure 1B), and this indicator of signaling function remained positive throughout DC generation. This finding is consistent with a recent report of *cis*-ligand binding of PIR-B in murine mast cells.[10]

Next we investigated the effect of continuous ligation of LILRB1 on the phenotype of the developing cells. We ligated this receptor with a physiological mimic of HLA class I molecules or with its pathological ligand, the human cytomegalovirus (HCMV) class I homologue UL18,[11] by respectively including anti-LILRB1 mAb or UL18-Fc fusion protein in the cultures. Subsequent flow cytometric analysis of the resultant cell populations after 6 days revealed profound differences between control and ligated cultures (Table 1). First, during normal DC differentiation, expression of the monocyte marker CD14 is markedly reduced and the cells acquire CD1a, but the ligated cells expressed the converse phenotype. Second, the ligated cells expressed almost no CD83 and relatively low levels of the costimulatory molecule CD86, both of which are normally up-regulated during DC differentiation and particularly after maturation. Third, they expressed constitutively high levels of the multifunctional ectoenzyme CD38, the costimulatory molecule

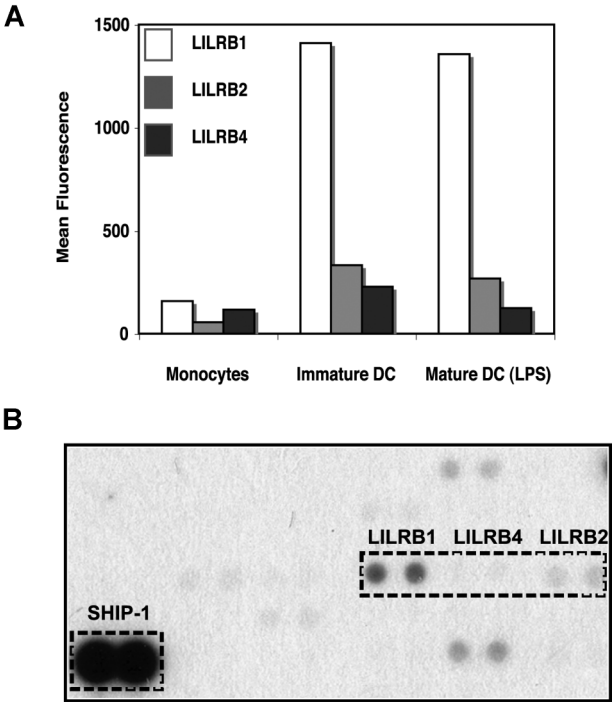

Figure 1. Expression and tyrosine phosphorylation status of inhibitory LILRs during differentiation of monocyte-derived DCs. (A) Cell-surface expression of LILRB1 (□), LILRB2 (▒), and LILRB4 (■) was analyzed by flow cytometry. The majority (> 95%) of each cell population expressed the respective inhibitory receptors. The y-axis represents the geometric mean fluorescence levels. Results shown are representative of 4 experiments. (B) Tyrosine phosphorylation status of LILRB receptors was examined using a commercial array. Negatively isolated monocytes cultured overnight or day-7 monocyte-derived DCs were lysed, incubated with the R&D Systems Human Phospho-Immunoreceptor Antibody Array, washed, and incubated with a phosphotyrosine-specific antibody, and results were visualized by chemiluminescence. LILRB1, LILRB2, and LILRB4 were positive throughout in vitro differentiation indicating continuous signaling function. A representative blot is shown.

Table 1. LILRB1 ligation during cellular differentiation results in a distinct and LPS-resistant DC phenotypic profile

