Rank ligand stimulation induces a partial but functional maturation of human monocyte-derived dendritic cells

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ABSTRACT. Mature dendritic cells (DC) are efficient, antigen-presenting cells required for the stimulation of naive T lymphocytes. Many members of the tumour necrosis factor (TNF) receptor family are involved in DC maturation, such as Fas, CD40, OX40L, LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes) or RANK (receptor activator of NFκB), with different, but often overlapping effects. We focused our attention on RANK DC stimulation, since RANK ligand (RL) is expressed on activated T lymphocytes with different kinetic and expression patterns from the other members of TNF family previously cited. After culture with RL-transfected cells, a significant percentage of immature DC generated from monocytes (Mo-DC) acquired a typical, mature DC morphology and phenotype characterised by up-regulation of CD83, DC-LAMP (lysosome-associated membrane glycoprotein), HLA class I, CD86 and CD54. The functional RL-mediated maturation was demonstrated by a decrease in DC macropinocytosis and acquisition of the capacity to stimulate allogenic T-cells. Among the various cytokines tested, we detected only a weak up-regulation of IL-12p40. Our results show that ligation of RANK on DC cell surfaces is not only a survival stimulus, but also induces a partial and specific mature DC phenotype, the physiological significance of which is under investigation.

Keywords: dendritic cell maturation, T cells, TNF receptors, rank ligand, cytokines

Dendritic cells (DC) play a crucial role in the initiation of the immune response, representing the most potent type of antigen- (Ag) presenting cell (APC) [1]. After antigen capture, DC migrate to the lymph nodes under the influence of inflammatory stimuli and receive a second stimulus that induces their maturation, *i.e*. the acquisition of efficient, antigen-presenting functions by HLA class I and II molecule upregulation and the capacity to deliver a second signal to T-lymphocytes *via* adhesion/costimulatory molecules (such as B7-1/B7-2) or cytokines (such as IL-12). Since this maturation step is required for the development of the immune response, the study of the various stimuli likely to drive this phenomenon is of pivotal importance. Various members of the TNF family are involved in DC maturation, such as TNFα, CD154 [1], Fas ligand [2] and LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes) [3]. These molecules have overlapping but not identical roles in DC maturation. For example, CD154 is expressed early and very transiently by activated $CD4^+$ T-lymphocytes, and induces a complete maturation of most DCs [1]. In contrast, LIGHT is expressed later, and for many days mainly by activated $CD8^+$ T-lymphocytes [4], induces maturation of only a small number of DC, and retains, as its major particularity, the capacity to enhance CD154-dependent IL-12 secretion [3]. Recent data have focused our attention on the putative role of another member of the TNF family, the receptor activator of NFκB (RANK)-ligand (RL) molecule, in DC physiology [5].

RL [6] is a member of the TNF superfamily that has two receptors: RANK and a "decoy" receptor, osteoprotegerin (OPG) [7]. The functions of the RL/RANK/OPG system involves bone remodelling while, in the immune system, the RANK/RL interaction is involved in lymphoid organs ontogenesis [7, 8]. In contrast with the ubiquitous distribution of RANK in tissue, in the immune system, expression of RL is restricted to activated T lymphocytes, but unlike CD154 or LIGHT, its expression equally concerns $CD4^+$ and $CD8^+$ lymphocytes [9]. The presence of RANK-L at activated T-cell surface and of its ligand at DC surface [5], prompts the question of the role of RANK stimulation in DC physiology. In human $CD34^+$ progenitor-derived DC,

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stimulation by soluble RL (sRL) induces cell cluster formation and up-regulation of HLA class I molecules, while no significant increase in other pivotal immune molecules (HLA class II, CD80, CD86, ICAM-1 or LFA-3) is observed [5], [10]. Moreover, in immature DC, constitutive RANK-L/RANK interaction is responsible for DC longevity [11]. In murine bone marrow-derived DC, RL stimulation sustains DC survival *via* the induction of bcl-xl [10], and increases the proliferation of unsorted allogenic T-cell in a mixed lymphocyte/DC reaction [12]. As regards cytokine secretion, in mice, RL stimulation of DC induces the transcription of IL-1β, IL-1α, IL-6 and IL-15 genes, and up-regulates IL-12p40 transcription [9, 12, 13]. In contrast, in human Mo-DC and using trimeric sRL, recent work failed to detect IL-12 transcription or secretion [14]. All these studies suggest a still-to-be-defined role for RL stimulation in DC physiology. In fact, additional data are required in man since; 1) many studies have been performed in murine models; 2) the model of human Mo-DC may more directly reflect clinical immunotherapy protocols in man [15]; 3) the putative capacity of RL stimulation to induce a mature DC phenotype has to be evaluated using the "gold standard" test of DC maturation, *i.e.* their capacity to stimulate naive T lymphocyte proliferation; 4) finally, experiments have been performed with soluble protein, while RL is a cell surface molecule. All these reasons prompted us to test the effects of RL stimulation on human Mo-DC using RL-transfected cells.

