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Characterization of Human Inducible Costimulator Ligand Expression and Function

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The inducible costimulator (ICOS) is the newest member of the CD28/CD152 receptor family involved in regulating T cell activation. We constructed a soluble-Ig fusion protein of the extracellular domain of human ICOS and used it as a probe to characterize expression patterns of the ICOS ligand (ICOSL). ICOSIg did not bind to CD80- or CD86-transfected Chinese hamster ovary cell lines, demonstrating that ICOSL is distinct from those ligands identified for CD80/CD152. ICOSIg showed selective binding to mononuclear and B cell lines, whereas binding was undetectable on unstimulated monocytes and peripheral blood T and B cells. Expression of ICOSL was induced on monocytes after integrin-dependent plastic adhesion. Pretreatment of monocytes with mAb to the β2-integrin subunit CD18 decreased adhesion and abolished ICOSL up-regulation but had no effect on CD80/86 (CD152 ligand (CD152L)) expression. Both ICOSL and CD152L were up-regulated on monocytes by IFN-γ but by distinct signaling pathways. Unlike CD152L expression, ICOSL expression did not change when monocytes were differentiated into dendritic cells (DCs) or after DCs were induced to mature by LPS, TNF-α, or CD40 ligation. Addition of ICOSIg to allogeneic MLRs between DCs and T cells reduced T cell proliferative responses but did so less efficiently than CTLA4Ig (CD152Ig) did. Similarly, ICOSIg also blocked Ag-specific T cell proliferation to tetanus toxoid. Thus, ICOSL, like CD80/86, is expressed on activated monocytes and dendritic cells but is regulated differently and delivers distinct signals to T cells that can be specifically inhibited by ICOSIg. The Journal of Immunology, 2000, 164: 4689 – 4696.

The CD28 receptor family plays a key role in regulating T cell activation. Interaction of CD80 and CD86 on APCs with the receptors CD28 and CD152 (CTLA4) on T cells results in signaling events that regulate immune responses, including the balance between Th1 and Th2 responses (1, 2). Costimulation of T cells through CD28 is important for the generation of Ag-specific immune responses (3). Unlike CD28, inducible co-stimulator (ICOS), a newly identified member of this family, is not constitutively expressed; rather, it is induced after T cell activation (4). Structural homology among CD28, CD152, and ICOS is significant, yet ICOS differs from CD28 and CD152 in the MYPPPY homology domain required for CD28/CD152 to bind to CD80 or CD86 (5). Because of these structural differences and because the functional effects of ICOS ligation differ from CD28 or CD152 ligation, it was suggested that the ligand for ICOS is different from the CD28/CD152 ligands (4). However, it is not known whether ICOS binds to CD80 or CD86 or whether it interacts with another distinct cell surface molecule. Ab binding to ICOS promotes T cell proliferation at levels similar to those observed for CD28 mAb but without the accompanying increase in production of IL-2 (4). Instead, ICOS up-regulates expression of IL-10 (4). ICOS expression on T cells varies depending on the lymphoid tissue from which the T cells derive and the activation state of the cells; T cells from tonsillar germinal centers (GCs) express high levels of ICOS, suggesting that ICOS may have a specialized function in regulating GC B cell differentiation (4). Follicular dendritic cells (DCs) and DCs also play a critical role in GC formation, Ag presentation, T cell priming, and subsequent B cell differentiation (6), suggesting that the ligand for ICOS might be expressed on either B cells or DCs.

In this study, we generated a soluble form of the ICOS receptor and then used it to detect and characterize the ICOS ligand (ICOSL). We found that ICOSL is expressed and regulated differently than the ligands for CD28 and CD152 (CD152L). Despite their distinct patterns of expression and regulation, ICOSL and CD80/CD86 are similar in that they can regulate T cell proliferation. ICOSIg inhibits both Ag-specific and polyclonal T cell responses by blocking interactions between T cells and APCs, although not as efficiently as CD152Ig.

Materials and Methods

Cell lines

The monocytic and B cell lines were maintained as described (7–9). The Hodgkin cell lines were cultured as described (10–13). The Chinese hamster ovary (CHO) DG44 cell line was kindly provided by Lawrence Chasin at Columbia University (New York, NY) (14).
Monoclonal Abs

Monoclonal Abs to CD40 (G28-5), CD18 (60.3), CD3 (64.1), CD8 (G10-1), CD20 (1F5), CD16 (FC-2), and HLA-DR (HB10a) were generated in our laboratory from in vivo passage of hybridoma cells.

