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 CD8+ 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,
stabilized 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 naïve 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, Becton, Dickinson), with the exclusion of cells 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/Neo (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/hygro (Invitrogen, Eugene, Oregon, 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 Gy) stimulator cells were cultured in triplicate with 5 × 10⁴ allogenic naive CD4+ T. Proliferation of T cells was monitored by measuring methyl-[³H]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), TNFa (sensitivity 5 pg/mL) (R&D systems, Minneapolis, MN, USA).

Macropinocytosis assay

For flow cytometry experiments, 10⁵ 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 analyzed. In confocal microscopy experiments, cells were deposited on coverslips at a concentration of 5.10⁵/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 × 10⁶ 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 MgCl₂, 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 GGC 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'- GTT GCT GTC GTC TCA TCA GC -3'. IL-4 detection; S 5'- TGC CTC CAA GAA CAC AAG TG -3' and AS 5'- AAT GTA TCC TGC TCG TGT TCT AC -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 CTT CGA GA -3' and AS 5'- AAA TCG ATG ACA GGC 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'.
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 2B, 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...
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

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**Table 1**

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

<table>
<thead>
<tr>
<th>CD83</th>
<th>CD86</th>
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<tr>
<td>% (median/range)</td>
<td>MFI (median/range)</td>
</tr>
<tr>
<td>Control</td>
<td>20 (1-13)</td>
</tr>
<tr>
<td>CD154</td>
<td>76 (5-94)</td>
</tr>
<tr>
<td>RL</td>
<td>20 (4-55)</td>
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**Figure 3**

A) RL reduces the macropinocytic activity of DC. On day 8, iMo-DC (co-cultured with CD32+ transfectants), CD154- or RL-matured Mo-DC 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 microscope (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.
RL stimulation of DC is a weak inducer of cytokine secretion

CD154-matured Mo-DC secrete various cytokines, some of which, such as IL-12, 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 \[ R = \frac{\text{IL}-12p40/\beta-actin} {\text{IL}-12p40/\beta-actin} \] 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 4A)). Using a specific ELISA test, we measured IL-12p40 secretion (figure 4B). 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 4B, 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, naïve 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 naïve 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-DRhigh/B7high). 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 IL-1β, IL-2, IL-6, IL-10, IL-4, IL-6, and TNFα, after a 72-hour incubation [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 T-cells, while CD154 or RL-matured CD83+ Mo-DCs had statistically equivalent capacities to induce γ-IFN secretion (figure 4B).
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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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, supernatants were then harvested and tested by ELISA for IL-12p40 and IL-12p70. Results are expressed as pg/mL (corresponding to 5 × 10⁵ 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.
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