

Tumor Necrosis Factor- α Regulates the Expression of Inducible Costimulator Receptor Ligand on CD34⁺ Progenitor Cells during Differentiation into Antigen Presenting Cells*

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The inducible costimulator receptor (ICOS) is a third member of the CD28 receptor family that regulates T cell activation and function. ICOS binds to a newly identified ligand on antigen presenting cells different from the CD152 ligands CD80 and CD86. We used soluble ICOSIg and a newly developed murine anti-human ICOS ligand (ICOSL) monoclonal antibody to further characterize the ICOSL during ontogeny of antigen presenting cells. In a previous study, we found that ICOSL is expressed on monocytes, dendritic cells, and B cells. To define when ICOSL is first expressed on myeloid antigen presenting cells, we examined ICOSL expression on CD34⁺ cells in bone marrow. We found that CD34^{bright} cells regardless of their myeloid commitment were ICOSL⁻, whereas ICOSL was first expressed when CD34 expression diminished and the myeloid marker CD33 appeared. However, acute myeloid leukemia cells were ICOSL-negative, whereas among B-cell malignancies only some cases of the most mature tumors such as prolymphocytic leukemia and hairy cell leukemia were positive. Next, we investigated purified CD34⁺ hematopoietic progenitor cells that did not constitutively express ICOSL but were induced to express ICOSL within 12 h after granulocyte/macrophage colony-stimulating factor/tumor necrosis factor α (TNF- α) stimulation. Interestingly, ICOSL was induced prior to CD80/CD86 induction on CD34⁺ cells so that ICOSL was expressed in the absence of CD80/CD86. This suggests that ICOSL is an early differentiation marker along the monocytic/dendritic maturation pathway. Induction of ICOSL was dependent on TNF- α and was regulated via NF- κ B as revealed by use of inhibitors specific for I κ B α phosphorylation such as BAY 11-7082 and BAY 11-7085. The antigen presenting capacity of TNF- α stimulated CD34⁺ cells was strongly inhibited by ICOSIg fusion proteins or by NF- κ B inhibition. Thus, TNF- α -induced ICOSL expression seemed to be functionally important for the costimulatory capacity of CD34⁺ hematopoietic progenitor cells.

Successful antigen-specific T cell stimulation via the T cell receptor (TCR)¹-CD3 complex (TCR-CD3) requires costimulatory signals by the CD28 receptor family. During this process, CD28 or CD152 (CTLA-4) expressed on T cells is engaged by the ligands CD80 (B7-1) or CD86 (B7-2) expressed on antigen presenting cells (1, 2). The inducible costimulator (ICOS) is a recently defined third member of the CD28 family, but unlike CD28, it is not constitutively expressed on T cells (3). ICOS expression requires the activation of T cells via the TCR-CD3 complex. ICOS shows structural homology to CD28 and CD152, but it differs in the MYPPPY homology domain necessary for binding of CD28/CD152 to CD80 or CD86 (4). Engagement of ICOS, like CD28, can mediate potent costimulation of T cells (3, 5), and promotes T cell proliferation at levels similar to those observed after CD28 triggering but without the accompanying increase in IL-2 production. Instead, ICOS up-regulates expression of IL-4, IL-5, GM-CSF, IFN- γ , TNF- α , and IL-10 (3, 6). Blocking the interaction of ICOS with its natural ligand by use of a soluble ICOSIg construct reduced the proliferative response of T cells (7). The expression of ICOS on T cells varies depending on the source of lymphoid tissue; T cells in tonsillar germinal centers express high levels of ICOS, suggesting a role in the regulation of germinal center B cell differentiation (3).

Among the ligands of the CD28 receptor family, CD80 is expressed on antigen presenting cells after induction by microbes, cytokines, or CD40 ligation and is also expressed on fibroblasts, whereas CD86 is constitutively expressed on monocytes and is inducible upon stimulation (8). Most lymphoma and leukemia cells lack CD80, but ~50% of cases express CD86 (9). Recently, new homologues of CD80 and CD86 were described. One of these, B7h (also designated B7RP-1, GL50, or B7-H2) binds to ICOS but not to CD28 or CD152/CTLA-4 (5, 10–14). The ligand for ICOS (ICOSL) belongs to the immunoglobulin family of genes, but shares only ~20% amino acid identity with CD80 and CD86. ICOSL is constitutively expressed on B cells, macrophages, and on murine spleen cells and can be induced by TNF- α or by inflammatory stimuli on

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¹ The abbreviations used are: TCR, T cell receptor; ICOS, inducible costimulator receptor; ICOSL, inducible costimulator receptor ligand; GM-CSF, granulocyte/macrophage colony-stimulating factor; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; PDTC, pyrrolidine dithiocarbamate; APC, allophycocyanin; PE, phycoerythrin; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; TPA, 12-O-tetradecanophorbol-13-acetate; mAb, monoclonal antibody; RT, reverse transcription; PCR, polymerase chain reaction; AML, acute myeloid leukemia; DC, dendritic cell; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay.

fibroblasts and peripheral tissue (5, 10, 13–14). TNF- α is a potent inflammatory cytokine that upon stimulation of TNF receptors leads to activation of transcription factors such as NF- κ B/Rel (15, 16). Expression of ICOSL has been shown to provide costimulation *in vitro* and enhance T cell-dependent antibody responses and cytokine production from CD4⁺ T cells *in vivo* (3, 5, 17). Moreover, recent results reveal that blockade of ICOS/ICOSL interaction also inhibits T_H1-regulated effector phases in acute allograft rejection and experimental induction of autoimmune encephalomyelitis, leading to increased allograft acceptance and prevention of the disease (18, 19). In addition, it appears that ICOS stimulation also has a prominent role in secondary cytotoxic CD8⁺ T cell responses, leading to effective mobilization of adoptively transferred T cells (20).

We reported previously that ICOSL is expressed on antigen presenting cells and can be up-regulated by IFN- γ but not TNF- α on monocytes, whereas expression levels remain constant on monocyte-derived dendritic cells (DC) (7). In this study, we have used soluble ICOSIg and a recently developed monoclonal antibody against human ICOSL to further characterize its expression, function, and regulation in bone marrow and CD34⁺ progenitor cells when differentiated into dendritic cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—U937 cells (monocytic) were cultured as described (7). Actinomycin D, cycloheximide, the inhibitors of I κ B α phosphorylation: 3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile (BAY 11-7082) and 3-[(4-*t*-butylphenyl)-sulfonyl]-2-propenenitrile (BAY 11-7085) (21) were purchased from Calbiochem (Schwalbach, Germany). The NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC) (22) and 12-*O*-tetradecanolphorbol-13-acetate (TPA) were purchased from Sigma (Deisenhofen, Germany).