	Isotype		Isotype + LPS		LILRB1		LILRB1 + LPS	
Marker	%*	MFI†	%*	MFI†	%*	MFI†	%*	MFI†
CD1a	46.5	45.7	41.3	30.9	4.6	16.4	3.7	15.5
CD11c	99.8	124.0	99.5	148.9	95.2	94.2	94.7	96.3
CD14	27.2	36.5	26.5	37.8	90.3	210.1	90.7	210.4
CD38	25.7	13.0	86.2	29.1	81.4	32.4	82.1	33.3
CD40	21.5	32.0	42.4	39.2	28.6	19.3	30.9	18.5
CD63	73.4	20.6	32.7	15.8	70.9	17.7	75.4	17.9
CD80	72.8	29.3	97.9	103.7	98.0	66.7	98.4	79.3
CD83	10.5	15.6	78.1	22.3	0.7	15.2	1.3	15.0
CD86	74.8	58.7	97.8	80.1	43.8	46.8	43.8	43.5
CCR7	9.3	19.4	21.1	26.0	22.2	28.4	26.7	22.1
HLA-DR	99.4	294.7	99.8	666.2	99.4	211.2	99.8	230.3

Cell populations cultured in the presence of anti-LILRB1 mAb or isotype control and subsequently exposed or not to LPS, as indicated, were analyzed by flow cytometry for expression of the indicated markers.

*Percentage of positive cells.

†Geometric mean fluorescence of positive cells. Results are representative of more than 12 experiments.

CD80, and the chemokine receptor CCR7 that approximated the levels expressed by LPS-matured DCs.

Remarkably, the phenotype of the ligated cells remained stable even after exposure to LPS (Table 1), whereas control cells showed the characteristic phenotypic changes associated with DC maturation noted above, including down-regulation of the endosomal marker CD63 (LAMP-3). Despite this, the ligated cells expressed TLR4, for which LPS is an agonist, at only slightly lower mean levels compared with control (MFI = 35.9 ± 10.1 vs 64.7 ± 17.2, respectively; n = 4; $P = .112$) together with high levels of CD14, as noted above, which normally partners TLR4 in LPS responses. Moreover, after 6 days of culture the ligated cells morphologically resembled immature DCs, but they did not develop the long dendritic processes typical of mature DCs after exposure to LPS (not shown).

In cultures derived from different donors, LILRB1 ligation often resulted in a noticeable increase in the acidity of the medium compared with controls, as reflected by a yellow color of indicator