DONORS AND METHODS

Blood samples and cell separation

Peripheral blood mononucleated cells (PBMC) from healthy donors were isolated on Ficoll-Hypaque gradients. T lymphocytes were positively selected by E-rosetting with sheep erythrocytes [16]. Monocytes were isolated by CD14-positive sorting using magnetic beads (Miltenyi-Biotec, Germany). Naive $CD4^+$ T cells were prepared from purified (E-rosetting) T cells by two rounds of negative depletion using magnetic beads (Beckman Coulter, Marseille, France) and incubated with mAbs to CD8 (8E1.7, kind gift of Pr PC Beverley, London, UK) and CD45RO (UCHL1, Olive D, INSERM U119, Marseille, France). Recovery of $CD83^+$ and $CD83^-$ -purified DC was performed by direct sorting by flow cytometry (Facs-Vantage, Becton, Dickinson), with the exclusion of cells with a very low CD83 expression that were not included in the $CD83^+$ or in the $CD83^-$ pools. In all cases, purity of the preparation was measured by flow cytometry re-analysis of sorted cells (anti-CD3, anti-CD14, anti-CD45RA or anti-CD83 staining) and was found to be ≥ 98%.

CD154 and RL transfected cell lines

Full length cDNA of human CD154 was cloned in pcDNA3.1/Neomycin (Invitrogen, Groningen, The Netherlands), and transfected by electroporation (960 µF, 220V) into LTK-murine fibroblasts. Full length cDNA of human RL was cloned in pcDNA (SmithKline Beecham, King of Prussia, CA, USA), and co-transfected with empty pcDNA3.1/hygromycin as describe above. Stably transfected cells, selected by resistance to Geneticin (Life Technology, Paisley, Scotland) or Hygromycin B (Invitrogen), were then selected for expression by three round of FACS Vantage sorting. CD32 transfected fibroblasts were a kind gift from Schering-Plough (Dardilly, France). All transfected cells expressed the molecule of interest, with a median MFI of 113 (range 93-195) for CD154 and a median MFI of 146 (range 89-249) for RL (using the 2A4 mAb).

Culture conditions and dendritic cell (DC) generation

Purified monocytes were cultured in RPMI 1640 (Biowhittaker, Verviers, Belgium) 10% FCS with GM-CSF (Novartis, Geneva, Switzerland) at 100 ng/mL and IL-4 (kind gift from Schering-Plough) at 10 ng/mL for five days. At day 5, final maturation was induced by the addition of irradiated (75 Gy) LTK cells with a LTK:DC ratio of 1:10.

Flow-cytometry studies

Cells were processed following standard procedures and analysis was performed on a FACSCalibur flow cytometer (Becton, Dickinson, Immunocytometry Systems, CA, USA). The mAbs to CD1a, CD3, CD4, CD8, CD14, CD19, CD25, CD40, CD54, CD56, CD83, HLA ABC and HLA DR were purchased from Beckman Coulter (Marseille, France). The mAb against CD80 was from Becton, Dickinson, and the mAbs against CD86 and CD154 were from Pharmingen (Heidelberg, Germany). The mAbs directed against RL (2A4, murine IgG2a) and OPG (8B4, murine IgG1) were generated at SmithKline Beecham.

Primary MLR

Serial dilutions of irradiated (25 Gys) stimulator cells were cultured in triplicate with 5×10^4 allogenic naive $CD4^+$ T. Proliferation of T cells was monitored by measuring methyl-[^{3}H]thymidine (1 µCi/well; Amersham Biosciences, Freiburg, Germany) with incorporation during the last 16 hours of a six-day culture. The thymidine uptake was assessed on a gas-phase beta counter (Matrix 9600, Packard Instrument, Meriden, CT, USA).

Cytokine determination

After 48 h of final maturation, cell-free supernatants of DC cultures were frozen. After thawing, cytokine concentrations were quantified by ELISA: IL-12p70, (Pharmingen, OPTEia kit, sensitivity 5pg/mL), IL-12p40 (sensitivity < 15 pg/mL), IL-1β (sensitivity 0.4 pg/mL) and IL-6 (sensitivity 3 pg/mL), TNFα (sensitivity 5 pg/mL) (R&D systems, Minneapolis, MN, USA).

Macropinocytosis assay

For flow cytometry experiments, 10^5 immature or mature DC were incubated for 1h at 37°C or 4°C (as negative control) in culture medium containing FITC-Dextran (molecular weight 400 000 daltons, 5 mg/mL, Sigma, Saint Quentin Fallavier, France). After staining with DC markers, cells were washed four times in cold PBS and analysed. In confocal microscopy experiments, cells were deposited on coverslips at a concentration of 5.10^5/mL in

serum-free medium with FITC-Dextran (0.5 mg/mL), and incubated for one hour at 37°C, fixed in 3% paraformaldehyde (Fluka, Saint Quentin Fallavier, France) and permeabilized with 0.1% TritonX100 (Sigma) for one minute. Then cells were indirectly stained with mouse anti-DC LAMP at 1 µg/ml (104G4, Immunotech, Marseille), followed by TRITC-GAM at 1/500 dilution (Molecular Probes, Oregon, USA). Serial optical sections were obtained using the TCS 4D laser scanning confocal microscope (Leica, Heidelberg, Germany). Microscope settings were adjusted in order to obtain black level values when cells were stained with the mouse isotypic Ig control.

