Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy

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Supplementary Design and Methods

Culture conditions and reagents

The complete culture medium used for the mixed lymphocyte culture (MLR) was X-Vivo 15 medium (BioWhittaker, Verviers, Belgium) supplemented with 5% pooled AB human serum (Biowhittaker) and 100 U/mL penicillin/streptomycin (Bristol-Myers Squibb, Sermoneta, Italy). The culture medium for analysis of cytotoxic T lymphocyte precursors (CTLp) was Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (Biowhittaker), 100 U/mL penicillin/streptomycin (Bristol-Myers Squibb), and 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD, USA). All cultures were incubated at 37°C in a humidified air incubator with 5% CO_2. Human recombinant transforming growth factor-β (TGF-β) was purchased from R&D Systems (Minneapolis, MN, USA). CD3-coupled magnetic beads were purchased from Dynal (Oxoid, Milan, Italy). Neutralizing anti-interleukin-10 receptor (clone 3F9) was obtained from BD PharMingen (San Diego, CA, USA) and anti-TGF–$\beta_{1,2,3}$ (clone 1D11) from R&D systems.

Antigen-specific responses

After 10 days of culture, cells were harvested, washed and plated at a density of 10^5/well in 96-well plates with 10^5/well autologous irradiated CD3-depleted cells in the presence of *Candida albicans* (10^6/well heat-inactivated spores, generously provided by Prof.ssa L. Romani, University of Perugia, Italy), tetanus toxoid at 5 μg/mL (Alexis Biochemicals, San Diego, CA, USA), and cytomegalovirus (CMV) (lysate of infected human fibroblasts; diluted 1:30; kindly provided by Dr. Chiara Bonini, Laboratory of Experimental Hematology, San Raffaele Scientific Institute, Milan, Italy).[1] In parallel cells were stimulated with TPA (10 ng/mL; Calbiochem, Bioscience, La Jolla, CA, USA) plus ionomycin (150 ng/mL; Sigma). After the indicated time, cells were pulsed for 16 h with 1 μCi/well ^{3}H-thymidine.

Cytotoxic T lymphocyte precursor frequency assay

MLR cells cultured in the presence of interleukin-10 (IL-10) (MLR/10) were tested for the frequency of CTLp for the original allogeneic stimulator cells. Results were compared to those obtained from a primary MLR in the absence of IL-10. The CTLp frequency assay was performed as previously described.[2] Briefly, MLR and MLR/10 responder cells were plated in 2- or 3-fold limiting dilution (starting concentration of 5×10^4 for haploidentical pairs and 5×10^3 for mismatched pairs) in 20 replicates for each dilution, in the presence of 5×10^4 irradiated (6000 rad) CD3-depleted or total peripheral blood mononuclear cells (PBMC) of the original stimulator. Cultures were performed in the presence of 90 IU/mL recombinant human interleukin-2 (hIL-2; Chiron Italia, Milan, Italy). In parallel, a T-cell line of the stimulator was established by plating 2×10^6/mL PBMC with 1 μg/mL phytohemagglutinin (PHA) (Sigma) in the presence of rhIL-2 (600 IU/mL). On day 10, individual wells were tested for cytotoxic activity against the ^{51}Cr-labeled PHA-T-cell line (10^3/well) of the stimulator, in the presence of a 30-fold excess of unlabeled K562 cells, in order to avoid detection of non-specific killing. According to a standard ^{51}Cr-release assay, effector and target cells were incubated for 4 h at 37°C; subsequently the supernatants were removed and counted in a γ-counter. The percentage of specific release was calculated using the following formula: 100*(51Cr-release in the supernatant-spontaneous release)/(total release into detergent-spontaneous release). For calculation of CTLp frequencies, the fraction of negative wells was plotted for each dilution on a semi-logarithmic scale and the dilution in which 37% of wells were negative corresponded to the calculated frequency.[3] A positive and a negative control were included in the CTLp assay of each donor pair. The positive control was the cytotoxic activity of an unrelated responder against the specific stimulator cells used in the MLR, whereas the negative control was the autologous cytotoxic activity. The mean of lytic activity [cpm + 3SD] of the 20 autologous control wells was used as the cut-off value for evaluation of positive wells. Epstein-Barr virus (EBV)-specific CTLp frequencies were analyzed and calculated as described above for alloantigen-specific CTLp, but autologous to the responder EBV-LCL (previously established incubating PBMC with B95.8 viral supernatant) were used as stimulators for the 10-day *in vitro* culture period (3×10^4 irradiated by 10000 Rad) as well as readout targets for the cytotoxic assay.