Flow cytometric analysis

Cells were incubated in binding buffer (2% FBS in PBS) and preblocked using mouse anti-human CD32 (Fab'), Ab (Ancell, Bayport, MN) at 5 μg/ml for 30 min at room temperature. Next, cells were washed once in binding buffer and incubated 45 min on ice with the fusion proteins (10 μg/ml). Then cells were washed once with binding buffer and incubated with FITC-conjugated goat anti-human IgG (Fab')2, Fc-specific, preadsorbed with mouse cells) 1:50 as a secondary step (Caltag, Burlingame, CA) for 45 min on ice. Finally, cells were washed two to three times in PBS and analyzed by FACScan (Becton Dickinson, San Jose, CA). Cells were fixed in 1% paraformaldehyde in PBS and refrigerated in the dark before analysis. For two-color immunofluorescence of DCs with ICOSlg or CD152lg, CD1a-FITC (Dako, Carpinteria, CA) and PE-labeled goat anti-human IgG (Fab')2, Ab (BioSource International, Camarillo, CA) were used. For the two-color analysis of peripheral B cells and tonsilar B cells, ICOSlg was biotinylated on free sulhydryl using Biotin-BMCC (Pierce, Rockford, IL) and was used at 75 μg/ml with Streptavidin-PE (Becton Dickinson, Mountain View, CA) as a secondary step. B cells were detected by using a FITC-labeled anti- IgM serum (Sigma, St. Louis, MO).

Cloning ICOS cDNA

RNA was prepared from PHA-activated (1 μg/ml, 5 days) peripheral blood T cell blasts, from human thymus, and from PMA + ionomycin (10 ng/ml PMA and 1 μg/ml ionomycin, overnight)-activated peripheral blood T cells using Trizol (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. cDNA was prepared using 1–3 μg RNA, random hexamers, and Superscript II Reverse Transcriptase (Life Technologies) according to manufacturer’s directions. ICOS cDNA was PCR-amplified from the random primed cDNA in 100 μl reactions containing 1 μg cDNA. 2.5 units (0.5 μl) ExTaq DNA polymerase (Takara Shuzo, Otsu, Japan), diluted buffers and nucleotides according to insert directions, and ICOS-specific primer sets. Reactions were amplified for 36 cycles, with an amplification profile of 94°C, 45 s; 50°C, 60 s; and 72°C, 60 s. The published protein sequence for ICOS (4) was used as a guide in designing partially degenerate oligonucleotides to amplify the ICOS cDNA. Several similar sense or antisense oligonucleotides were pooled for amplification reactions. PCR products of the expected size for ICOS full-length and extracellular domains were obtained, and the fragments were cloned into pT-Advantage vectors (Clontech, Palo Alto, CA). Clones with inserts were sequenced using the BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA) on an ABI Prism 310 sequencer (PE Biosystems). Constructs that contained sequence corresponding to the published protein sequence were used as templates in secondary amplification reactions to attach appropriate restriction sites and adapter sequences for construction of an -Ig fusion gene. The 5′ oligonucleotide contained a HindIII site and Kozak sequence from the internal methionine, whereas the 3′ oligonucleotide contained a BamHI site at the junction between the ICOS extracellular domain and the -Ig tail encoding human IgG1 Fc. The DNA sequence has been submitted to GenBank with accession no. AF218312.

Production of ICOSlg fusion protein

The ICOSlg cDNA construct was inserted into the mammalian expression vector pD18, a derivative of pcDNA3, as described previously (15). Constructs initially were transfected by DEAE-Dextran transient transfections as described above. CHO lines expressing ICOSlg were selected by high-copy electroporation in the pD18 vector (15, 17) and selection of methotrexate-resistant clones by limiting dilution in Excell 302 CHO media (JRH Biosciences, Denver, PA) containing 0.5 μg/ml recombinant insulin (Life Technologies), sodium pyruvate (Irvine Scientific, Santa Ana, CA), 4 mM l-glutamine (Irvine Scientific), 2% nonessential amino acids for MEM (Irvine Scientific), and 100 mM methotrexate (Sigma). Culture supernatants were harvested from large-scale cultures, and ICOSlg was purified by protein A affinity chromatography over a 2-ml protein A-agarose (Repligen, Cambridge, MA) column. Fusion protein was eluted from the column as 0.8-ml fractions in 0.1 M citrate buffer (pH 2.7) and neutralized using 100 μl of 1 M Tris-HCl (pH 7.4). Eluted fractions were assessed for absorbance at 280 nm, and fractions containing fusion protein were pooled, dialyzed overnight in several liters of PBS (pH 7.4), and filter sterilized through 0.2-μm syringe filter units (Millipore, Bedford, MA).