Nucleic acid preparation, RT-PCR amplification

Briefly, total RNA was isolated from 0.5 to 5×10^6 cells for each sample that was suspended in Trizol (GIBCO-BRL, France), and extracted by phenol-chloroform, as recommended by the manufacturer. Total RNA (2.5 µg) was reverse-transcribed using Moloney murine leukemia virus superscript reverse transcriptase and random hexamers according to the manufacturer's instructions (Life Technologies, USA). Each PCR reaction was performed using 1/20 of the cDNA prepared, in a total volume of 25 µL containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.5, 200 µM each of dNTP, 1 pmol/µl of primers and 1.25 U of Taq polymerase (Perkin-Elmer, Courtaboeuf, France). The amplification was performed in a Touchdown Temperature Cycling System thermal cycler (Hybaid, Teddington, UK). The standard procedure was; first cycle 94°C for three mins, then annealing at 65°C (except mentioned below) for 30 s, extension at 72°C for 30 s, and then denaturation at 94°C for 30 s terminating with 10 min at 72°C. Modification of incubation times and temperatures was performed when necessary in order to optimise PCR conditions depending on the various primers used (data available on request). For semi-quantitative RT-PCR, a variable number of PCR rounds were performed; 18, 20 and 22 cycles for β-actin, 28, 32 and 35 cycles for IL-12p40. Then, comparative evaluation of the amounts of transcript was performed by gel analysis using the Bio-Imaging Analyzer MacBAS V2.5 (Fuji Photo Film Co, Ltd, Koshin Graphic Systems Inc, Tokyo, Japan).

Primers

The detection of the housekeeping gene β-actin was used to assess RT and PCR efficiency, with the following primers; sense (S) 5′- GGC ATC GTG ATG GAC TCC G-3′ and the antisense (AS) 5′- GCT GGA AGG TGG ACA GCG A -3′ (22 cycles). The following primers were used for IL-1β detection; S 5′- GGA TAT GGA GCA ACA AGT GG -3′ and AS 5′- ATG TAC CAG TTG GGG AAC TG 3′. For IL-2 detection: S 5'- GTC ACA AAC AGT GCA CCT AC -3' and AS 5'- ATG GTT GCT GTC TCA TCA GC -3'. IL-4 detection: S 5'- TGC CTC CAA GAA CAC AAC TG -3' and AS 5'- AAC GTA CTC TGG TTG GCT TC -3'. IL-6 detection: S 5' - TCA ATG AGG AGA CTT GCC TG –3' and AS 5'- GAT GAG TTG TCA TGT CCT GC –3'. IL-10 detection: S 5'- ATG CTT CGA GAT CTC CGA GA –3' and AS 5'- AAA TCG ATG ACA GCG CCG TA –3'. IL-12p40 detection: S 5'- ATT GAG GTC ATG GTG GAT GC –3' and AS 5'- AAT GCT GGC ATT TTT GCG GC –3'.

Statistical analysis

Statistical analysis was performed using the SPSS software [17]. The Kolmogorov-Smirnov test was used to determine if the data fitted a normal distribution. As this test rejected the assumption of normality for all variables, comparisons were made using the non-parametric, Wilcoxon's matched-pairs signed-rank test.

RESULTS

Stimulation of immature Mo-DC with RL-expressing transfectant cells induces cluster formation and maturation-related morphological changes

As seen in *figure 1*, immature i Mo-DC co-cultured with RL-expressing LTK cells, formed clusters (first column, third row), but not when co-incubated with CD32-expressing transfectants (first column, first row). With RL-expressing LTK cells, the number and size of clusters were lower than in CD154-stimulated Mo-DC (first column, second row). From the morphological point of view (second column), the incubation of iMo-DC with CD32-expressing transfectants did not induce major modifications with monomorph, small round cells (first row). In contrast, RL-stimulated Mo-DC showed a larger size, with the acquisition of dendrites (third row), and were quite similar to CD154-stimulated Mo-DC (second row).

RL stimulation of Mo-DC induces a partially mature phenotype

In order to achieve more insight in the morphological data we observed, the Mo-DC surface phenotype was analysed

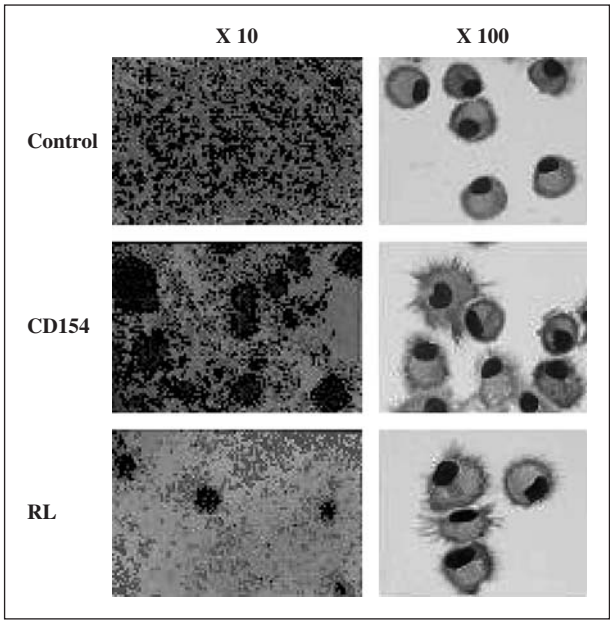

Figure 1
Stimulation of Mo-DC by RL-transfected cells induces cluster formation and maturation-related morphological changes. The iMo-DC generated as described above were incubated for 72 hours with the various transfectants (ratio of 1 stimulator: 10 DC). Photomicrographs were taken on day 8 of culture with a resolution of × 10 (left column) or × 100 (right column, interferential contrast), and are representative of seven experiments.