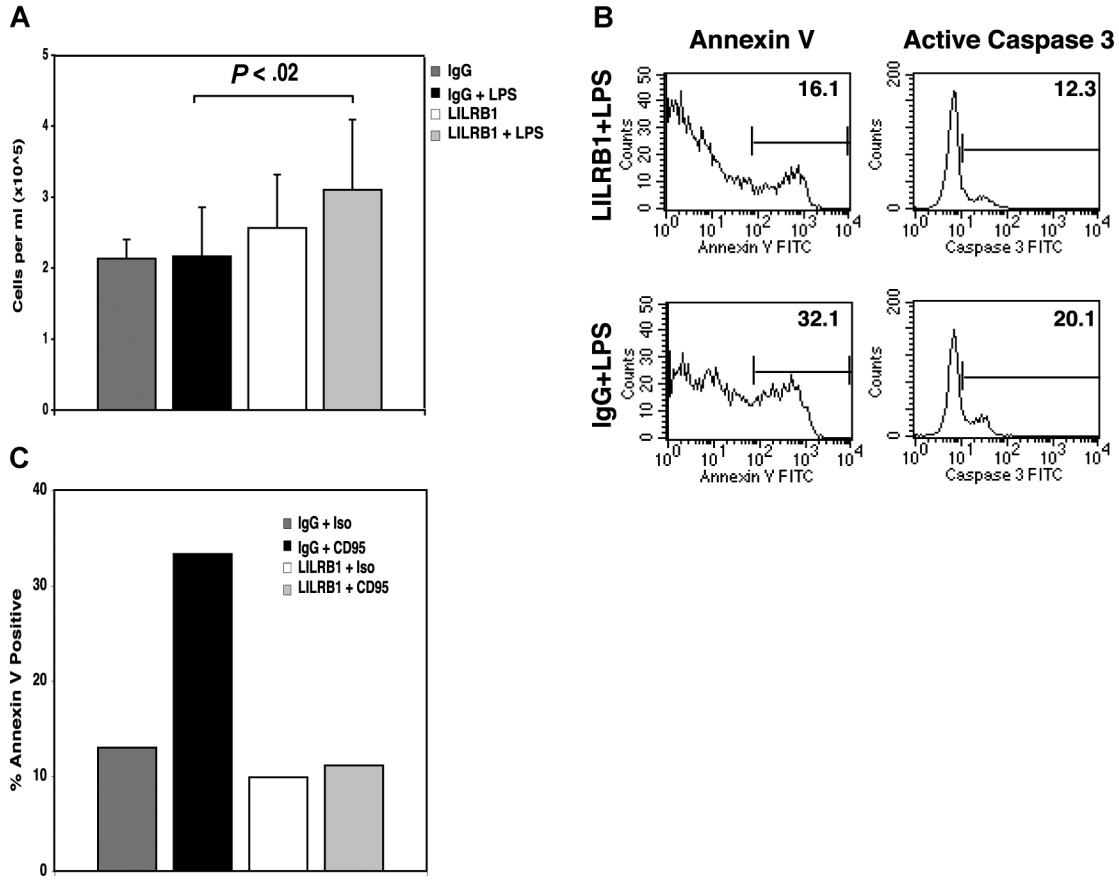

Figure 2. LILRB1 ligation renders DCs resistant to CD95-mediated cell death. (A) Viable cells were counted at the end of culture under the indicated conditions by trypan blue exclusion. Results show means (± SD) of 6 experiments. (B) Annexin V and caspase-3 expression was analyzed by flow cytometry and histograms show percentage positive cells above control. Results are representative of 7 experiments. (C) Cell populations were cultured in the presence or absence of anti-CD95 mAb as indicated and analyzed for expression of annexin V. Results are representative of 2 experiments.