For *in vitro* induction of effector cells specific for the immunodominant influenza protein-derived peptide MP.58-66, CD8-enriched T cells from HLA-A*0201$^+$ healthy donors were obtained from PBMC depleted of CD19$^+$ and CD4$^+$ cells by immunomagnetic beads. Mixed-lymphocyte-peptide stimulations were performed as follows: 2×10^6/well T cells were seeded in 24-well plates with 10 μg/mL MP.58-66 peptide, in the presence or absence of IL-10 (10 ng/mL). On day 2, IL-2 (10 IU/mL) was added. Responder CD8$^+$ T cells were stimulated weekly as described with autologous irradiated monocytes (CD3$^-$ CD19$^-$ PBMC) previously pulsed with the MP.58-66 peptide. The responder populations were analyzed after the second stimulation for lytic activity and interferon-γ

release against MP.58-66 pulsed and unpulsed T2 cells. MP.58-66-specific $CD8^+$ T cells in culture can be traced by Vβ17 expression, the dominant Vβ chain used by MP.58-66-specific T cells[4] or by A2/MP.58-66 tetramers.[5]

Flow cytometric analysis

Anti-CD4, -CD8, CD14, -CD25, -CD28, HLA-DR, -CD14, -CD1a, and -CD83 monoclonal antibodies, directly coupled to fluoroscein isothiocyanate or phycoerythrin, were purchased from BD Bioscience. Cells were incubated with the indicated monoclonal antibodies for 20 min at 4°C in phosphate-buffered saline 2% fetal calf serum, washed twice and analyzed using a FACScan flow cytometer (BD Biosciences), and data were analyzed with CellQuest software (BD Biosciences).

RNA sampling and SuperAmplification

Total PBMC were activated with allogeneic monocytes in the absence (MLR) or in the presence of IL-10 (MLR/10) or with allogeneic DC-10 (MLR/DC-10) or mature dendritic cells (MLR/mDC). After 10 days, cells were collected and lysed using SuperAmp™ lysis buffer (Miltenyi Biotec GmbH, Germany) following the manufacturer's instructions and stored at -80°C. DNA microarray analysis of gene expression was performed at Miltenyi Biotec's Genomics Service facility (Bergisch Gladbach, Germany), using Agilent technology (Agilent Technologies, Inc.). SuperAmplification was performed according to the manufacturer's instructions. Briefly, cell samples were collected in 6.4 µL lysis buffer (including detergent, tRNA and protease), the mRNA extracted using magnetic beads and transcribed into cDNA using tagged random and oligo(dT) primer. First strand cDNA was 5' tagged using terminal deoxynucleotidyl transferase (Fermentas). Tagged cDNA was globally amplified (Expand Long Template PCR System DNA Pol Mix, Roche) using primer complementary to the tag sequence. Amplified cDNA were quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc). The integrity of the cDNA was checked via the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Inc.).

Hybridization of Agilent whole human genome oligo microarrays

Two hundred and fifty nanograms of the purified polymerase chain reaction product (NucleoSpin® Extract II Kit, Macherey & Nagel) were labeled with Cy3-dCTP (GE Healthcare) in a Klenow Fragment reaction. Finally, 1.25 µg Cy3-labeled and purified (CyScribe GFX Purification Kit, GE Healthcare) cDNA in hybridization buffer were hybridized overnight (17 h, 65°C) to 4x44K Agilent Whole Human Genome Oligo Microarrays using Agilent's recommended hybridization chamber and oven. Following hybridization, the microarrays were washed once with Agilent gene expression wash buffer 1 for 1 min at room temperature followed by a second wash with preheated (37°C) Agilent gene expression wash buffer 2 containing 0.005% N-lauroylsarcosine for 1 min. The last washing step was performed with acetonitrile for 30 sec.