Monoclonal Antibodies and Production of ICOSIg Fusion Protein—Allophycocyanin (APC)- or phycoerythrin (PE)-conjugated monoclonal antibodies against CD1a (SK9), CD3 (SK7), CD4 (SK3), CD11c (S-HCL-3), CD13 (L138), CD14 (MΦP9), CD15 (MMA), CD19 (4G7), CD20 (L27), CD22 (S-HCL-1), CD33 (P67.6), CD34 (8G12), CD38 (HB-7), CD56 (My31), CD80 (L307.4), HLA-DR (L243), and isotype control mAb (MOPC-21) were purchased from Becton Dickinson (Heidelberg, Germany). Antibodies against CD83 (HB15a) and CD86 (FUN-1) were from Coulter Immunotech and PharMingen (both Hamburg, Germany), respectively. The ICOSIg and CD152Ig fusion proteins were produced as described previously (7). Briefly, stable Chinese hamster ovary lines expressing ICOSIg or CD152Ig were grown in Excell 302 Chinese hamster ovary media (JRH Biosciences, Lenexa, KS) containing 0.5 mg/ml recombinant insulin (Life Technologies, Inc.), sodium pyruvate (Irvine Scientific, Santa Ana, CA), 4 mM L-glutamine (Irvine Scientific), 23 nonessential amino acids for minimal essential medium (Irvine Scientific), and 100 nM methotrexate (Sigma). Spent supernatants were harvested from large scale cultures, and Ig fusion proteins were purified by protein A affinity chromatography over a 2-ml protein A-agarose column (Repligen, Cambridge, MA). 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 ml of 1 M Tris-HCl (pH 7.4). Eluted fractions were assayed 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- μ m syringe filter units (Millipore, Bedford, MA). Staining capacity was tested by serial dilutions on U937 cells that have been shown to be positive for ICOSIg and CD152Ig binding. Optimal staining was usually obtained with 1–2 μ g of fusion protein/10⁶ cells.

Preparation of Bone Marrow and Isolation of CD34⁺ Cells—Bone marrow cells were collected after informed consent from patients with breast cancer or non-Hodgkin's lymphoma undergoing evaluation for bone marrow involvement and found to be negative. Cells were isolated by Ficoll-Hypaque (Seromed, Berlin, Germany) density gradient centrifugation. Interphases were harvested and washed three times and subjected directly to flow cytometry analysis. For positive selection of T cells, B cells, and CD33⁺ and CD34⁺ cells from bone marrow, cells were labeled with anti-CD3 mAb (SK7), anti-CD19 mAb (HD37), anti-CD33 mAb (P67.6), or anti-CD34 (My10), respectively and then positively selected with goat anti-mouse IgG immunomagnetic beads according to

the manufacturer's instructions (DynaL, Hamburg, Germany). Purity was greater than 95%, as assessed by flow cytometry.

For purification of CD34⁺ cells for dendritic cell differentiation assays, mononuclear cells were collected by leukapheresis from peripheral blood of breast cancer patients in the context of a high dose chemotherapy program using an AS104 cell separator (Fresenius, Wiesbaden, Germany). CD34⁺ cells were enriched by use of an Isolex 300 device (Baxter Biotech, München, Germany) as described previously (23). The positively selected cell fraction (purity > 90%) was cryopreserved and stored within the vapor phase of liquid nitrogen.

Stimulation of CD34⁺ Cells and Allogeneic Mixed Lymphocyte Reaction (MLR)—For stimulation CD34⁺ cells were plated in six-well Nunclon plates (Nunc, Naperville, IL) at a density of 1×10^6 cells/ml in 2 ml of RPMI 1640 medium with L-glutamine, 2-mercaptoethanol, antibiotics, and 10% fetal calf serum. Culture medium was supplemented with different cytokines at the following concentrations depending on the stimulation mixture used: 100 ng/ml human GM-CSF (Leukomax[®], Novartis), 1000 units/ml human IL-4 (kindly provided by Dr. Satwant Narula, Schering Plough Research Institute, Kenilworth, NJ), 50 ng/ml human TNF- α (Bender, Vienna, Austria), and 1000 units/ml human IL-3 (R&D Systems, Wiesbaden, Germany). Every other day, 50% of the medium was removed and the same volume of fresh medium containing twice the amount of cytokines was added. Cells were analyzed at different time points for differentiation markers and for ICOSIg or CD152Ig binding.

Responder T cells for the MLR were obtained from normal donors after centrifugation over Ficoll-Hypaque and subsequent positive selection via CD4 positive isolation kit with immunomagnetic beads according to the manufacturer's instructions (DynaL). To induce ICOS expression, CD4⁺ T cells (purity > 95%) were preactivated before use in the MLR by soluble anti-CD3 (OKT3, Ortho Pharmaceuticals, Raritan, NJ) for 12 h at 37 °C at a concentration that does not induce proliferation (0.5 μ g/ml).

MLRs were set up by culturing 5×10^3 γ -irradiated CD34⁺ cells (3,000 rad ¹³⁷Cs) that had been precultivated for 12 h under the conditions indicated in the results, with 5×10^4 prestimulated CD4⁺ T cells. Cells were cocultured in 96-well round-bottom microtiter plates for 3 days. T cell proliferation was assessed after addition of 1 μ Ci/well [³H]-thymidine (Amersham Pharmacia Biotech, Freiburg, Germany) 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 counts/min \pm S.E.

Hematologic Malignancies—Tumor cells were obtained after informed consent from freshly diagnosed or cryopreserved samples of patients with acute and chronic leukemia or follicular non-Hodgkin's lymphoma undergoing routine phenotype analysis. All lymphoma cases were leukemic, and cell populations contained at least 75% neoplastic cells according to FACS analysis. Lymphoma diagnoses had been independently confirmed by routine histology; acute myeloid leukemia (AML), acute lymphoblastic leukemia, chronic lymphocytic leukemia, hairy cell leukemia, and prolymphocytic leukemia cases had been diagnosed by a combination of standard morphology, histochemistry, and phenotype analysis. All the AML cases were positive for CD13 and/or CD33; all B cell leukemias and lymphomas were positive for CD19.

RNA Preparation and Polymerase Chain Reaction (PCR)—For reverse transcription-PCR (RT-PCR), cells were lysed in Trizol[®] (Life Technologies, Karlsruhe, Germany), RNA prepared and converted to first strand cDNA by use of random hexamer or oligo(dT) primers and murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences, Weiterstadt, Germany) according to the manufacturer's instructions. Success of cDNA synthesis was monitored by PCR with β_2 -microglobulin-specific primers 5'-CCAGCAGAGAATGGAAAGTC-3' and 5'-GATGCTGCTTACATGTCTCG-3', amplifying after 27 cycles a PCR product of 268 base pairs in size. Primers used for analysis of ICOSL expression were derived from B7-H2 sequence (GenBank[®] accession no. AF289028; Ref. 13) and were 5'-GGTTACACTGCATGTGCGAGC-3' and 5'-GTGAGCTCCGGTCAAACGTGG-3'. PCR synthesis was run for 40 cycles, amplifying a 534-base pair product.