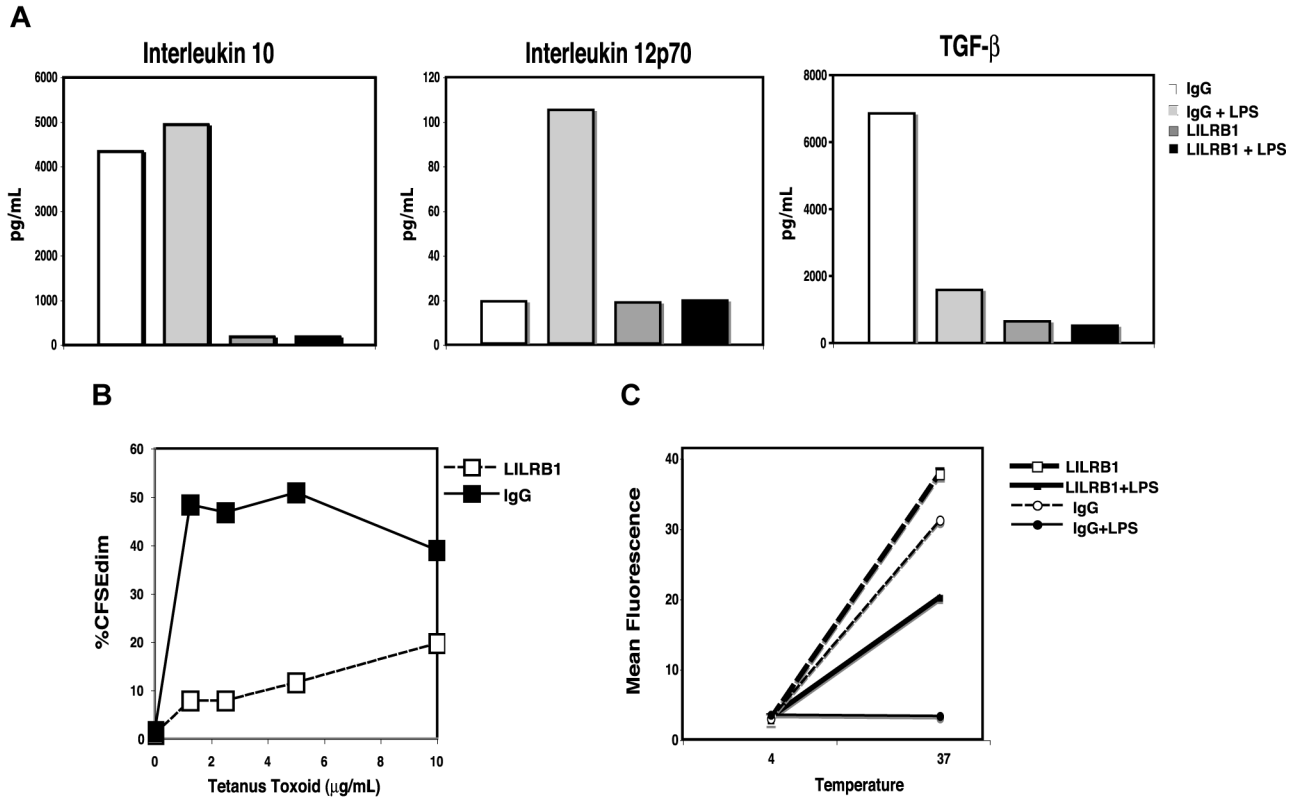

Figure 3. LILRB1 ligation inhibits cytokine secretion and stimulatory capacity for memory responses. (A) Levels of cytokines in supernatants from cells cultured as indicated were analyzed by ELISA. Results are representative of 4 experiments. Similar results were obtained when LILRB1 was ligated with HCMV UL18-Fc (not shown). (B) Cell populations were cultured with graded doses of tetanus toxoid, irradiated, and used to stimulate CFSE-labeled autologous T-cell proliferation for 7 days. Proliferation was assessed by flow cytometric analysis of CFSE levels in responder $CD3^+$ T cells. Results are representative of 2 experiments. (C) Cell populations were incubated with FITC-labeled 40K-dextran at either 4°C or 37°C, washed, and analyzed by flow cytometry. Results shown are the averages of 2 experiments.

dye (not shown). This was indicative of increased survival and/or metabolism of the ligated cells, in contrast to the general paradigm that monocytes simply differentiate and do not proliferate in culture. Total cell counts revealed small but significantly increased yields of ligated cells compared with controls after 6-day culture and exposure to LPS ($3.1 \pm 1.0 \times 10^5$/mL vs $2.2 \pm 0.7 \times 10^5$/mL; n = 6; $P = .014$), indicative of increased cell survival (Figure 2A). We therefore examined apoptotic markers and observed a dramatic difference between the 2 conditions. By flow cytometry, levels of annexin V staining as a surrogate marker of apoptosis in LILRB1-ligated cultures were approximately half those of control cultures (12.8% ± 4.2% vs 24.9% ± 9.8%; n = 7; $P = .005$), roughly correlating with a similar difference in levels of intracellular active caspase-3, the downstream effector of apoptosis (Figure 2B). We also assessed relative susceptibility to Fas-dependent cell death by incubating the cells with anti-CD95 mAb. While there was a significant increase in the percentage of annexin V–positive cells in control cultures, there was little if any change in the ligated cultures (Figure 2C). Hence LILRB1-ligated cells are relatively resistant to apoptosis induced by cross-linking of cell surface Fas/CD95, whereas conventional DC maturation is normally associated with an increased susceptibility to this death-inducing stimulus. The LRC-encoded activating receptor OSCAR can also confer resistance to apoptosis on DCs,[12] but this is the first report of an LRC inhibitory receptor performing a similar function in myeloid cells.

The above findings demonstrate that continuous ligation of LILRB1 has a profound effect on cellular differentiation, and results in a population of cells with a unique and LPS-resistant phenotype as well as increased survival. We also examined the effects of LILRB1 ligation on the secretion of 3 key cytokines by the resultant cells. Cells in control cultures produced considerable amounts of the anti-inflammatory cytokines IL-10 and TGF-β, and after LPS stimulation the secretion of TGF-β was considerably reduced, whereas that of IL-12p70 was much enhanced as expected (Figure 3A). In contrast, LILRB1-ligated cells produced low or undetectable levels of these cytokines either before or after exposure to LPS. LILRB1-mediated inhibition of IL-12 secretion has previously been reported by Cella et al.[13]