by flow cytometry after a 72-hour incubation with transfectant cells at a 1:10 ratio of iMo-DCs *(figure 2A)*. A significantly higher percentage of RL-stimulated DC (third column) acquired the expression of the CD83 maturation marker (first row) in comparison with iDC stimulated by CD32-expressing transfectants ($p < 0.05$, paired sample test, seven independent experiments with different healthy donors, see *table 1*). Increased HLA-DR expression (second row) was observed following RL stimulation, but this did not reach statistical significance. In comparison with the CD32-transfected control cells, RL-stimulated DC significantly up-regulated CD86 (third row, $p < 0.05$, see *table 1*), HLA class I molecules (fourth row, $p < 0.05$), CD54/ICAM-1 (fifth row, $p < 0.05$) and CD40 (sixth row, $p < 0.05$). The increased CD54/ICAM-1 expression observed following RL stimulation provides a direct explanation for the DC clustering we observed *(figure 1)*. We found no significant changes in CD58/LFA-3 or CD80 expression (data not shown). As positive control for our maturation conditions, we performed CD154 stimulation of iDC. In comparison with RL stimulation, a significantly higher percentage of DC stimulated by CD154, acquired a mature phenotype as demonstrated by the expression of the CD83 molecule ($p < 0.05$), HLA class I and II molecules ($p < 0.05$), CD86 ($p < 0.05$) and CD40 ($p < 0.05$).

The specificity of the RL stimulation was verified by incubating RL-stimulated DC with the 2A4 anti-RL blocking mAb. As shown in *figure 2*B, anti-RL blocking (third row) completely inhibited the phenotypic changes observed in the RL condition (second row), and induced a phenotype that was identical to the DC stimulated by CD32-expressing transfectants (first row). In addition, the 2A4 mAb did not interfere with CD154-induced Mo-DC maturation (data not shown).

RL stimulation down-regulates pinocytic activity in a subpopulation of Mo-DC

DC lose their macropinocytosis capacity when the maturation process occurs, and, in turn, acquire potent Ag-presenting capacity. We tested the macropinocytosis capacity of Mo-DC by measuring the cellular entrance of FITC-coupled dextran in flow cytometry *(figure 3A)* and confocal microscopy *(figure 3B)* experiments. Most iMo-DC (CD32 stimulated, first column of panel A and B) were positive for dextran uptake (X axis in panel A and green fluorescence in panel B), did not express CD83 (Y axis, first row of panel A) or the other maturation marker DC-LAMP (panel B, red fluorescence), and weakly expressed CD86 (Y axis, second row of panel A). Most mature Mo-DC (CD154-stimulated, second column of panel A and B) no longer incorporated dextran, and expressed CD83, and high-levels of CD86/B7-2 and DC-LAMP. Stimulation of Mo-DC by RL induced two cellular populations. The immature one was still able to capture dextran and did not express CD83 (panel A) or DC-LAMP (panel B), while the mature one was negative for dextran incorporation, and positive for CD83 (panel A) and DC-LAMP expression (panel B). Concerning the DC population that was negative for dextran incorporation, a small proportion of cells did not express CD83 or CD86. Nonetheless, the percentage of dextran-negative/CD83 negative

