Design and Methods

Monoclonal antibodies (mAb) and reagents.

The anti-CD69 mAb (clone 31C4, IgG2a), anti-CD45 mAb (TA218/12, IgM; T205/23; IgM), anti-CD2 mAb (clone γδ54, IgG1), anti-CD31 mAb (89D3), anti-CD16 mAb (NK1, IgG1; NK54, IgG2a), anti-CD18 mAb (70H12, IgG2a), anti-CD28 (CK248, IgM), anti-CD54 mAb (ICAM1, clone SM89, IgM), and anti-CD44 mAb (T61/2, IgG1, TA153/G8, IgG2a) were obtained in our laboratory as described elsewhere.[35,36] The anti-CD3 mAb (UCHT-1, IgG1) was from Ancell (Bayport, MN55003, USA). The anti-HLA class-I W6/32 (IgG2a), anti-SH2 (CD105, IgG1), the anti-SH3 (CD73, IgG2b), anti-SH4 (IgG1), anti-CD34 (clone IgG1), anti-CD11a (LFA1α, TS1.22, IgG1), anti-CD18 (LFA1β, TS1.18, IgG1) and anti-CD40 producing hybridomas were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Anti-HLA class I mAb (clone 3A3, IgM) and anti-CD14 mAb (63D3, IgG1) were kindly provided by E. Ciccone (Institute of Anatomy, University of Genoa, Italy) and D. Vercelli (Scientifc Institute San Raffaele, Milan, Italy) respectively. The anti-β1 integrin (CD29) mAb (3E1, IgG1) was a kind gift from Dr L. Zardi (IST-Genoa, Italy). Anti-ICAM2 and anti-ICAM3 mAb were from Bender MedSystem (CA 94010, USA). The anti-prolyl-4-hydroxylase mAb (clone 5B5, IgG1) was purchased from Dako (Denmark). Anti-human CTLA4 (CD152, clone 48815, IgG2a) mAb PE-conjugated and anti-human glucorticoid-induced TNFR-related gene GITR/TNFRSF18 mAb (Clone 109101, IgG1), anti-IL10 (MAB217, clone 23738, IgG2b) and anti-TGF-β (MAB1835, clone 1D11, IgG1) mAb were from R&D System Europe Ltd. (Abingdon, UK). Complete medium was composed of RPMI1640 (Biochrom, Berlin, Germany) with 10% fetal calf serum (FCS, Biochrom) supplemented with penicillin, streptomycin and L-glutamine (Biochrom).

Phytohemagglutinin (PHA) was from Sigma Chemicals Co. (St. Louis, MO) and interleukin (IL) 2 from Pepro Tec EC (London, UK). The COX-2 inhibitor NS398 and cyclosporin A (CSA) were from Calbiochem (Merck Biosciences GmbH (Bad Soden, Germany).

Isolation of PBMC, T lymphocytes and $CD4^+$ or $CD8^+$ T-cell subsets and co-cultures with MSC

PBMC were obtained by Ficoll Hypaque (Biochrom) gradient centrifugation from venous blood samples of healthy volunteers. Unfractionated T lymphocytes or $CD4^+$ or $CD8^+$ subsets were isolated from whole blood with the specific RosetteSep negative selection kit (StemCell Biotechnologies, Vancouver, Canada). The resulting T or $CD4^+$ or $CD8^+$ cell populations were 100% $CD45^+$, 99-100% $CD3^+$ or 95-98% $CD3^+CD8^+$ or 97-99% $CD3^+CD4^+$ (n=10) as determined by staining with specific mAb and FACS analysis. No natural killer (NK) cells ($CD3^-CD16^+CD56^+$), monocytes ($CD14^+$) or B lymphocytes ($CD20^+$surface IgM^+) were present in these cell populations. PBMC, or T or $CD4^+$ or $CD8^+$ cell subsets were used immediately in co-culture experiments with autologous or allogeneic MSC. These co-cultures were performed using different ratios of MSC and responder lymphocytes (1:2, 1:20, 1:200, 1:2000 and 1:20000). The ratio of 1:2000 was subsequently chosen as it was the optimal condition for generating regulatory T cells.

Generation of MSC

MSC were obtained, upon informed consent, from eight patients with acute myeloid leukemia (AML) and one with acute lymphoblastic leukemia (ALL) in complete remission following chemotherapy and from four healthy donors (from the Clinical