Western analysis of ICOSlg fusion proteins

Protein samples were resolved by SDS-PAGE electrophoresis on 8–16% gradient gels and transferred by semidyli blotting onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked to prevent nonspecific Ab binding by incubation in 5% nonfat dry milk (Carnation) in PBS/0.25% Nonidet P-40 or TBST (50 mM Tris- HCl (pH 7.6), 0.15 M NaCl, and 0.05% Tween 20) overnight at 4°C. The membranes were incubated with HRP-goat anti-human IgG (1/10,000) or with HRP-streptavidin (1/5000) (Caltag) in TBST for 1 h at room temperature or 4°C, with gentle agitation. Alternatively, membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG (I/5000). After two rinses and four washes with PBS/Nonidet P-40 or TBST, the membrane was incubated in Western Blue for alkaline phosphatase substrate development (5 min) (Promega, Madison, WI) or in ECL (Amer sham, Little Chalfont, U.K.); reagent for HRP (60 s) and exposure to autoradiograph film for visualization of the bands.

Monocyte-derived human DCs and tonsillar B cells

PBMC were isolated from peripheral blood samples by centrifugation over Ficoll-Hypaque (Robbins Scientific, Sunnyvale, CA). After sheep erythocyte rosetting to deplete T cells, CD14+ cells were obtained by positive selection with magnetic anti-CD14 microbeads according to the manufacturer’s instructions (Miltenyi Biotech, Auburn, CA). CD14+ cells (93–99% pure as assessed by flow cytometry) were cultured in RPMI 1640 medium plus 100 ng/ml human GM-CSF (RDI, Flanders, NJ) and 30 ng/ml human IL-4 (RDI) (18). Every other day, 50% of the medium was replaced and the same volume of fresh medium containing twice the amount of cytokines was added. After 5–7 days, cells exhibited an immature DC phenotype, i.e., CD14+, CD86+CD80−, HLA-DR+ (95%), CD1a+, and CD46+ (18). Lymphocytes from tonsillar cell suspensions were isolated by centrifugation over Ficoll-Hypaque and then were subjected to T cell depletion by sheep erythrocyte rosetting or panning on anti-CD3-coated plates. Dense B cells were prepared by Percoll gradient fractionation as described (19).

Stimulation of monocytes and monocyte-derived DCs

Monocytes were stimulated with 1 μg/ml IFN-γ (RDI) for 24 h. Sometimes monocytes were preincubated with 10 μg/ml anti-CD18 for 30 min or with 50 mM staurosporine (Calbiochem, La Jolla, CA) for 1 h. Maturation in monocyte-derived DCs was induced by addition of 100 ng/ml LPS (Sigma), 100 ng/ml TNF-α (RDI), or 10 μg/ml anti-CD40 for 2 days.

Responder T cells for the MLRs were obtained after centrifugation over Ficoll-Hypaque by sheep erythrocyte rosetting. After lysis of RBCs with water for 10 s, cells were washed with PBS and then depleted of CD8+ T cells, NK cells, activated T cells or monocytes, and B cells by panning with anti-CD8, anti-CD16, anti-HLA-DR, and anti-CD20 for 2 days.

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Monocytes were cultured with 5,000 gamma-irradiated DCs (3,000 rad 137Cs) with 50,000 prestimulated (soluble anti-CD3 (64.1) for 12 h at 37°C) CD4+ T cells. These cells were cocultured in 96-well round-bottom microtiter plates for 3 days. T cell proliferation was assessed after the addition of 1 μCi/well [3H]thymidine (Amersham) for the final 9 h. [3H]Thymidine incorporation was measured by liquid scintillation counting. All determinations were performed in triplicate and measured as the mean cpm ± SEM.