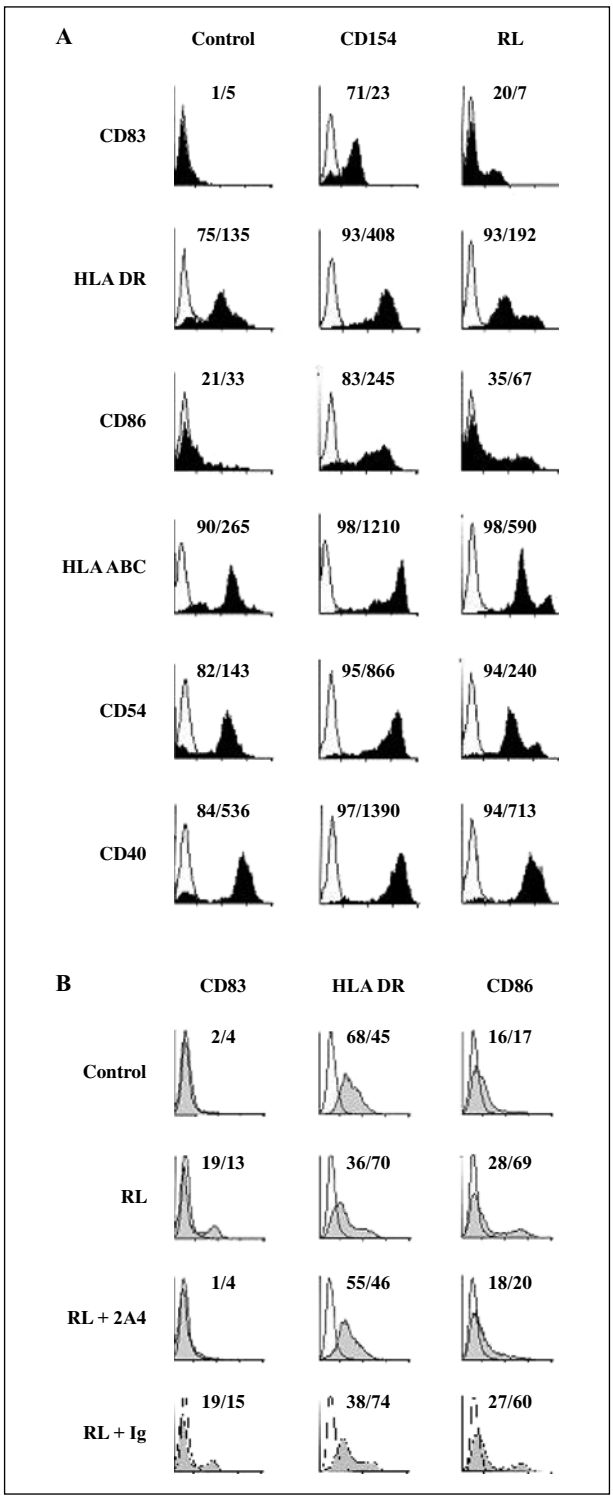

Figure 2

A) RL induces maturation of Mo-DC assessed as changes in surface phenotype. Final maturation of iMo-DC, cultured as previously described in *figure 1*, was induced by incubation for 72 hours with $CD32^+$ (negative control), $CD154^+$ or RL^+ tranfectants. Cells were analysed by flow cytometry using PE-conjugated mAb. Values represent the percentage of positive cells (left), and mean fluorescence intensity (right) subtracted from the value of matched isotype control mAb (open histogram). These data correspond to one representative experiment from seven performed using different, healthy, blood donors. **B**), RL-induced Mo-DC maturation is inhibited by anti-RL blocking mAb. Final maturation of Mo-DC was induced by control or RL-expressing transfectants in the presence of isotype control mAb or anti-RL (2A4) blocking mAb for 72 hours. The mAbs were used at 50 µg/mL. These data correspond to one representative experiment of three performed using different, healthy, blood donors.

Table 1
Data from seven independent experiments using different, healthy donors (adapted from Schiano de Colella *et al.*)

	CD83		CD86	
	% (median/range)	MFI (median/range)	% (median/range)	MFI (median/range)
Control	2(0-13)	3 (3-9)	25 (17-41)	30 (15-47)
CD154	76 (52-94)	23 (10-53)	87 (63-95)	256 (111-332)
RL	20 (4-55)	9 (5-29)	45 (20-61)	88 (43-257)

or CD86-negative DC was not statistically different between the four culture conditions tested (data not shown). Finally, no statistically significant difference in the immature/mature DC ratio was observed whatever the technique used, *i.e.* flow cytometry *versus* confocal microscopy (data not shown).

RL enhances DC-mediated alloreactivity of naive T cells

A major aspect of the functional characterisation of mature DC is their ability to stimulate naive T cell proliferation [1]. Thus, we investigated the Ag-presenting capacity in a primary allogeneic MLR *(figure 4)*. RL-matured Mo-DC showed an equivalent capacity to activate T-cells compared with CD154-matured Mo-DC, which was significantly higher ($p < 0.05$, for all numbers of stimulating cells, Wilcoxon's matched-pairs test) than iMo-DC obtained by co-culture with CD32-expressing transfectants *(figure 4A)*. As another marker of T-cell activation in MLR, we tested the gamma interferon (γ-IFN) secretion of Mo-DC-stimulated naive T-cells. Immature ($CD83^-$) Mo-DCs failed to induce any significant γ-IFN secretion by naive