Hematology Department, University of Genoa) subjected to bone marrow aspirates, during IRB-approved conventional diagnostic procedures. Bone marrow cell suspensions were cultured in six-well plates ($5x10^6$ cells/well) in RPMI 1640 complete medium for 3 days. After this period, non-adherent cells were washed away and adherent cells cultured for an additional 7 days. On day 10, small groups of adherent cells (100-200 cells/group) were detectable under microscopic examination, the medium was changed and the cells cultured for an additional 7 days. Confluent adherent cells were then harvested and expanded in 25 cm^2 flasks. At this time, the surface phenotype of the adherent cells was the following: 99%SH3/CD73$^+$SH4$^+$SH2/CD105$^+$ 100%CD44$^+$β1-integrin(CD29)$^+$ ICAM1(CD54)$^+$, 100%HLA-I$^+$ and CD40$^+$, 98-100% CD45$^-$CD31$^-$CD34$^-$CD33$^-$CD3$^-$CD2$^-$CD16$^-$CD14$^-$ ICAM2$^-$ICAM3$^-$CD80$^-$CD86$^-$CD83$^-$HLA-DR$^-$. The constitutive expression of CD40 and CD54 was confirmed by analysis with specific primers of mRNA coding for these molecules by reverse transcriptase polymerase chain reaction (RT-PCR) (*not shown*). These cells were spindle-shaped, morphologically resembling fibroblasts, and can be considered *bona fide* MSC as they were able to differentiate into adipocytes, chondroblasts or osteoblasts under appropriate culture conditions [1-16,37] (*not shown*). Only MSC populations which were homogeneously positive or negative for the indicated markers and with similar morphological features were used in functional experiments. These MSC strongly inhibited (range 50-90%, mean 70%, n=6) the proliferation of T lymphocytes in MLR when added as a third party at the MSC:T responder ratios of 1:2 to 1:5. Thus, some phenotypic and functional features of MSC, obtained under our culture conditions were consistent with previous observations.[1-16,37] In functional experiments, we observed that similar results were obtained with MSC from healthy donors (n=4) and leukemic patients (n=9).

Immunofluorescence and cytofluorimetric analysis

Immunofluorescence was performed on cells incubated with specific mAb followed by the addition of an anti-isotype specific goat anti-mouse (GAM) antisera (Southern Biotechnology, CA, USA) conjugated with phycoerythrin (PE) or with fluorescein isothiocyanate (FITC) as indicated. Control aliquots were stained with isotype-matched irrelevant mAb (Becton Dickinson) followed by GAM-PE or GAM-FITC. To analyze intracytoplasmic staining for CTLA4 expression some samples were fixed in formaldehyde and permeabilized with NP-40 (Sigma) before staining with mAb. Samples were run on a flow cytometer (FACSsort, Becton Dickinson) equipped with an argon ion laser exciting PE at 488 nm. Data were analyzed using the CellQuest computer program and results are expressed as log green fluorescence intensity or vs. number of cells or as mean fluorescence intensity (MFI) in arbitrary units (a.u.). Calibration was assessed with CALIBRITE particles (Becton Dickinson) using the AutoCOMP computer program.

MLR, recall to alloantigen, lymphocyte stimulation with PHA or through CD3/TCR engagement.

MLR were performed by culturing 10^5 PBMC for 7 days with 10^5 allogeneic irradiated PBMC in 96 U-bottomed-well microplates. To perform the recall to alloantigen tests, secondary MLR were set up with cells from the primary MLR (harvested after 7-10 days of culture) re-stimulated for 3-5 days with 10^5 allogeneic PBMC in 96 U-bottomed-well microplates. In some experiments, 10^5 PBMC were cultured with 10 ng/mL of anti-CD3 (JT3A, Ig2a) mAb (this optimal stimulating concentration was

determined by preliminary experiments using 1000, 100, 10 and 1 ng/mL) or with 1μg/mL of PHA for 3-5 days.

Inhibition of lymphocyte proliferation by cells generated from co-cultures of MSC with PBMC or $CD25^-$ or $CD4^+$ or $CD8^+$ T cell subsets

To analyze the regulating effect of Reg_c derived from PBMC-MSC co-cultures, PBMC were incubated with MSC for 4 days, then the Reg_c were harvested, subjected to Ficoll Hypaque gradient centrifugation to eliminate dying cells and added at the onset of MLR, recall to alloantigen (secondary MLR), anti-CD3 mAb- or PHA-stimulation at the Reg_c:PBMC ratio of 1:1, 1:10, 1:100, 1:250, and 1:500. In these experiments, PBMC and Reg_c were either autologous or allogeneic. The ability of MSC to induce the generation of Reg_c from different lymphocyte subsets was tested by setting up co-cultures of MSC with $CD25^-$ PBMC obtained after depletion of $CD25^+$ cells with immunomagnetic beads, (Oxoid, Milan, Italy) (94-99% $CD25^-$) or with $CD4^+$ or $CD8^+$ T cells using $CD4^+$ or $CD8^+$ T cell enrichment RosetteSep negative selection kit (Stemcell Biotechnologies) (100% $CD3^+$, 95-99% $CD4^+$ or 97-99% $CD8^+$).

In additional experiments, Reg_c obtained from MSC-PBMC co-cultures were depleted by $CD4^+CD105^+$ or $CD8^+CD105^+$ cells by negative selection using specific mAb and immunomagnetic beads (Oxoid, Milan, Italy). The resulting Reg_c subsets were 95-98% $CD8^+$ 1-5% $CD25^+$ or 85-95%$CD4^+$ 20-55%$CD25^+$ respectively and no residual MSC ($CD105^+SH3^+SH4^+$ cells) could be detected as assessed by staining with specific mAb and FACS analysis.