The unique phenotype of LILRB1-ligated cells, and their incapacity to induce key regulatory cytokines, suggested that they might have distinct functions compared with conventional DC populations. We tested their capacity to stimulate recall responses to the soluble antigen tetanus toxoid. Compared with controls, the ligated cells appeared markedly deficient at stimulating these T-cell proliferative responses (Figure 3B). This could have been due to defects in antigen uptake, processing, and/or presentation. Although we have not formally excluded these possibilities, the cells were at least as efficient as the control cells at endocytosing the soluble tracer FITC-dextran (Figure 3C), and they possessed endocytic compartments (high intracellular CD63) and expressed only slightly lower levels of HLA-DR molecules (Table 1). Moreover the ligated cells also appeared to be markedly deficient at stimulating primary T-cell responses when tested in the allogeneic MLR (Figure 4A), which does not depend on uptake or processing of exogenous antigens. Despite the LPS-resistant phenotype of these cells (Table 1), their stimulatory activity in the MLR was enhanced following exposure to LPS albeit only to the level of conventional "immature" DCs (Figure 4A), and their endocytic

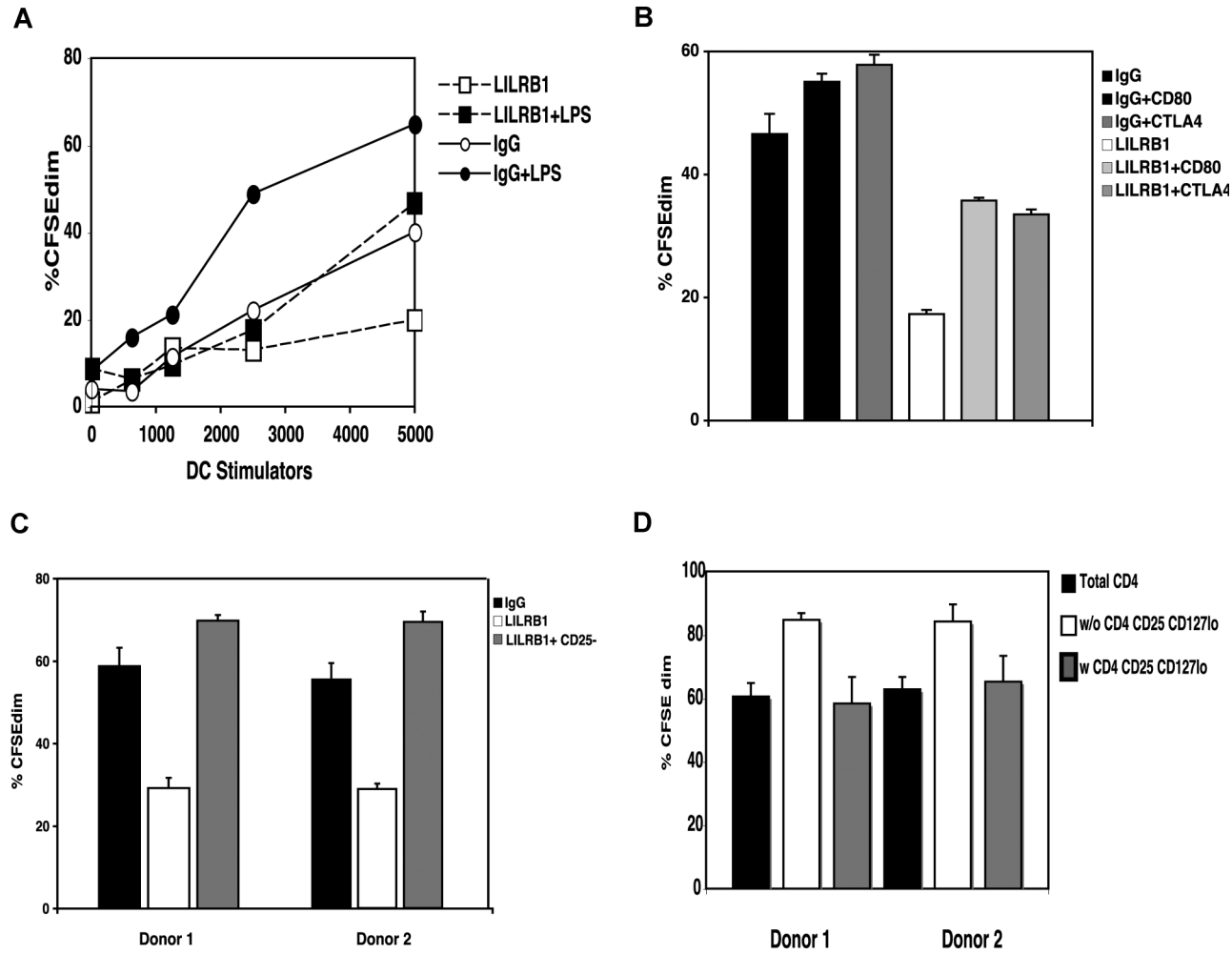

Figure 4. LILRB1 ligation inhibits immunostimulatory function in allogeneic MLRs that is overcome by CD80 or CTLA-4 blockade or by removal of CD4$^+$ CD25$^+$ or CD4$^+$ CD25$^+$ CD127^{lo} Tregs. (A) Cell populations were irradiated and used to stimulate CFSE-labeled allogeneic T-cell proliferation in MLRs for 7 days. Proliferation was assessed by flow cytometric analysis of CFSE levels in responder CD3$^+$ T cells. Results are representative of 4 experiments. (B) Cell populations were irradiated and used to stimulate CFSE-labeled allogeneic T-cell proliferation in MLR in the presence of mAbs specific for CTLA-4 or CD80, or isotype controls for 7 days. Proliferation was assessed as above. Error bars depict the standard deviation of triplicate cultures. Results are representative of 2 experiments. (C) Cell populations were irradiated and used to stimulate CFSE-labeled allogeneic CD4$^+$ T cells, from which CD25$^+$ cells had been depleted by negative