Scanning and data analysis

Fluorescence signals of the hybridized Agilent microarrays were detected using Agilent's microarray scanner system (Agilent Technologies). The Agilent feature extraction software (FES, v.9.5.1.1.) was used to read out and process the microarray image files. For determination of differential gene expression FES-derived output data files were further analyzed using the Rosetta Resolver gene expression data analysis system (v.7.1.0.0. Rosetta Inpharmatics LLC). Ratios were computed for each sample/control pair and transformed to a $\log_2$ scale. Additionally, *P* values indicating the reliability of an observed difference between a sample and its corresponding control were calculated for each gene applying the universal error model implemented in the Rosetta Resolver software.[6] Only expression values with *P* values less than 0.01 were considered statistically significant. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[7] and are accessible through GEO Series accession number GSE17493 (*http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17493*). Genes which were at least 1.7-fold up- or down-regulated in at least two of the three biological replicates (and the same trend of expression in the third replicate) of both the comparisons "MLR/10 *versus* MLR" and "MLR/DC-10 *versus* MLR/mDC" were selected and analyzed for a statistically significant enrichment of biological pathway annotation terms. Term enrichment relative to the expected background distribution was scored using Fisher's exact test. Annotations were derived from publicly available and literature-derived data sources, e.g. Gene Ontology (GO, *www.geneontology.org*), signaling pathway membership, sequence motifs, chromosomal proximity, literature keywords, and cell-specific marker genes.

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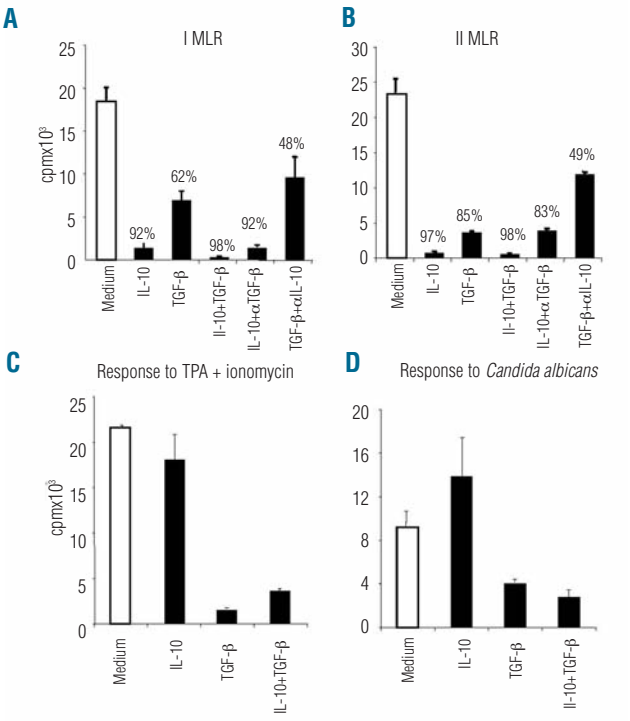
Online Supplementary Table S1. Genes up-regulated in IL-10-anergized cultures in comparison to in control cultures.