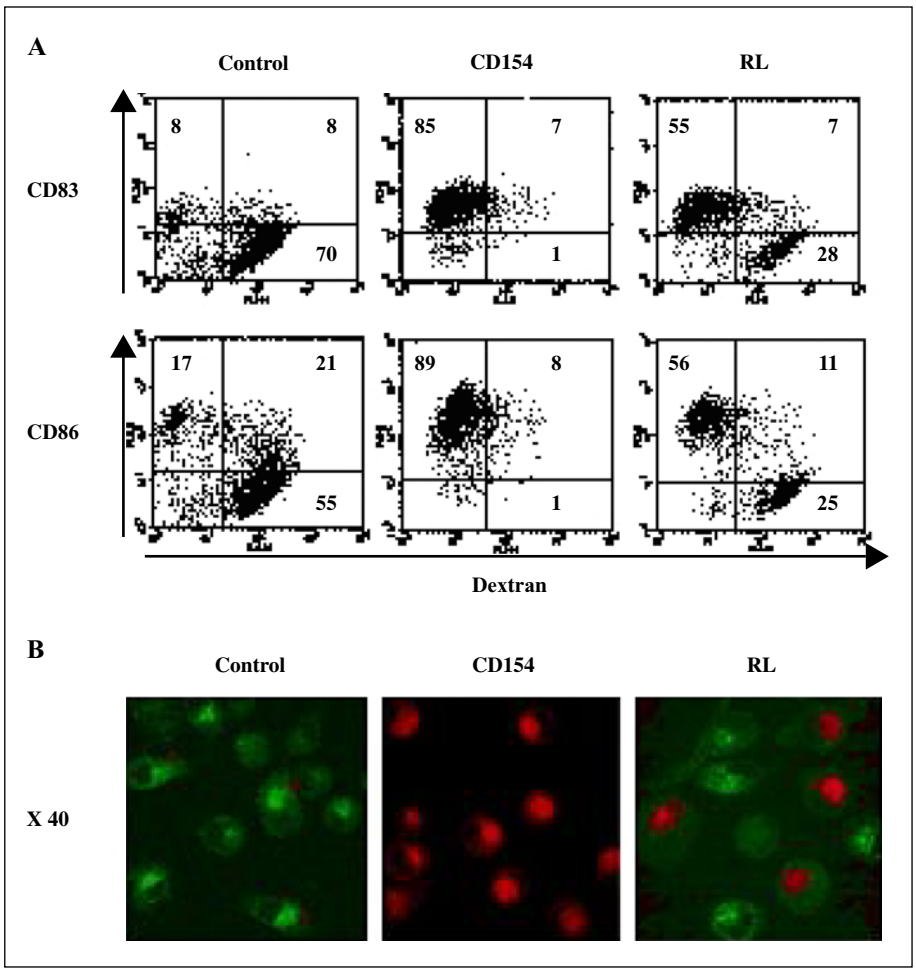

Figure 3
A) RL reduces the macropinocytic activity of DC. On day 8, iMo-DC (co-cultured with $CD32^+$ transfectants), CD154- or RL-matured MoDC were incubated at 37°C or 4°C (negative control) for 1 h in the presence of FITC-dextran (1 mg/mL). Then, Mo-DC were stained with CD83 or CD86 mAbs, fixed in 2% formaldehyde and immediately analysed by flow cytometry. These data correspond to one representative experiment of three performed using different, healthy, blood donors. **B**) RL stimulation induces the reciprocal down-regulation of dextran uptake, and up-regulation of DC-LAMP. The Mo-DC cultured as mentioned above, were plated on coverslips coated with polylysin (to enhance adhesion), and incubated with FITC-dextran. Then, cells were washed, fixed in paraformaldehyde, permeabilised with 0.1% triton and marked with DC-LAMP plus GAM TRITC. Serial optical sections were obtained using the TCs4D laser scanning confocal microspcope (Leica). Microscope settings were adjusted to black level values when cells were stained with the isotypic controls. These data correspond to one representative experiment of three performed using different, healthy, blood donors.