Electromobility Shift Assay (EMSA)—For isolation of nuclear proteins, 2×10^6 CD34⁺ cells were washed in cold PBS before addition of 200 μ l of cold low salt buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μ M leupeptin). After a 15-min incubation on ice, 12 μ l of 10% Nonidet P-40 was added and samples were vortexed. Nuclei were spun down and resuspended in 25 μ l of high salt buffer (20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μ M leupeptin). For extraction of proteins, samples were vigorously rocked

at 4 °C for 20 min. After subsequent centrifugation, nuclear extracts were collected as supernatants and stored at -80 °C until use. The NF- κ B oligonucleotide (Santa Cruz Biotechnology, Heidelberg, Germany) was end-labeled with [γ -³²P]ATP (Amersham Pharmacia Biotech) in the presence of T4 polynucleotide kinase (Promega, Mannheim, Germany). Unincorporated [γ -³²P]ATP was removed by a NICK[®] Sephadex column (Amersham Pharmacia Biotech). Binding reactions were performed for 30 min on ice with 3 μ g of protein and 20,000 cpm of radiolabeled NF- κ B oligonucleotide in 20 μ l of binding buffer (4% Ficoll, 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.25 mg/ml bovine serum albumin). The DNA-protein complexes were then separated from unbound oligonucleotides on nondenaturing 4.5% polyacrylamide gels in 0.5 \times TBE buffer, fixed, and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Flow Cytometry Analysis—As described previously (7), cells were incubated in staining buffer (2% fetal calf serum in PBS, 0.05% Na₂S₂O₈), nonspecific Fc-binding was blocked by preincubation with mouse anti-human CD32 F(ab')₂ antibody (Ancll, Bayport, MN) at 1 μ g/ml for 30 min at 4 °C. When evaluating AML cells, it was important to use human Ig to block nonspecific Fc receptor binding, as CD32 antibody treatment was not sufficient because of high expression of other Fc receptors in AML cells. After Fc blocking, cells were washed once in staining buffer and incubated 45 min on ice with 10 μ g/ml Ig fusion protein. Cells were again washed once with binding buffer and subsequently incubated with second step reagent FITC-conjugated goat anti-human IgG F(ab')₂ (Jackson) at 1:50 dilution for 45 min on ice. Finally cells were washed three times in staining buffer, resuspended in PBS, and analyzed on a FACScalibur (Becton Dickinson, Heidelberg, Germany). For staining of DC generated from CD34⁺ cells, APC- and/or PE-conjugated mAbs directed against CD1a, CD3, CD4, CD11c, CD14, CD15, CD33, CD34, CD38, CD54, CD58, CD80, CD83, CD86, HLA-DR, and their corresponding isotype controls (Becton Dickinson, Mountain View, CA) were used. In addition, for two/three-color immunofluorescence of bone marrow and CD34⁺ cells, cells were preblocked with 50 μ g/ml human Ig (Biotest Pharma, Dreieich, Germany) instead of CD32 antibody and stained with a murine mAb against the extracellular domain of human ICOSL developed with the help of Genovac (Freiburg, Germany).

RESULTS

CD34^{bright} Hematopoietic Progenitor Cells in Human Bone Marrow Are ICOSL-negative—Previously, we found that the ligand of ICOS (ICOSL) is expressed on antigen presenting cells of myeloid origin and on ~40% of peripheral blood CD19⁺ B cells (7). In this study we wanted to determine whether ICOSL is expressed early during ontogeny of myeloid antigen presenting cells. Human bone marrow mononuclear cells were isolated and stained with mouse anti-human ICOSL mAb followed by goat anti-mouse FITC, and then counterstained with APC- and PE-conjugated mAb. Fig. 1 shows the staining of ICOSL on CD34-positive cells according to their CD33 expression. When gating on the small lymphoid cells in the bone marrow (Fig. 1A, upper part), almost all cells were ICOSL-negative. Only a few cells in this population coexpress CD33. In total bone marrow, a large proportion of the CD34⁺ cells coexpressed CD33 (Fig. 1A, lower half). Only the CD34^{dim}CD33⁺ fraction (R9 in Fig. 1A) showed some staining for ICOSL (25 \pm 4%), suggesting that ICOSL expression is acquired as soon as hematopoietic progenitor cells have a clear myeloid commitment, as indicated by strong CD33 expression and beginning loss of CD34 antigen expression. The amount of CD34⁺CD38⁻ cells in our preparations was too small to allow for a clear evaluation of ICOSL expression on this fraction of more primitive hematopoietic precursor cells (data not shown).

We also examined ICOSL expression on purified bone marrow fractions by RT-PCR. We isolated bone marrow cells according to their expression of CD34 and lineage markers for B cells, T cells, and myeloid cells by immunomagnetic separation for CD34, CD19, CD3, and CD33, respectively. Primers for RT-PCR were designed from the sequence of the B7-H2 gene to