Ag-specific proliferation assays

PBMCs were isolated over Ficoll-Hypaque and frozen in human AB serum. For the assays, 100,000 PBMCs were cultured with 10 μg/ml fusion protein and 10 μg/ml tetanus toxoid (TT; Chiron Behring, Liederbach, Germany) or influenza hemagglutinin (HA; produced according to previously described procedures (21)) in medium with 10% human AB serum in 96-well plates for 4 days; 1 μCi/well [3H]thymidine (Amersham) was added for the final 24 h. [3H]Thymidine incorporation was measured by liquid scintillation counting. All determinations were performed in triplicate and measured as the mean cpm ± SEM.

Results

Construction and expression of a soluble form of the ICOS receptor

The published protein sequence for ICOS (4) was used as a guide in designing partially degenerate oligonucleotides to amplify the
Expression of ICOSIg and CD152Ig fusion proteins from different hematopoietic cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ICOSIg Binding</th>
<th>CD152Ig Binding</th>
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<tr>
<td>HL-60 (promyelocytic leukemia)</td>
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</tr>
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<td>U937 (histiocytic lymphoma)</td>
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<td>CEM (acute T-lymphoblastic leukemia)</td>
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<td>Jurkat (acute T cell leukemia)</td>
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</table>

*Binding data are recorded as the fold increase in relative brightness compared with that of the isotype control.

ICOSIg binds specifically to its ligand but not to CD152L (CD80 or CD86)

To determine whether ICOS shares the ligands CD80 and CD86 with CD28 and CD152, we examined ICOSIg binding to CHO cells transfected with CD80 or CD86 and compared the binding pattern with CD152Ig (Fig. 1C). Both CD80CHO and CD86CHO lines showed specific, high-affinity binding to CD152Ig. The cells also exhibited a weak but specific binding interaction with CD28Ig (data not shown). No detectable binding was observed using ICOSIg, even at concentrations of 100 μg/ml. Similar binding results were observed in ELISAs performed by coating plates with ICOSIg, CD152Ig, or CD28Ig, binding to biotinylated CD80Ig and CD86Ig, and detection with streptavidin-HRP (data not shown).

ICOSL is strongly expressed on monocytic cell lines and to a lesser extent on B cell lines

To study the expression of ICOSL, we first examined several B, T, and monocytic cell lines by flow cytometry for ability to bind the ICOSIg fusion protein. We compared the binding profiles for ICOSIg with those for CD152Ig, which binds to CD80 and CD86.
Monocytic cell lines such as HL-60 (pro-myelocytic) and U937 (pro-monocytic) showed the highest levels of binding to ICOSIg fusion protein, with more than 7.5-fold increases in relative brightness compared with isotype controls (Table I). Both of these cell lines are also strongly positive for CD152Ig binding. Some B cell lines expressed ICOSL as well, including the immature B cell lymphoma B104 and, to a lesser extent, REH (pre-B cell leukemia), MP-1 and T51 (B-lymphoblastoid cell lines), Raji and Ramos (Burkitt’s lymphomas), and 8226 (myeloma cell line). Further, we tested several Hodgkin cell lines that were all negative for ICOSL, whereas the majority of these cell lines were very strongly positive for CD152Ig binding (22). We also stained a Hodgkin cell line, HD-MyZ, with a more myelomonocytoid immunophenotype; we expected to see expression of ICOSL due to the myeloid features of this cell line, but HD-MyZ was negative for both ICOSL and CD152L. Two T lymphoblastic cell lines, CEM and Jurkat, did not express either the ICOS or CD152 ligands.