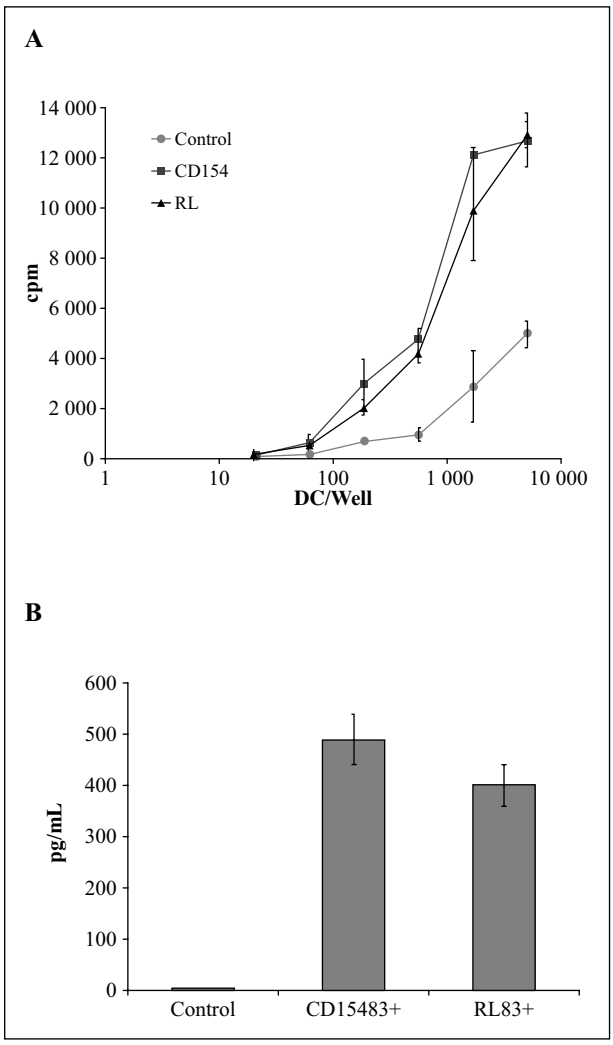

Figure 4

A) RL enhances the capacity of Mo-DC to activate allogeneic naive T cells. A total of 10^5 purified, peripheral, naive T cells ($CD4^+CD45RA^+CD45RO^-$) were stimulated by serial dilutions (20/well to 5×10^3/well) of irradiated (25 Gy) iMo-DC, CD154- or RL-stimulated Mo-DC on day 8. The proliferative response was measured by [^{3}H]thymidine incorporation during the last 16 h of a six-day culture. Background T cell proliferation was <100 cpm. Results are expressed as mean cpm ± SD and are representative of seven independent experiments. Data corresponding to identical stimulating cell numbers were compared using Wilcoxon's non-parametric, matched-pair test. **B**) RL-stimulated mature Mo-DC enhance IFNγ secretion by allogeneic naive T cells. iMo-DC (control) or $CD83^+$ (separated by Facs-Vantage sorting) Mo-DC matured by CD154 or RL stimulation were incubated (5×10^3/well) in a final volume of 200µl with 5×10^4 allogeneic naive T cells. After two days of culture, supernatants were frozen and analysed for IFNγ secretion by an ELISA assay. Data are from one representative of three experiments performed using different, healthy donors, results are expressed as mean pg/mL ± SD of duplicates. Data were compared using Wilcoxon's non-parametric, matched-pair test.

T-cells, while CD154 or RL-matured $CD83^+$ Mo-DCs had statistically equivalent capacities to induce γ-IFN secretion *(figure 4B)*.

RL stimulation of DC is a weak inducer of cytokine secretion

CD154-matured Mo-DC secrete various cytokines, some of which, such as IL12, contribute to T stimulation. The evaluation by semi-quantitative RT-PCR of IL-1β, IL-2, IL-10, IL-4, IL-6, and IL-3 gene transcription in RL-stimulated Mo-DC did not show any significant induction in comparison with iMo-DC (data not shown). Using a specific ELISA assay, we verified the absence of secretion of IL-1β, IL-6, IFNγ and TNFα after a 72-hour incubation with RL-expressing transfectants (data not shown). We detected an up-regulation of a IL12p40 transcript in semi-quantitative RT-PCR, since the ratio [R = IL-12p40/β-actin] was higher for RL stimulation compared to the negative control CD32 stimulation (respectively $R = 0.81$ *versus* $R = 0.16$, $p < 0.05$), while a higher induction was measured for CD40L stimulation in comparison with RL ($R = 1.3$ *versus* $R = 0.81$, $p < 0.05$) *(figure 5A)*. Using a specific ELISA test, we measured IL-12p40 secretion (*figure 5B*, left figure). Although IL-12p40 secretion was significantly higher in RL-stimulated $CD83^-$ or $CD83^+$ Mo-DC in comparison with the negative control CD32-stimulated cells ($p < 0.01$), it remained weak and significantly lower ($p < 0.01$) than in CD154-stimulated $CD83^-$ or $CD83^+$ Mo-DC. No difference in IL12p70 secretion was observed between CD32- and RL-stimulated Mo-DC, in contrast with the potent increase in IL12p70 secretion in CD154-matured Mo-DC (*figure 5B*, right figure).

DISCUSSION

In this study, we demonstrate that, in man, RL stimulation with its membrane form induces not only DC survival [5], but also maturation, as proved by both descriptive (cell morphology, CD83 or DC-LAMP expression) and functional (loss of endocytosis properties, naive T-lymphocyte-stimulating capacities) changes. In line with the expression of RANK that is restricted to 20-30% of DC [5], only a fraction of DC acquired a mature phenotype following RL stimulation, in contrast with CD40L that induces maturation of nearly all DC. In a mixed lymphocyte reaction (MLR), using naive T-cells, RANK-L mediated DC stimulation induced the same APC properties as CD40L, despite the lower efficiency at inducing mature DC phenotype ($CD83^+$/DC-$LAMP^+$/HLA-DR_{high}/$B7_{high}$). This demonstrates that true APC capacities are not limited to this *bona fide* DC population and extends the previous observation of a B7-independent T-cell priming in $CD34^+$-derived DC [5]. In order to more precisely define the basis of RL-induced APC capacities, we focused our attention on cytokine secretion. CD154-matured DC secrete many cytokines that are central for the immune response such as IL1-β, IL-2, IL-6, IL-10, TNFα and IL-12 [1, 3]. We failed to demonstrate any induction of these cytokines at RNA or protein levels. The exception was IL-12, since the IL-12p40 subunit was up-regulated at both mRNA and protein levels, although only slightly, while IL-12p35 was not up-regulated in comparison with negative control. Although mRNA transcription of the two subunits is often closely coordinated, this discrepancy in IL-12 subunit regulation has already been observed in DC, since the terminal maturation of murine bone-marrow DC by CD40L induces IL-12p40, but not IL-12p35 mRNA up-regulation [18]. In addition, using trimeric sRL, Yu *et al*. [14] observed an up-regulation of 12p40 mRNA, while the IL-12p35 mRNA level was not affected. An additional origin of this heterogeneity in IL-12 secretion relies on DC origin, since the stimulation by RL of splenic