selection, for 7 days. Proliferation was assessed as above. Each panel depicts an individual responder-stimulator allogeneic combination, and error bars indicate SD of triplicate cultures. (D) LILRB1-ligated DCs were irradiated and used to stimulate CFSE-labeled allogeneic CD4$^+$ T cells (■) or allogeneic CD4$^+$ T cells from which the CD25$^+$ CD127^{lo} population had been depleted (□) or were added back to cultures (▩) for 10 days. Each set of 3 values depicts an individual responder-stimulator allogeneic combination, and error bars indicate SD of triplicate cultures.

activity was also reduced although not to the same extent as that of conventional "mature" DCs (Figure 3C).

One particularly striking feature of LILRB1-ligated cells was their unusually high expression of CD80 prior to LPS exposure (Table 1). Recent studies of in vitro human T-cell proliferation,[14–15] molecular binding affinities,[16] and murine T-cell synapse formation[17] strongly indicate that this molecule preferentially binds CTLA-4, the inhibitory homologue of CD28 constitutively expressed by regulatory T cells (Tregs). We therefore investigated whether the ligated cells induced the differentiation and/or function of Tregs that, in turn, suppressed in vitro T-cell responses. Blockade of CD80 or CTLA-4 by antagonist mAbs resulted in a significant increase in T-cell proliferation when LILRB1-ligated cells were used as stimulators in the MLR and even in a small increase in control cultures (Figure 4B). Importantly, depletion of CD4$^+$ CD25$^+$ putative Tregs from responder populations also increased T-cell proliferative responses in the MLR (Figure 4C). This finding was consistent when a more refined population of Tregs (CD4$^+$ CD25$^+$ CD127^{lo}) was studied.[18-20] Removal of this population of responder lymphocytes resulted in an increase in MLR proliferation that was reduced when the putative Treg population was added back to MLR cultures (Figure 4D). Hence LILRB1-ligated cells may preferentially induce and/or interact with Tregs that maintain T-cell unresponsiveness. This would account for their apparently weak immunostimulatory activity. The interaction with Tregs appears to be mediated through binding of CD80 on LILRB1-ligated cells to its preferred ligand CTLA-4 on the T cells.