		Donor #1			Donor #2			Donor #3			Enriched pathway annotation terms								
		MLR	MLR-10	MLR-mDC	MLR-DC10	MLR	MLR-10	MLR-mDC	MLR-DC10	MLR	MLR-10	MLR-mDC	MLR-DC10	Immune responses	Response to wounding	Inflammatory responses	Monocytes/macrophages	Cytokines and cytokine receptors	Chemokines and chemotaxis	Cell proliferation	Signal transduction	NEUTROPHILS
Scavenger receptors																						
CD163	hemoglobin scavenger receptor		6.3		39.2		34.5		100.0		100.0		16.6									
SCARB2	scavenger receptor class B, member 2		5.5		4.1		3.4		5.3		24.7		1.7									
Enzymes																						
CHIT1	chitinase 1 (chitotriosidase)		3.0		25.9		12.4		13.0		3.8		3.5									
GPX3	glutathione peroxidase 3 (plasma)		1.9		6.1		3.0		2.7		2.6		3.4									
CES1	carboxylesterase 1 (monocyte/macrophage serine esterase 1)		1.6		7.6		5.2		7.4		3.1		2.5									
NAGA	N-acetylgalactosaminidase, alpha-		1.9		2.5		5.5		4.5		3.7		4.5									
SIK1	salt-inducible kinase 1		1.8		1.5		2.9		3.7		1.6		1.8									
MTMR4	myotubularin related protein 4		1.8		3.6		1.8		2.4		2.0		2.4									
Lipid metabolism																						
FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)		1.7		8.5		6.7		8.0		2.2		2.2									
LRPAP1	low density lipoprotein receptor-related protein associated protein 1		2.9		2.0		3.3		1.9		1.6		1.7									
PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)		1.6		15.8		7.1		13.5		30.1		2.0									
APOE	apolipoprotein E		1.5		11.8		6.6		19.7		3.3		1.8									
CLN8	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)		1.6		2.3		2.4		1.8		10.1		2.6									
IRS2	insulin receptor substrate 2		1.6		1.7		2.7		1.7		2.5		3.4									
ABHD12	abhydrolase domain containing 12		7.8		2.8		2.5		2.4		1.8		5.9									
Antigen processing and presentation																						
CTSS	cathepsin S		1.6		2.2		2.2		3.1		2.2		4.4									
CTSB	cathepsin B		1.9		5.2		7.0		4.7		1.7		1.5									
Cytokines and chemokines																						
IL1B	interleukin 1, beta		1.2		2.4		2.6		2.2		1.8		1.8									
CCL2	chemokine (C-C motif) ligand 2		1.6		7.0		1.8		18.0		26.5		2.7									
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)		2.7		10.8		3.4		13.3		11.6		4.1									
CXCL16	chemokine (C-X-C motif) ligand 16		3.4		1.5		3.3		7.9		2.8		1.8									
C-type lectins																						
CLEC4E	C-type lectin domain family 4, member E		23.2		100.0		6.9		3.8		6.0		22.5									
CLEC7A	C-type lectin domain family 7, member A		7.1		4.5		2.0		3.0		30.2		3.8									
Fc immunoreceptors																						
FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)		3.6		3.6		2.2		2.3		1.6		2.2									
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide		1.6		3.2		2.5		4.8		4.5		1.5									
Immunoglobulin domain containing																						
IGHA1	immunoglobulin heavy constant alpha 1		1.6		1.7		2.0		2.7		2.1		1.5									
IGHV	immunoglobulin heavy variable group		2.8		1.7		2.8		3.0		1.9		7.6									
CEACAM21	carcinoembryonic antigen-related cell adhesion molecule 21		1.9		5.9		2.0		2.5		4.6		10.7									
OSCAR	osteoclast associated, immunoglobulin-like receptor		2.0		8.0		3.2		12.5		1.6		4.7									
NFAM1	NFAT activating protein with ITAM motif 1		5.0		21.0		7.5		6.5		1.8		22.8									
Coagulation																						
PROS1	protein S (alpha)		1.8		17.5		5.6		2.5		2.1		1.9									
C1QB	complement component 1, q subcomponent, B chain		1.8		28.5		6.8		30.2		3.4		3.5									
Transcriptional regulation																						
CIITA	class II, major histocompatibility complex, transactivator		2.0		2.7		1.9		2.1		1.7		3.0									
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog		3.3		3.0		2.5		5.3		3.1		2.1									
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)		1.1		1.2		2.0		2.1		5.9		2.4									
Signal transduction																						
GPR183	G protein-coupled receptor 183		1.5		2.4		4.6		4.2		3.6		3.7									
RASGEF1B	RasGEF domain family, member 1B		3.0		2.4		2.9		3.0		2.0		4.2									
RGL1	ral guanine nucleotide dissociation stimulator-like 1		2.7		10.9		4.3		2.3		1.9		4.7									
GPNMB	glycoprotein (transmembrane) nmb		2.4		38.5		16.1		36.1		15.0		9.9									
TNS1	tensin 1		11.4		100.0		28.1		8.1		24.4		10.5									
Calcium binding																						
ANXA2	annexin A2		3.9		4.9		2.7		3.4		1.6		1.6									
ANXA2P3	annexin A2 pseudogene 3 (non-coding RNA)		15.5		28.2		5.2		4.3		3.2		2.0									
S100A8	S100 calcium binding protein A8		2.5		6.0		2.5		2.1		9.5		1.6									
Others																						
NDFIP1	Nedd4 family interacting protein 1		2.7		2.4		1.5		1.9		1.8		2.0									
CPNE8	copine VIII		2.8		2.7		2.2		1.7		1.8		2.6									
FTH1	ferritin, heavy polypeptide 1		1.6		5.2		3.1		3.3		2.4		1.9									
HC1	HC1 mRNA (DNA repair protein)		1.6		5.5		3.7		2.6		3.7		6.3									
KRT72	keratin 72		2.6		10.2		4.0		5.4		5.1		1.7									
LHFPL2	lipoma HMGIC fusion partner-like 2		2.0		29.0		3.9		5.9		6.8		5.1									
DFNA5	deafness, autosomal dominant 5		1.6		7.7		9.7		2.1		3.6		5.1									
TPCN1	two pore segment channel 1		3.2		1.9		2.6		1.8		1.8		1.5									
IFI27	interferon, alpha-inducible protein 27		1.7		3.2		2.1		3.1		1.3		1.8									
Unknown function																						
KIAA0146	Unknown		2.6		2.4		3.4		1.7		1.6		2.0									
C2orf61	chromosome 2 open reading frame 61		47.4		3.5		2.5		4.0		4.3		9.8									
BG057295	Unknown		4.9		5.5		3.5		4.5		1.5		3.7									
C16orf81	chromosome 16 open reading frame 81		3.9		3.2		2.4		2.2		2.7		14.7									
TMEM176A	transmembrane protein 176A		4.0		17.4		7.3		13.6		20.0		2.7									