Induction of expression of ICOSL in CD14+ monocytes

Because some ligands for T cell signaling receptors depend on activation (23), we next tested whether peripheral blood cells with or without different stimuli expressed ICOSL. Unstimulated peripheral blood B cells from most donors were negative for ICOSIg binding though binding was detectable in a few donors on as many as 40% of the CD14+ B cells. Stimulation with anti-CD40 and anti-IgM did not induce ICOSL expression (data not shown). Total T cells or CD4+ T cells were also negative for ICOSIg binding. ICOSIg binding was still undetectable after stimulation with either anti-CD3 or anti-CD3/anti-CD28 for 12 h. To eliminate background staining from surface Ig, we stained peripheral blood and tonsillar B cells with a highly sensitive detection assay using a sulfhydryl-biotinylated ICOSIg fusion protein and streptavidin-PE. We found that ICOSIg binding was detectable at low levels on a subpopulation of viable, unstimulated, dense tonsillar B lymphocytes (data not shown). CD14+ monocytes that had no contact with plastic surfaces expressed ICOSIg at very low levels if at all, depending on the donor. After contact with plastic surfaces in cell culture dishes, ICOSL expression as well as CD152L expression increased (Fig. 2A). Because the activation of monocytes by plastic adherence is mediated via integrins, we tested whether the CD18 mAb 60.3 could block adhesion and induction of ICOSL (Fig. 2B). In the presence of CD18 mAb, cells no longer adhered to plastic, and ICOSL up-regulation was strongly inhibited. However, up-regulation of CD80/86 via plastic adherence was not significantly altered by CD18 mAb (Fig. 2B). We also tested several stimuli to determine their effects on ICOSL expression in CD14+ monocytes. Only IFN-γ, but not IFN-α, TNF-α, LPS, anti-CD40, PMA,
or macrophage-inflammatory protein-1α, increased ICOSL expression on monocytes after incubation for 24 h (Fig. 2C). ICOSL was significantly up-regulated by IFN-γ in a dose-dependent manner. Similarly, ligands for CD152 were elevated after IFN-γ stimulation of monocytes (Fig. 2C). Because a previous study showed that a NF-κB inhibitor (N-tosyl-L-phenylalanine chloromethyl ketone) could block LPS-induced up-regulation of CD86 (24) and because IFN-γ activates NF-κB in mouse peritoneal macrophages (25), we tested whether a NF-κB inhibiting peptide could alter the up-regulation of ICOSL and CTLA4 ligand mediated by IFN-γ (data not shown). We did not detect an effect, suggesting that the ICOSL induction by IFN-γ is independent of NF-κB regulation. Next, we preincubated monocytes with the broad-spectrum protein kinase C (PKC) and protein tyrosine kinase (PTK) inhibitor staurosporine. Staurosporine blocked both integrin-mediated and IFN-γ-induced up-regulation of ICOSL; in contrast, integrin-mediated expression of CD152 ligands was increased by staurosporine, and IFN-γ induction of CD152 ligands was not changed (Fig. 3). We also tested the combination of IFN-γ with IL-2 on activation of monocytes, but we did not detect any significant changes in ICOSL expression using these stimuli. These results imply that the expressions of ICOS and CD152 ligands on monocytes are regulated differently.

Expression of ICOSL in monocyte-derived DCs

To find out whether ICOSL expression is altered when monocytes undergo further differentiation into DCs, we examined ICOSL expression in monocyte-derived DCs that were kept in GM-CSF and IL-4 for 7 days. ICOSL was coexpressed with anti-CD1a, a marker that is strongly expressed on DCs (Fig. 4A). Expression levels of ICOSL on DCs did not differ from the expression levels on monocyte precursors; thus, differentiation into DCs did not further change ICOSL expression (Fig. 4A). However, DCs showed a much stronger expression of CD152L compared with their monocyte precursors. This effect apparently is due to the influence of IL-4 because IL-4 alone induced increases in CD152L binding (Fig. 4B). Next, we induced immature DCs to mature to a more active Ag presentation phase using LPS, TNF-α, or CD40 mAb (25, 26). Mature DCs are much more efficient at stimulating T cell proliferation, in part due to an up-regulation of CD80 and CD86. Therefore, it seemed likely that ICOSL might be induced during DC maturation. After maturation through LPS, TNF-α, or CD40 mAb, CD152L expression increased, whereas ICOSL expression did not change. Thus, although IFN-γ induced ICOSL expression in monocytes, no further up-regulation could be detected in DCs.

ICOSIg partially blocks T cell proliferation in CD4+ T cells preactivated with soluble anti-CD3

To study the potential role of ICOSL as a costimulatory molecule, we performed allogeneic MLRs with DCs and CD4+ T cells in the