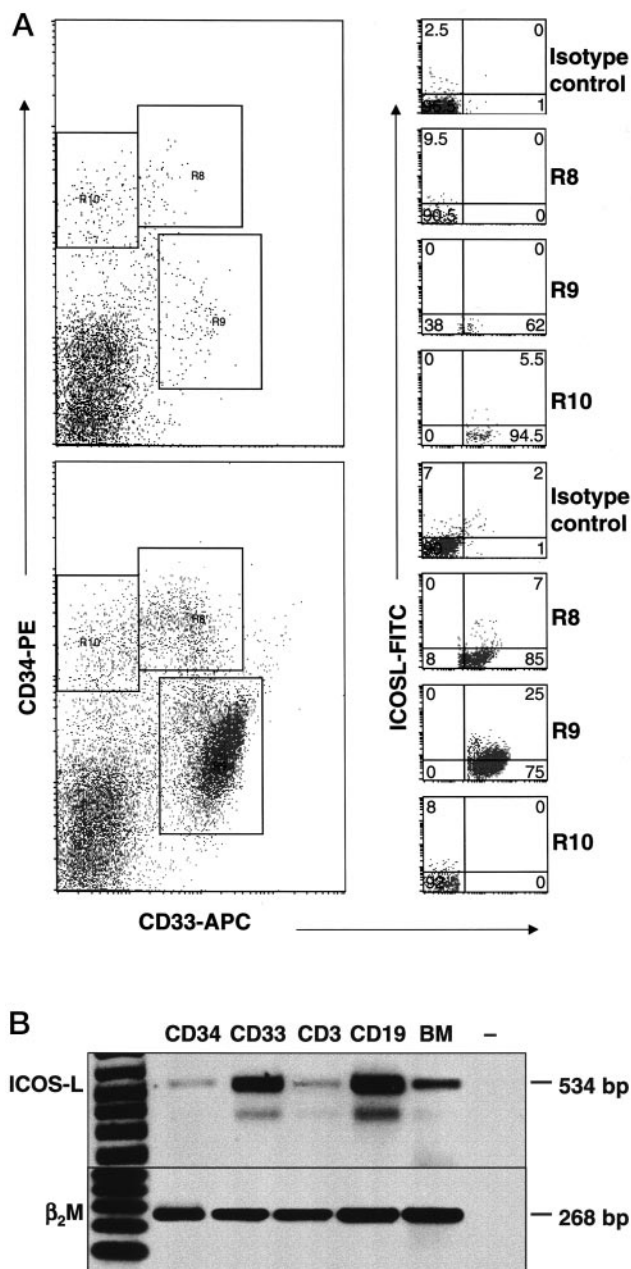


FIG. 1. ICOSL expression in the bone marrow. A, for three-color FACS analysis, a newly developed murine anti-human-ICOSL mAb was used and detected by goat anti-mouse FITC (see "Experimental Procedures"), followed by counterstaining with directly labeled CD34-PE and CD33-APC. Different populations identified by their level of CD33 and CD34 expression were gated (left), and their ICOSL expression is shown (right). Quadrant settings were done with respective isotype controls. In the upper half, the small lymphocyte gated population is shown; the lower half represents staining for total bone marrow. B, RT-PCR of bone marrow subpopulations for ICOSL expression (upper panel). CD3⁺, CD19⁺, CD33⁺, and CD34⁺ subpopulations were isolated with immunomagnetic beads. Expression was compared with whole bone marrow (BM); - is PCR control without cDNA. Use of approximately equal amounts of cDNA in each case was monitored with β_2 -microglobulin (β_2 M)-specific PCR (lower panel).

which protein binding of ICOSIg recently has been shown (13). Calibrated cDNA of purified cell populations (see "Experimental Procedures") confirmed the lack or very weak expression in the CD34^{bright} fraction that was isolated and revealed a clear presence of the ICOSL transcript in the CD33⁺ myeloid cells and in the CD19⁺ B cell population, consistent with the ICOSL expression on peripheral blood cells (Fig. 1B). The weak expres-

TABLE I

ICOSL versus CD80/CD86 expression on leukemias and lymphomas

AML indicates acute myelogenous leukemia; B-ALL, lymphoblastic leukemia of B cell origin; B-CLL, B cell type chronic lymphocytic leukemia; B-PLL, B cell type prolymphocytic leukemia; HCL, hairy cell leukemia; FL, follicular lymphoma. The cell populations contained more than 75% malignant cells.

Tumor type	Marker	CD80 ^a	CD86 ^a	ICOSL ^a
AML	CD13/CD33	0/7	3/7	0/9
B-ALL	CD19	1/6	5/6	0/6
B-CLL	CD19	0/5	2/5	1/5
B-PLL	CD19	1/4	2/4	2/4
HCL	CD19	2/6	3/6	4/6
FL	CD19	1/7	3/7	0/7

^a Number of positive cases/cases tested.

sion of ICOSL transcripts in the CD3⁺ T cell fraction is consistent with a weak expression on a small number of T cells by flow cytometry (data not shown) and deserves further investigation. Thus, the lack of ICOSL staining on CD34^{bright}CD33⁻ and CD34^{bright}CD33⁺ cells suggested that hematopoietic progenitors do not express the ligand for ICOS and that its expression is acquired later during differentiation into myeloid committed CD34^{dull}-CD33^{bright} cells.

Leukemic Cells Corresponding to Immature Myeloid and Lymphoid Cells Do Not Express ICOSL—We reasoned that ICOSL is first acquired when hematopoietic cells differentiate into antigen presenting cells of myeloid (monocytes and DC) or lymphoid (B cells) origin. Because the phenotype of leukemia and lymphoma cells may correspond to frozen stages of lymphoid and myeloid differentiation, we examined leukemic cells for ICOSIg binding. None of seven cases of CD13⁺CD33⁺ AML nor any of six cases of B-lineage acute lymphoblastic leukemia examined was bound by ICOSIg (Table I). These data suggest that the very early myeloid cells and B cell progenitor cells do not express ICOSL. Surprisingly, some of the leukemias stained positive for CD80 and/or CD86. Four of five cases of chronic lymphocytic leukemia, which is thought to represent a disease of immature, virgin B-lymphocytes, were ICOSL⁻, whereas two of four prolymphocytic leukemia and four of six hairy cell leukemia were ICOSL⁺. Because both prolymphocytic leukemia and hairy cell leukemia correspond to mature, almost preterminally differentiated B cells, the results suggest that ICOSL is expressed relatively late during B cell maturation. None of seven cases of follicular lymphoma (which correspond to germinal center B cells) reacted with our ICOSIg reagent.

ICOSL Is Expressed Early during Differentiation of CD34⁺ Progenitor Cells into Dendritic Cells—Although ICOSL is absent from CD34^{bright} hematopoietic progenitor cells, it is present on mature monocytes and dendritic cells. Therefore, we evaluated ICOSL expression during *in vitro* maturation of purified CD34⁺ cells. These cells were enriched from mobilized peripheral blood progenitor cells by immunomagnetic separation. The CD34 population obtained with this procedure is more uniform than the heterogeneous CD34 population in the bone marrow, according to forward scatter and side scatter profiles and marker expression. All cells were uniformly bright CD34-positive, CD38-positive, and weakly CD33-positive (Fig. 2, A and B). Cells were grown in the presence of different cytokine mixtures including GM-CSF/TNF- α , GM-CSF/IL-4/TNF- α , and GM-CSF/IL-3. The combination of GM-CSF and TNF- α induces differentiation of hematopoietic progenitor cells into DCs (24). IL-4 prevents terminal monocytic differentiation, allowing for generation of large numbers of DC even from mature monocytes (25, 26). Purified CD34⁺ cells were cultivated for 12 days and analyzed at day 3, 6, 9, and 12 for surface marker expression (Table II). At day 9 DCs differentiated from