Discussion

We have shown that continuous ligation of LILRB1 during culture of monocytes, under conditions that would otherwise generate conventional DCs, generates a population of cells that can apparently induce the differentiation or function of Tregs that suppress T-cell responses in vitro. The CD14$^+$ subset of monocytes is believed to represent a pool of precursors that can be recruited to inflamed tissues where they may differentiate into DCs.[21] However, as these cells differentiate, LILRB1 receptors would presumably be

ligated by HLA class I molecules that are up-regulated in response to inflammation. Obviously the cellular response could be further modulated by intrinsic stimuli such as inflammatory cytokines. It is intriguing that we observed marked up-regulation of CCR7 (Table 1) in response to continued ligation of LILRB1. There is evidence for a "tick-over" migration of relatively immature DCs from peripheral tissues in the steady state that is markedly increased in response to inflammation.[22] If this also applied to the putative in vivo homologues of the cells we have derived, it would seem reasonable to assume that they would have the potential to migrate into secondary lymphoid tissues and interact with T cells. Their capacity to induce Tregs (Figure 4) and their resistance to Fas-mediated cell death (Figure 2; eg, induced by FasL expressed on activated and/or central memory T cells) could then lead to relatively long-lasting dampening of adaptive T-cell responses. A recent report has described the inhibition of maturation that occurs when DCs are treated with HCMV UL18-Fc fusion proteins.[23] This supports our findings and suggests that one function of the viral UL18 class I–like molecule may be to modulate myeloid cell functions leading to the generation of suppressive HCMV-specific regulatory T cells.[24]

The relative resistance of ligated cells to LPS stimulation, in terms of their phenotype, cytokine secretion, and resistance to CD95-mediated cell death, implicates LILRB1 as a regulator of TLR4 signaling events. LILRB1 is associated with the hematopoietic phosphatase SHP-1, which is known to inhibit activation by receptors such as FCGR in monocytes,[25] OSCAR in DCs,[26] and the TCR in CD8 T cells.[27] The inhibitory kinase Csk has also been demonstrated to be recruited by the cytoplasmic tail of LILRB1[28] and may also regulate the TLR4 pathway.[29] These putative intracellular signaling events require further investigation.

In a limited number of experiments, we also evaluated the effect of ligating LAIR1, which is closely related to LILR and also encoded in the LRC, since this receptor has been reported to inhibit the differentiation of monocytes induced by culture in GM-CSF.[30] The resultant cells displayed a small, rounded morphology that did not resemble classic DCs, as previously reported, and they expressed very low levels of CD80, which were dramatically up-regulated on LPS stimulation (not shown). It has also been reported that a population of $CD8^+$ $CD28^-$ "suppressor T cells" can increase the expression of LILRB2 and LILRB4 on DCs that did not express CD80 and that were able to anergize CD4 T cells.[3,31] Taken together, these observations and related studies in mice[32-34] suggest that ligation of different LRC receptors results in distinct functional outcomes.

Whether or not ligation of LILRB1 occurs in vivo, our study suggests that LILRB1 may represent an attractive new therapeutic target for ex vivo strategies to overcome aberrant or unwanted immune responses. There is considerable interest in the use of ex vivo–derived DCs to enhance immunotherapy of cancer and infectious diseases. Attention is now turning to the potential of ex vivo–derived DCs that can suppress immune responses[35] for therapy of autoimmune diseases and allergic reactions, and to overcome transplantation complications. Further studies of the modulation of the cells we have described in this report are warranted.

Acknowledgments

We would like to thank I-Hsuen Chen and Damien Brown for technical assistance, and Prof Miguel Lopez-Botet for providing HPF1 monoclonal antibody.

This work was supported by awards from the Leukemia Research Fund and the Association for International Cancer Research (N.T.Y.), and The Wellcome Trust and Medical Research Council (J.T.). N.T.Y. is a Leukemia Research Fund Bennett Senior Research Fellow.

Authorship

Contribution: N.T.Y. and J.M.A. designed research; N.T.Y., E.C.P.W., R.P., and A.R. performed research and analyzed data; and N.T.Y., J.M.A., and J.T. drafted the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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