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**FIGURE 4.** Maturation of monocytes to DCs differentially affects ICOSL and CD152L expression. A, CD1a+ monocyte-derived DCs (used after 7 days of culture) express ICOSL and CD152L, as can be shown by two-color analysis with anti-CD1a-FITC and ICOSIg or CD152L-PE. B, Differentiation of monocytes into DCs after culture for 4 days in 100 ng/ml GM-CSF and 30 ng/ml IL-4 did not alter ICOSL expression, whereas GM-CSF + IL-4 strongly up-regulated CD152L expression. Up-regulation of CD152L was dependent on the presence of IL-4. C, When maturation of 7-day-old DCs was induced by culture with LPS (100 ng/ml), TNF-α (100 ng/ml), and anti-CD40 (10 μg/ml) for another 2 days, ICOSL expression did not increase further, whereas CD152L expression was up-regulated. Although IFN-γ increased ICOSL expression on monocytes, this effect of IFN-γ could not be seen on DCs.
The presence of ICOSIg, CD152Ig, and control isotype-Ig (Fig. 5). T cells were prestimulated with anti-CD3 (1 μg/ml) to induce ICOS expression on T cells so that ICOS was already present before contact with DCs. The low dose of anti-CD3 used did not induce T cell proliferation (data not shown). DCs and T cells were then cultured with graded doses of ICOSIg, CTLA4Ig, or isotype-Ig for 3 days. In the presence of CD152Ig, maximal blocking of proliferation was observed at 5 μg/ml. The highest dose (20 μg/ml) gave a 75% reduction of T cell proliferation from the isotype-treated control cells. ICOSIg also significantly reduced T cell proliferation levels, but even the highest concentration of ICOSIg reduced T cell proliferation by only about 50%. Thus, soluble ICOSIg partially blocks T cell proliferation in allogeneic MLRs, suggesting that ICOSL delivers a costimulatory signal to T cells.

**ICOSIg blocks Ag-specific T cell proliferation**

The results found in MLRs were further confirmed using Ag-specific T cell proliferation assays (Fig. 6). The presence of TT increased T cell proliferation by three-fold. This T cell proliferation was inhibited about 50% in the presence of ICOSIg. Similar results were observed using influenza HA. However, in this case T cell proliferation by HA was augmented by two-fold and could be blocked by only ~30%. The presence of CD152Ig efficiently blocked T cell proliferation in both cases by more than 80%.

**Discussion**

In this study, we compared the expression pattern of ICOSL, whose receptor ICOS is a recently identified inducible T cell marker related to CD28 and CD152 (24), with the expression patterns of CD80 and CD86. ICOSIg bound at high levels to monocytic cell lines and also to several B cell lines, but not to T cell lines. Ligands of other inducible T cell Ags such as 4-1BB (CD137) (23) show similar expression patterns for monocytic and B cell lines. All the cell lines with strong expression of ICOSL, such as HL-60, U937, and B104, were also positive for CD152Ig binding. In contrast, some lines, such as the Burkitt’s lymphoma BJAB and the Hodgkin cell lines tested, expressed high levels of CD152 ligands but not ICOSL. Despite the strong expression of CD80/CD86 in neoplastic cells of Hodgkin’s disease, these cells appear to derive not from myeloid precursors but from B cells (22, 27).

Due to the high expression levels in myelomonocytic cell lines, we tested whether ICOSL is expressed on CD14+ monocytes, where we found little or no expression of ICOSL or CD152 ligands. However, after contact with plastic surfaces, both ICOSL and CD152 ligands were up-regulated. Adhesion of monocytes to endothelial cells or extracellular matrices plays a critical role in triggering monocyte activation in extracellular sites of infection or tissue damage and induces calcium signaling, phosphorylation of signaling and cytoskeletal proteins, and induction of inflammatory cytokines (28–31). Monocyte adhesion to surfaces is inhibited by mAbs to the leukocyte integrin β2-subunit CD18 (32, 33). Using CD18 mAb, we could selectively block integrin-mediated up-regulation of ICOSL but not of CTLA4 ligands, suggesting that ICOSL vs CD80/CD86 are differentially regulated after adhesion to the plastic substrate. IFN-γ can selectively induce expression of CD80 on monocytes, whereas CD80 is not inducible by either TNF-α or LPS (34, 35). In this study we demonstrate that ICOSL expression in monocytes is also IFN-γ inducible. Besides IFN-γ, we tested several stimuli such as IFN-α, TNF-α, LPS, PMA, and chemokines such as macrophage-inflammatory protein-1α, and all of these agents had no effect on monocyte expression of ICOSL. CD40 ligation seemed to slightly down-regulate ICOSL expression (data not shown).