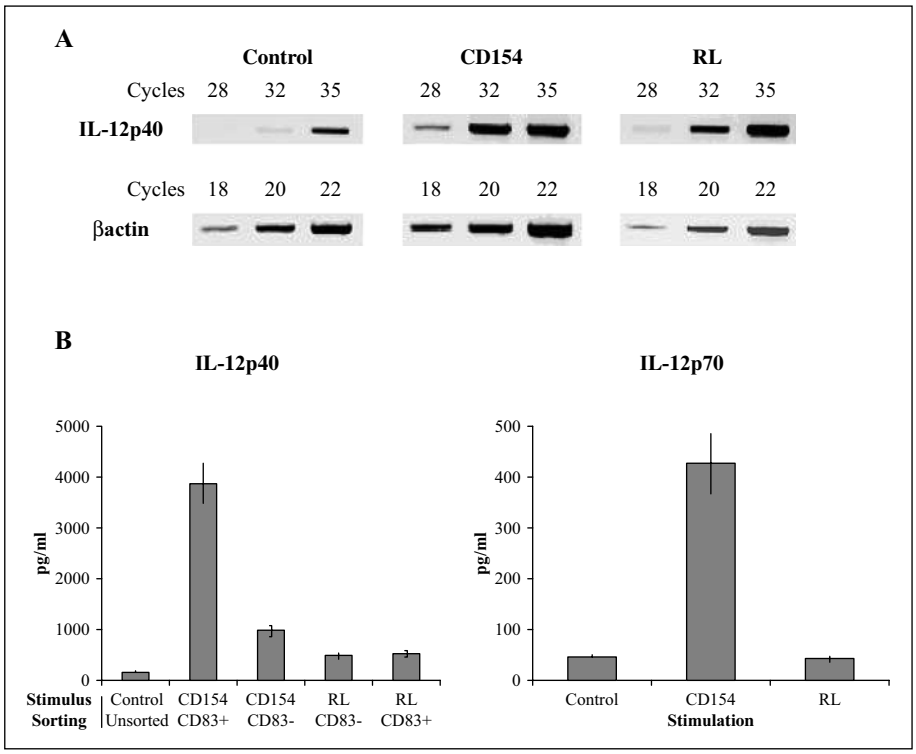

Figure 5

A) RL stimulation of Mo-DC increases transcription of the IL-12p40 gene. Mo-DC were triggered by the various stimuli as previously described. At day 8, cells were analysed for IL-12p40 specific transcript by RT-PCR. We used variable numbers of cycles to compare specific signals to control β-actin in non-saturating conditions, and the modification of transcripts were evaluated by checking the IL-12p40 transcript/β-actin ratio, under non-saturating conditions for both signals (20 cycles for β-actin, 32 cycles for IL-12p40). **B**) RL stimulation of Mo-DC weakly induces IL-12p40, but not IL-12p70, secretion. The iMoDC were stimulated for 72 h by CD32 (control), CD154- or RL-expressing transfectants. Then, the $CD83^+$ and $CD83^-$ subpopulations were separated by Facs-Vantage sorting and cultured for an additional two days. Supernatants were then harvested and tested by ELISA for IL-12p40 and IL-12p70. Results are expressed as pg/mL (corresponding to 5×10^5 MoDC/mL). Data are from one representative of three experiments performed using different, healthy donors. Results are expressed as the mean pg/mL ± SD of duplicates.

DC, but not of mucosal or peripheral node DC, induces IL-12p40 mRNA expression [19]. The IL-12p40 has been recently shown to engage a p19 protein to form a novel cytokine, IL-23, that stimulates IFNγ production and proliferation in PHA blast T cells [20]. The T cell proliferation and IFNγ secretion we observed in MLR following RL-mediated DC stimulation could be related to IL-23. Although IL-23 stimulates memory T lymphocytes rather than naïve cells [20], we used cell-sorted T-cells from adult blood in our MLR, instead of cord-blood T cells (which are truly naïve lymphocytes), so that IL-23 might have stimulated a memory T-lymphocyte activity.

Among the pivotal TNF superfamily members involved in DC maturation, three molecules are expressed by activated T lymphocytes although with quite different patterns. CD154 is expressed on $CD4^+$ T lymphocyte upon stimulation; its expression is quite rapid and transient (a few hours). LIGHT expression is predominantly found in $CD8^+$ T-cells, is more delayed after stimulation and lasts for days. In contrast with these two molecules, RL expression is detected on both $CD4^+$ and $CD8^+$ T lymphocytes, and remains stable for days [9]. While CD154 stimulation induces most DC to mature, either LIGHT or RL stimulation induces maturation of only a small fraction of DC. The most drastic effect of DC stimulation by LIGHT is co-stimulation of CD154-induced IL-12 secretion [3], but RL probably "prepares" DC for IL-12 release by intracellular accumulation. Finally, while LIGHT mainly acts as a co-stimulator, RL stimulation induces as potent APC functions as CD154, despite the lack of classical mature DC markers. Notably, increased RL expression has been extensively demonstrated in multiple myeloma patients (for a review, see Theill *et al.* [21]). Moreover, abnormal immunological functions have also been described in myeloma [22]. Abnormal RL expression by plasma cells or stroma could positively modulate antigenic presentation by APC and so participate in tumor escape.

In conclusion, RL-mediated DC stimulation has a role complementary to the two other TNF family members, CD40L and LIGHT, since the effects of these different molecules are distinct from each other, although they partially overlap. Co-ordinated engagement of all these TNF family members may act to optimise APC functions in DC and have important implications for the improvement of DC-mediated immunotherapy.

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