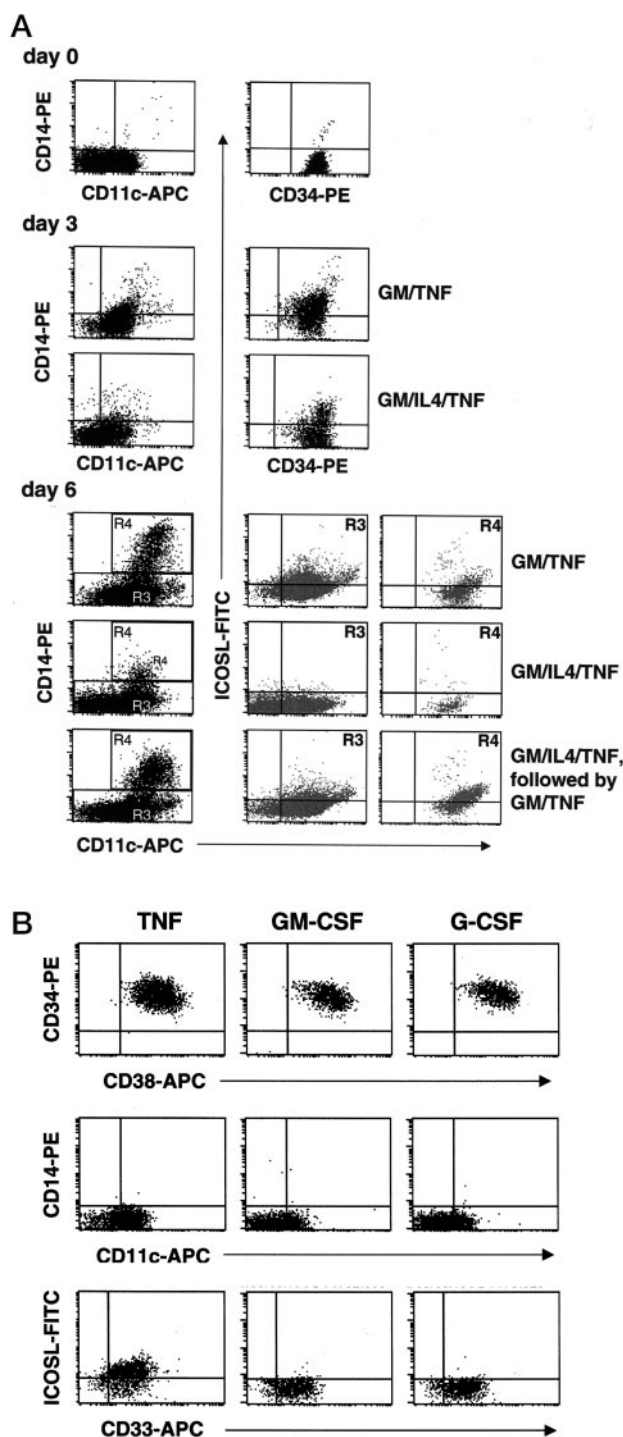


FIG. 2. Differentiation of CD34⁺ progenitor cells into dendritic cells results in early up-regulation of ICOSL. A, purified CD34⁺ cells from mobilized peripheral stem cells were cultured with GM-CSF/TNF- α , GM-CSF/IL4/TNF- α , or GM-CSF/IL-4/TNF- α until day 3, followed for another 3 days by GM-CSF/TNF- α (see text) to induce differentiation into dendritic cells. Kinetic studies of FACS phenotype were analyzed on days 0, 3, and 6 (B). To identify the ICOSL-inducing cytokine on purified CD34⁺ cells, cells were cultivated for 18 h with G-CSF, GM-CSF, or TNF- α , respectively. FACS analysis after 18 h was performed with murine anti-human ICOSL mAb detected by goat anti-mouse FITC and counterstained with APC- or PE-conjugated mAbs (see "Experimental Procedures"). A fraction of the cells was further cultivated to confirm terminal differentiation of DC (see "Results," Table II).

CD34⁺ cells in the presence of GM-CSF and TNF- α were positive for CD11c, CD33, CD54, CD58, CD80, CD86, and HLA-DR; in part positive for CD1a and CD14; and negative for CD15

TABLE II
 ICOSL expression during DC differentiation

 Values are given as percentage \pm standard deviation.

	Day 0	Day 3	Day 6	Day 9	Day 12
GM-CSF/TNF					
CD1a	2.9 \pm 2.8	1.7 \pm 2.0	11.9 \pm 5.4	24.3 \pm 12.1	18.0 \pm 6.7
CD11c	40.3 \pm 4.5	67.3 \pm 26.9	86.7 \pm 5.4	92.4 \pm 3.7	94.4 \pm 1.8
CD14	2.7 \pm 2.5	1.4 \pm 1.0	19.6 \pm 8.8	34.0 \pm 10.0	32.4 \pm 14.8
CD33	73.3 \pm 28.0	85.2 \pm 14.1	82.8 \pm 10.6	68.7 \pm 5.9	84.7 \pm 12.5
CD83	0.5 \pm 0.5	8.5 \pm 5.0	4.4 \pm 1.5	26.7 \pm 21.8	11.9 \pm 2.2
HLA-DR	98.3 \pm 0.5	95.5 \pm 0.2	94.7 \pm 2.3	96.1 \pm 0.6	94.6 \pm 2.2
ICOSI_g	2.6 \pm 2.0	65.0 \pm 6.6	56.6 \pm 17.9	66.2 \pm 6.6	40.6 \pm 5.9
CD152I _g	0.9 \pm 1.2	9.8 \pm 3.0	37.5 \pm 10.9	57.2 \pm 2.5	65.4 \pm 3.8
GM-CSF/IL-3					
CD1a		2.9 \pm 5.0	9.5 \pm 9.8	9.8 \pm 8.5	1.6 \pm 1.2
CD11c		53.2 \pm 11.9	70.4 \pm 1.6	90.8 \pm 2.2	86.9 \pm 4.4
CD14		0.5 \pm 1.6	10.6 \pm 6.6	13.6 \pm 14.5	8.1 \pm 1.1
CD33		87.6 \pm 2.3	92.3 \pm 4.3	89.8 \pm 2.1	93.5 \pm 3.1
CD83		5.2 \pm 2.5	10.7 \pm 9.7	3.6 \pm 4.5	1.9 \pm 3.5
HLA-DR		92.9 \pm 2.2	95.6 \pm 1.0	93.6 \pm 1.5	69.9 \pm 13.3
ICOSI_g		26.9 \pm 18.1	38.7 \pm 4.4	52.4 \pm 19.2	42.1 \pm 14.9
CD152I _g		7.7 \pm 5.8	24.8 \pm 1.6	32.1 \pm 6.3	63.7 \pm 5.6

(Table II, and data not shown). CD83 expression was achieved only after the 6th day of culture (Table II).

DC and monocyte populations can be distinguished by the expression of CD11c and CD14 (24, 27). Therefore, we examined ICOSL expression together with CD11c and CD14. ICOSL was equally expressed in the GM-CSF/TNF- α -induced CD11c⁺CD14⁻ DC and CD11c⁺CD14⁺ monocyte fraction (Fig. 2A). Both at day 3 and day 6, cells cultured in GM-CSF, TNF, and IL-4 had a lower expression of ICOSL as compared with cells cultured with GM-CSF and TNF, suggesting that IL-4 partially counteracted the induction of ICOSL and reduced the percentage of ICOSL⁺ by ~30–40%. However, this effect was reversible when IL-4 was washed out at day 3 and replaced by GM-CSF/TNF- α for another 3 days and measured again at day 6 (Fig. 2A). Significant expression of the costimulatory molecules CD80/CD86 (as judged by binding of the CD152I_g fusion protein (or anti-CD80 and CD86 mAb; data not shown) appeared later at day 6 (Table II) and were not affected by IL-4 (data not shown). The expression of ICOSL on maturing DC peaked at day 3 of the culture and was not further up-regulated later (Table II).