IFN-γ-induced signaling pathways thus play a role in regulating expression of both ICOSL and CD80/CD86. A possible explanation might be that the promoter region of ICOSL has IFN-γ-responsive elements similar to those of CD80/CD86. With addition of the broad-spectrum protein kinase inhibitor staurosporine, we noticed a reversal of both the integrin-mediated and the IFN-γ-induced up-regulation of ICOSL. This effect was specific for

![Figure 5](http://www.jimmunol.org/)  
**FIGURE 5.** Allogeneic MLRs of DCs and CD4+ T cells pretreated with soluble anti-CD3 in the presence of graded doses of ICOSIg, CD152Ig, or isotype-Ig. Proliferative T cell responses can be significantly inhibited by ICOSIg and, to a stronger extent, by CD152Ig compared with isotype-Ig. Data are shown as mean values ± SEM from three independent experiments.

![Figure 6](http://www.jimmunol.org/)  
**FIGURE 6.** Ag-specific proliferation assays in the presence of ICOSIg, CD152Ig, or isotype-Ig. A. Proliferative T cell responses of PBMCs were strongly increased when TT was added as a recall Ag. T cell activation could be inhibited significantly in the presence of ICOSIg and even more strongly in the presence of CD152Ig. B. When an influenza HA-specific peptide was used instead of TT, CD152Ig still strongly inhibited T cell proliferation, whereas ICOSIg was less efficient in blocking T cell proliferative responses. One representative experiment from three independent experiments is shown. Each bar on the graph represents the average of quadruplicate cultures, with an SEM <7%.
ICOSL expression. Indeed, induction of CD152L via adhesion was enhanced by staurosporine treatment, whereas IFN-γ induction remained unaffected. Because staurosporine is an inhibitor of PKC but is also known to inhibit PTKs in some cell types at higher doses (36), these results suggest a potential role for a PKC-signaling pathway or a requirement for PTKs when ICOSL is expressed after monocytes become activated by adhesion or IFN-γ. In addition, the observation that ICOSL is not detectable on resting human peripheral blood T or B cells from most donors, but is expressed only at low levels by a subset of dense tonsillar B cells suggests that ICOSL may have a specialized function for monocyte/DC APCs.

After monocytes were further differentiated into monocytode-derived DCs, they did not alter ICOSL expression. In other words, differentiation of monocytes into DCs does not further change expression levels of ICOSL. Likewise, maturation of CD83-immature DCs into CD83+ mature DCs with known potent inducers of maturation such as TNF-α, LPS, or anti-CD40 increased levels of CD80/CD86 but not of ICOSL. We also stimulated DCs with IFN-γ but were not able to further induce ICOSL expression. Whereas IFN-γ further up-regulates adhesion-induced ICOSL expression levels in monocytes, this up-regulation was inhibited in the presence of GM-CSF and IL-4. The immature DC used in our experiments are reported to be IFN-γR (CD119)-positive, whereas mature DCs show a reduced responsiveness to IFN-γ (37). Furthermore, these DCs clearly do react to IFN-γ by up-regulating CD152 ligands.

When ICOSIg was added to the cultures of T cells and DCs, a significant reduction of maximal proliferative responses was observed. This is comparable to the inhibition that can be obtained by other fusion proteins to newly defined TCRs (38), whereby higher concentrations of the fusion protein were needed to get a 50% inhibition of T cell proliferative responses. Therefore, it will be interesting to test in further studies whether both fusion proteins might show an additive effect on the inhibition of T cell proliferation. Initial experiments suggest that there is an additive effect between CD152l and ICOSIg on T cell proliferation but that this effect is both donor- and activation-dependent. We plan to perform additional experiments to help characterize the conditions under which these two fusion proteins can interact to inhibit proliferation. It also remains to be determined whether the simultaneous stimulation of ICOS and CD152 on T cells may lead to a different outcome than ligation of a single receptor. DCs clearly express ligands for both ICOS and CD152, and activated T cells express both ICOS and CD152, suggesting that ICOS-mediated signals may influence CD28 or CD152 signaling pathways. Similarly, because DCs express both ICOSL and CD40 whereas activated T cells express CD154 and ICOS, the ICOS-ICOSL and CD40-CD154 stimulation pathways may also interact to generate novel outcomes as part of the immune response.

Results similar to those in the MLRs were obtained in Ag-specific proliferation assays with TF. The differences in the ability of ICOSL to more effectively block TT-specific T cell proliferation vs ICOSIg could prove useful in determining whether this receptor-ligand pair is involved in autoimmune disease or inflammatory reactions. Our results also suggest that ICOSIg and molecules that bind to this receptor may have a future therapeutic potential.

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References