<100
100-500
500-2000
2000-5000
>5000

Genes which were at least 1.7 fold up-regulated in at least two of the three biological replicates (with the third replicate showing the same trend) of both MLR/10 vs MLR and MLR/DC-10 and MLR/mDC were selected and analyzed for a statistically significant enrichment of biological pathway annotation terms. Mid columns: the color range reflects the absolute level of gene expression. Differential expression between IL-10- anergized and control cultures is represented in fold-change scale for each individual patient. Right hand columns: assignment of the differentially expressed genes to significantly enriched biological pathway annotation terms. Term enrichment relative to the expected background distribution was scored using Fisher's exact test.



Online Supplementary Figure S1. Effect of exogenous and endogenous TGF-β on IL-10-mediated inhibition of primary and secondary responses. A. PBMC were stimulated with allogeneic monocytes in the absence or presence of IL-10, of TGF-β, of anti-TGF-β, and of anti-IL10R monoclonal antibodies. Proliferative responses were evaluated after 4 days of culture by adding ^{3}H-thymidine for an additional 16 h. (B) PBMC were stimulated with allogeneic monocytes in the absence or presence of IL-10, of TGF-β, of anti-TGF-β, and of anti-IL10R for 10 days. At the end of culture, T cells were tested for their ability to respond to the same allogeneic monocytes used in the primary stimulation. Proliferative responses were evaluated after 48 h of culture by adding of ^{3}H-thymidine for an additional 16 h. Alternatively, T cells were tested for their ability to proliferate in response to *Candida albicans* (C) or to polyclonal stimulation (TPA+ionomycin) (D). Data represent the mean±SD cpm of triplicate replications of at least four experiments. Numbers indicate % inhibition of proliferation (A) and % of anergy (B), calculated as follows: 100-[(cpm of control MLR–cpm of anergized MLR)/MLR*100].