Having shown that the combination of GM-CSF and TNF strongly induces the expression of ICOSL in culture, we then evaluated which cytokine is responsible for this effect; TNF- α appears to be the crucial cytokine for induction of expression of ICOSL, whereas GM-CSF alone and G-CSF alone were not able to induce ICOSL expression (Fig. 2B). Interestingly, the TNF-mediated ICOSL induction on purified CD34⁺ cells was already observed as early as 18 h of culture. All CD34⁺ cells coexpressed CD38, a marker that is not expressed on rare more primitive CD34-positive hematopoietic progenitor cells. In comparison with GM-CSF and G-CSF alone, TNF- α increased the expression of both ICOSL and CD11c, whereas cells remained negative for CD14 expression within this time.

Dendritic cells distinctive from those that give rise to Langerhans cells can be generated by stimulation with IL-3 or IL-3 + GM-CSF (28). These cells are also called "lymphoid dendritic cells" and are positive for CD4, CD33, CD54, CD58, CD86, and HLA-DR, but negative for CD1a and CD80 (Table II, and data not shown). The combination of GM-CSF and IL-3 induced an up-regulation of ICOSL that was earlier and stronger as compared with CD80/CD86 (Table II). Highest ICOSL expression however was achieved at a later time than in TNF-containing cultures. Moreover, expression of CD14 and CD83 was consistently lower in GM-CSF/IL-3-stimulated cells.

The TNF-induced Up-regulation Is Mediated via NF- κ B Ac-

tivation—Twelve hours after stimulation with TNF- α alone, CD34⁺ cells expressed high levels of ICOSL (Fig. 3A). The rapid and strong induction of ICOSL on CD34⁺ cells by TNF- α cannot be explained simply by release of ICOSL from intracellular stores, because inhibitors of *de novo* protein or RNA synthesis, such as cycloheximide or actinomycin D, completely abrogated ICOSL expression (Fig. 3A). More striking was the complete suppression of TNF- α -induced ICOSL by the inhibitors of NF- κ B-mediated signal transduction PDTC and, in particular, BAY 11-7082 and BAY 11-7085 (which interfere with NF- κ B by inhibiting I κ B α phosphorylation) strongly inhibited TNF- α -induced ICOSI_g binding. BAY 11-7082 and 11-7085 were already effective at a low dose level of 2 μ M. To demonstrate that the strong suppression of TNF- α -induced ICOSL on CD34⁺ cells by these inhibitors indeed affects NF- κ B-mediated transcription, we performed EMSAs of these cells. The EMSAs emphasized a strong TNF-mediated induction of NF- κ B that was completely blocked by the inhibitors BAY 11-7085 (Fig. 3B). Subsequently, a complete suppression of TNF-induced ICOSL transcription by BAY 11-7085 was also observed by RT-PCR (Fig. 3C). Although we cannot formally exclude that these inhibitors affect other pathways, the up-regulation of ICOSL on CD34⁺ cells seemed to be mainly dependent on the NF- κ B pathway, because we tested various other inhibitors of protein kinases and mediators of TNF-induced signal transduction (inhibitors of protein kinase C (staurosporine, Ro318220, and CGP41251) inhibitors of mitogen-activated protein kinase pathways such as U0126, an inhibitor of the extracellular signal-regulated kinase pathway (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 inhibitor), and the p38 mitogen-activated protein kinase inhibitors SB203580 and SB202190, inhibitors of tyrosine phosphorylation such as genistein, inhibitors of G protein signaling such as NF023, and inhibitors of phospholipase A₂ such as methyl arachinodoyl fluorophosphonate, all of which were negative in our experimental setting but active on control cells or in different stimulation assays. Furthermore, comparable amounts of the solvents of the different inhibitors had no influence on the TNF-mediated induction of ICOSL on CD34⁺ cells (data not shown).

TNF-activated CD34⁺ Cells Are Potent Stimulators of Allogeneic T Cells in MLR—To assess the possible function of ICOSL during early differentiation of myeloid antigen presenting cells, we tested the stimulatory capacity of ICOSL⁺ cells in allogeneic MLRs with purified CD4⁺ T cells in the presence or absence of ICOSI_g (Fig. 4). T cells had been prestimulated with

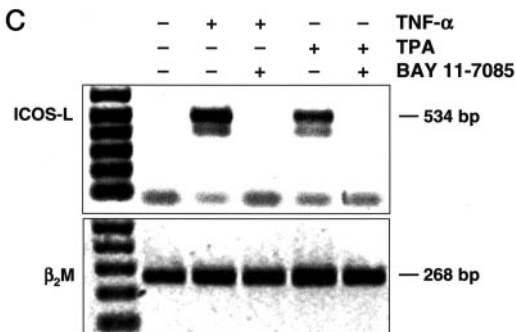
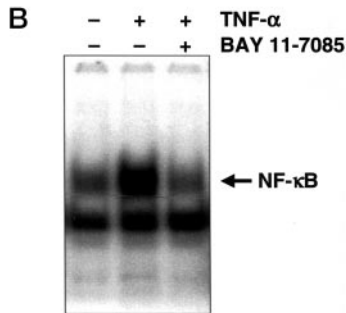
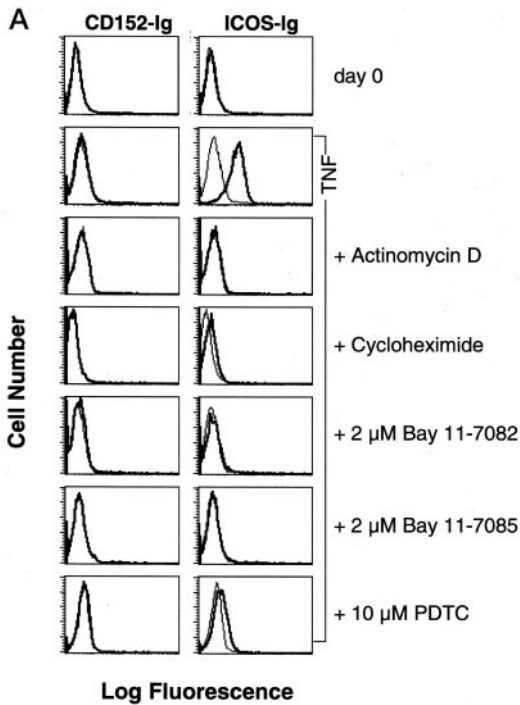