Control experiments were performed by adding to MLR or to anti-CD3 mAb- or to PHA-triggered PBMC, the autologous PBMC, or lymphocytes briefly stimulated (4

days) with anti-CD3 mAb or PHA or with long term (30 days) $CD4^+$ or $CD8^+$ cultured lymphocytes. To test the need of contact between Reg_c and PBMC to induce inhibition of proliferation, Reg_c and PBMC were separated from each other by a Millicell well (Millipore Corporation, Billerica, MA,USA) well with 0.3 μm pores in order to prevent contact between the Reg_c and PBMC while allowing the diffusion of cytokines produced by one or other of the cell populations. ^{3}H-thymidine uptake was measured during the last 18 h of culture incubation and results are given as stimulation index (S.I. calculated as the ratio between cpm $x10^{-3}$ of stimulated cultures and cpm $x10^{-3}$ of unstimulated cells) and are the mean±SD of at least five independent experiments. Some experiments were performed with PBMC labeled with carboxy fluorescein succiminidyl ester (CFSE, Molecular Probes, Carlsbad, CA, USA). Briefly, PBMC labeling with CFSE was performed according to manufacturer's instructions and then cells were cultured as described for the analysis of proliferation with ^{3}H-thymidine[36]. Results from CFSE-labeled cultures were analyzed with the ModFit computer program to determine the percentage of cells in parental and in any subsequent generations. Proliferation was indicated by the progressive reduction of CFSE content in a given cell population.

$CD4^+CD25^+$ T_{reg} were isolated from $CD4^+$ cells by positive selection using a custom-made EasySep isolation kit for $CD25^+$ cells. The resulting populations was 100% $CD4^+CD25^+$ ($CD25^{+high}$, range 35-65%, n=3). The $CD4^+CD25^+$ cells were used in functional experiments at 1:1, 1:10, 1:100, 1:250, 1:500 ratios with responding PBMC. In some experiments, anti-IL10 mAb, anti-TGF-β mAb (5μg/mL) and NS398 (15μM) were added at the onset of the MSC-lymphocyte co-culture or regulatory assay. MSC-lymphocyte co-cultures were treated with decreasing amounts of CSA (500-50-5 ng/mL) to evaluate the effect of CSA on the generation of Reg_c. In other experiments,

functionally active Reg_c were treated for 4 h with CSA (500 ng/mL), washed and used in regulatory assays to assessed whether CSA can inhibit the Reg_c-mediated immunosuppressive effect. That the dose of CSA used in these experiments was able to block T-cell activation was demonstrated by the finding that PBMC incubated with CSA did not proliferate in response to any mitogenic stimulus applied (anti-CD3 mAb, PHA or alloantigen) (*data not shown*).

Quantitative analysis of Foxp3 mRNA expression by real-time PCR

Total RNA was extracted from *ex vivo* isolated PBMC or $CD25^-$ PBMC alone or cultured for 4 days with MSC, $CD8^+(CD4^-CD105^-)$ or $CD4^+(CD8^-CD105^-)$ Reg_c and PHA-activated PBMC with TriPure isolation reagent (Roche, Indianapolis, USA) and cDNA was synthesized with RT-kit plus (Amplimedical, Turin, Italy). Quantitative real-time PCR was performed on an iCycler iQ (Bio-Rad, Milan, Italy) using Platinum SYBR Green qPCR SuperMix UDG reagent (Invitrogen Corp, Carlsbach, CA, USA), according to the manufacturer's instruction. The expression of Foxp3 was calculated as change in fold increase relative to RNA polymerase II polypeptide A levels with the $\Delta\Delta C_t$ method[38]. The PCR products were confirmed with melting curve analysis during real-time PCR.

The specific oligonucleotides were 5'-ATGGTACAGTCTCTGGAGCAG-3' and 5'-GATGAAGCCTTGGTCAGTG-3' for Foxp3 (101 bp) and 5'-GACAATGCAGAGAAGCTGG-3' and 5'-GCAGGAAGACATCATCATCC-3' for RNA polymerase II polypeptide A (112 bp). All primers were intron flanking to minimize genomic DNA amplification.

Evaluation of production of immune regulating cytokine: IL10 and TGF-β

In 24-well plates, $2x10^6$/mL PBMC or highly purified T or $CD4^+$ or $CD8^+$ cells were cultured for 24 h alone or with MSC at a 1:2000 MSC:responder cell ratio in RPMI1640 complete medium at 37°C. Some experiments were performed by seeding $2x10^6$/mL PBMC or highly purified T, $CD4^+$ or $CD8^+$ lymphocytes in Millicell wells with 0.3 μm pores (Millipore) in order to avoid contact between the MSC and lymphocytes but allow the diffusion of cytokines produced by either of the cell population. Supernatants were analyzed with a Cytokine Kit (Bender MedSystem GmbH, Vienna, Austria) for detection of IL10 while TGF-β was analyzed using a Cytotest assay kit from Biosource Europe SA (B-1400, Nivelles, Belgium) (detection limit for TGF-β: <30pg/ml) according to manufacturers' instructions.

Statistical analysis

Statistically significant differences in the parameters tested were assessed by applying Student's *t*-test statistics to the experimental data. The cut-off value of statistical significance was $p<0.01$.