FIG. 3. ICOSL induction on CD34⁺ progenitor cells is mediated via NF-κB activation. *A*, TNF-α alone induces ICOSL expression on purified CD34⁺ cells as measured by FACS analysis with ICOS-Ig together with FITC-conjugated goat anti-human IgG F(ab')₂ (upper panel). For inhibition assays CD34⁺ cells were pretreated for 30 min with the different inhibitors shown and subsequently stimulated for another 12 h with TNF-α in the presence of the inhibitors. ICOSL induction on CD34⁺ cells by TNF-α required *de novo* protein (cycloheximide) or RNA (actinomycin D) synthesis. Complete suppression of ICOSL expression is observed in the presence of inhibitors of the NF-κB-mediated signal transduction BAY 11-7082, BAY 11-7085, and PDTC. *B*, inhibition of TNF-α-induced NF-κB binding in the nuclear extracts of CD34⁺ cells by the compound BAY 11-7085 as revealed by EMSA. *C*, inhibition of TNF-α- or TPA-induced ICOSL-expression by 2 μM BAY 11-7085 shown at the transcriptional level by RT-PCR (upper panel). Use of approximately equal amounts of cDNA in each case was monitored with β₂-microglobulin (β₂M)-specific PCR (lower panel).

A Stimulator cells

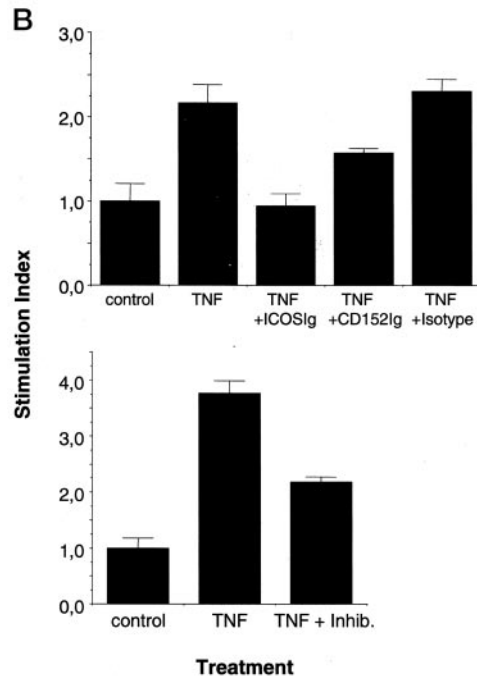
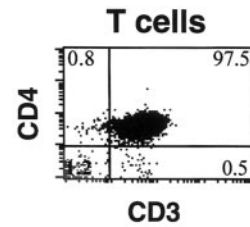
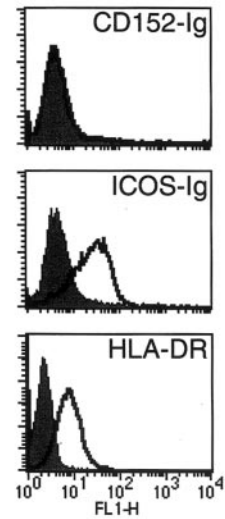


FIG. 4. ICOS:ICOSL interaction is involved in the allostimulatory capacity of TNF-activated CD34⁺ cells. *A*, purified CD34⁺ cells, which were used as stimulator cells in MLR assays, were pretreated for 18 h with TNF-α and stained for ICOSL, CTLA4-Ig binding, and HLA-DR expression (upper panel). Additional phenotypic markers are shown in Fig. 2*B*. Purity of CD4⁺ T cells is shown (lower panel). *B*, for MLR assays CD34⁺ cells were pretreated for 18 h with TNF-α or medium alone in the presence (lower panel) or absence (upper panel) of the NF-κB specific inhibitors BAY 11-7082 or BAY 11-7085 (TNF + *Inhib.*). Purified CD4⁺ T cells were pretreated with soluble anti-CD3. Cells were washed and then cocultured for 3 days in the presence or absence of different Ig constructs. Cells without TNF pretreatment (-) do not stimulate in the MLR. Data are shown as stimulation index ± S.E. One of four different experiments is shown.

CD3 to induce ICOS expression, because ICOS is not constitutively expressed on T cells. The dose of CD3 antibody used was not sufficient to induce T cell proliferation (data not shown). CD34⁺ cells were pretreated 12 h with TNF- α in the presence or absence of the NF- κ B-specific inhibitors BAY 11-7082 or BAY 11-7085. At this point, CD34⁺ cells stimulated with TNF expressed HLA-DR and ICOSL, but not CD80/86 (Fig. 4A). Moreover, at this time point, TNF-stimulated CD34⁺ cells express CD38 and lack CD14 (Fig. 2B). Cells were then washed, irradiated, and cocultured with T cells for 3 days in the presence or absence of a blocking dose of ICOSIg or CD152Ig, shown previously to block proliferation of allogeneic CD4⁺ T cells (7). As expected, ICOSIg reduced T cell proliferation to background levels (Fig. 4B, upper panel), whereas inhibition of CD80/86:CD28 interaction by CD152Ig in this situation was less effective. Indeed, we reproducibly observed some degree of inhibition by CD152Ig, which may be a result of the fact that during the coculture some degree of up-regulation of CD80/CD86 expression does occur despite irradiation. A consistent amount of inhibition (~50%) was also observed by preventing ICOSL expression via NF- κ B-specific inhibition (Fig. 4B, lower panel). Together, these results suggest that the TNF-mediated ICOSL induction on CD34⁺ cells delivers a costimulatory signal to allogeneic T cells.

DISCUSSION

In this study we have further characterized the expression of the ligand of the newly described costimulatory molecule ICOS during differentiation of antigen presenting cells and compared its expression to that of the other costimulatory ligands CD80 and CD86. ICOSL is not present on CD34^{bright} hematopoietic precursor cells, but is acquired later during ontogeny of both myeloid antigen presenting cells and B lymphocytes. In contrast, both AML and acute lymphoblastic leukemia cases were also found to be negative; only a few leukemia cases with the phenotype of most mature B lymphocytes were ICOSL⁺. Furthermore, it is surprising that all seven cases of leukemic follicular lymphoma cases tested were ICOSL⁻.

The prominent role of ICOSL in B cell responses has been demonstrated in transgenic mice overexpressing soluble ICOSL (5). In addition, ICOS-deficient mice show severely impaired T cell-dependent B cell responses and germinal center formation (29–31). In our previous paper we found a heterogeneous ICOSL distribution in lymphoma cell lines of different maturation stages, so altogether it is unlikely that in B cells ICOSL expression directly correlates with the maturation stage of B cells. However, the same appears to be true for CD80 and CD86.

Along the differentiation pathway of CD34⁺ cells toward the monocytic/dendritic lineage, ICOSL appears on cells as early as 12 h after stimulation with TNF- α . It is not further up-regulated during terminal differentiation of dendritic cells. We have found that IL-4 down-regulates the TNF-mediated ICOSL expression, an effect that seems to be reversible. This may be part of a regulatory loop, because ICOS expression on T cells appears to be crucial for IL-4 secretion (29) and will be a matter of further investigation. Although TNF is a crucial stimulus to induce ICOSL, as shown by stimulation with TNF- α or GM-CSF/TNF- α , GM-CSF/IL-3 was also relatively effective at stimulating ICOSL expression, although ICOSL expression peaked at a later time point. It has been proposed that this cytokine mixture differentiates CD34⁺ cells into “lymphoid” DCs (28). If this is the case, then ICOSL apparently is expressed on both myeloid and lymphoid DCs. However, we cannot formally exclude the possibility that, during the culture period, TNF- α was somehow produced, thereby providing an indirect source of ICOSL induction. In all these cultures, up-regulation of ICOSL

was consistently observed much earlier than for the CD152Ig binding molecules CD80/CD86. The functional reason for this is not yet clear. One intriguing possibility is that there are ICOS⁺ cells in the bone marrow, which can be influenced by maturing myelo-monocytic cells via ICOS-ICOSL interaction, a possibility we are currently exploring.

The early expression of ICOSL during myeloid ontogeny/differentiation of antigen presenting cells is rather unique among costimulatory molecules; Rondelli and co-workers (32) reported that CD86 is expressed constitutively on a small subset (~6%) of CD34⁺ human marrow cells. Subsequently Ryncarz *et al.* (33) showed that CD34⁺CD86⁺ cells are committed precursors of macrophages and dendritic cells that have already lost the ability to differentiate into granulocytes. In CD34⁺CD86⁻ cells, CD86 started to appear within 2 days in the presence of TNF- α and stem cell factor with a peak of expression after 6 days. Our CD34-enriched fraction was negative for CD152 binding at the beginning of the culture. This could be a result of the different source of CD34⁺ cells in our experiments; we used peripheral blood-derived CD34⁺ cells, which may reflect a more homogeneous population of immature progenitor cells as compared with bone marrow mononuclear cells. Most of the cells became ICOSL-positive within a very short period of time, and the levels of ICOSL were not further up-regulated during later maturation toward DCs.

The CD34⁺CD86⁺ marrow cell population described by Ryncarz *et al.* (33) has antigen presenting capacity and mediates allostimulation of T lymphocytes. Blocking ICOS:ICOSL interaction reduced the amount of T cell stimulation in both alloantigenic assay and antigen-specific assays (7). The present work suggests that ICOSL may also be important for the antigen presenting capacity of immature myeloid cells. Stimulation of purified CD34⁺ cells with TNF- α for 12 h generated a population of cells that expressed ICOSL but neither CD80 nor CD86. These TNF-activated CD34⁺ cells are potent stimulators in an alloantigen-specific MLR, suggesting that the CD28 costimulatory pathway is not necessary for ICOSL costimulation. Indeed, Yoshinaga and colleagues (5) found that T cells from CD28^{-/-} mice still could be stimulated via ICOSL. Preventing ICOSL expression by cocultivation of the cells in the presence of NF- κ B inhibitors reduced the allostimulatory capacity of the cells by ~50%. A reduction down to background levels was found by adding ICOSIg. In agreement with the observed phenotype, CD152Ig was less effective than ICOSIg in this experimental set-up. However, we were not able to “freeze” the cells at this differentiation stage, where they express ICOSL but not CD80 and CD86. Thus, it is likely that during the culture time some expression of CD80/86 was induced. Interestingly, when using mature monocytes or DC as antigen presenting cells, we had detected a 50% reduction of T cell stimulation by ICOSIg, but a complete inhibition by CD152Ig (7).

In our experiments TNF- α was the key regulator of ICOSL expression. In mammalian cells triggering of TNF receptor-I initiates activation of I κ B kinase and p38 mitogen-activated protein kinase, resulting in increased activity of the transcription factors NF- κ B and AP-1 (34). The TNF-mediated NF- κ B induction and the complete inhibition of ICOSL expression by all three inhibitors of NF- κ B activation clearly show that the TNF-induced ICOSL up-regulation is mainly mediated via the NF- κ B pathway. This observation fits with the finding that TPA can induce ICOSL expression as efficiently as TNF- α (Fig. 4C). These results altogether suggest a very fast TNF-induced and NF- κ B-dependent up-regulation of ICOSL, but not of the CD152-binding molecules CD80/CD86, on CD34⁺ hematopoietic precursor cells. ICOSL and CD80/CD86 are also differently regulated in mature monocytes where both antigens are super-

induced by IFN- γ , but by distinct signaling pathways (7).

Among several TNF- α -induced genes that are regulated by NF- κ B/Rel transcription, Swallow *et al.* (10) recently isolated a gene (B7h) that is a close homologue of CD80/CD86 and almost certainly is the murine homologue of ICOSL (5, 12). Murine ICOSL is induced in 3T3 cells and in embryonic fibroblasts upon TNF- α treatment, and is up-regulated in peripheral tissue under the influence of lipopolysaccharide, a potent activator of TNF- α . By analogy to these findings, we show that induction of ICOSL expression and its signaling pathway differ in the various antigen presenting cells types investigated. In our previous study, we could show that ICOSL expression on monocytes was dependent on stimulation with IFN- γ but not TNF- α , IFN- α , lipopolysaccharide, or phorbol 12-myristate 13-acetate (7). Signaling pathways after IFN- γ stimulation specifically involved protein kinase C but not NF- κ B activation, the protein kinase C inhibitor staurosporine could only block the IFN- γ -mediated ICOSL up-regulation but not that of CD152 binding molecules. In contrast, in the present study, we now demonstrate that induction of ICOSL expression on purified CD34⁺ progenitor cells is dependent on stimulation by TNF- α and phorbol esters and is mediated mainly by NF- κ B. Expression of ICOSL on mature, monocyte-derived DCs seems to be independent of TNF- α or lipopolysaccharide (7) and at a certain stage of DC maturation may even inhibit ICOSL expression, as demonstrated by others (13, 14).

Further experiments should reveal if efficient costimulation in early hematopoietic precursors requires sequential expression of the various costimulatory molecules. Furthermore, it would be important to find out whether memory T cells preferentially use this costimulatory molecule that is constitutively expressed on different types of antigen presenting cells, as suggested by a recent work of Wallin *et al.* for CD8⁺ CTL (20).

Finally, it will be important to analyze whether ICOSL plays a role in regulating hemopoiesis. Its early expression before CD80/CD86 may suggest that this molecule has a function beyond simply stimulating activated T cells. Functional assays of colony forming cells and distribution studies in pathological conditions such as autoimmune cytopenias or transplant rejection will possibly help to clarify the role of this molecule.

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