Modulated rat dendritic cells in renal transplantation models : immune regulation and graft outcome

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CHAPTER 2

CD40L stimulation of rat dendritic cells specifically favors the IL-12/IL-10 ratio resulting in a strong T cell stimulatory capacity

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Abstract

Dendritic cells (DC) play an important role in immune responses and have been studied extensively in human and mouse models. CD40 triggering of DC has a pivotal role in their maturation process, obtaining the unique capacity to induce strong CD4 and CD8 T cell activation. Although rat models are frequently used for the understanding of the underlying mechanism of human diseases, relatively little is known about rat DC. To investigate the effect of CD40 triggering on rat DC, we cloned the rat CD40L gene and generated murine fibroblasts with stable expression (L-rCD40L). DC stimulated by L-rCD40L cells exhibited a strong T cell stimulatory capacity, associated with higher amounts of IFN-γ as compared to LPS-stimulated DC. Analysis of cytokine production showed that LPS induced both IL-12 and IL-10 production, whereas CD40L induced high amounts of IL-12, but little IL-10 production by rat DC. This implies that the difference found in T cell stimulatory capacity by the stimulated DC is due to the cytokine profile of the DC at the time of T cell activation.

Introduction

Dendritic cells (DC) are professional antigen presenting cells that play a key role in the initiation and regulation of immune responses. DC have the capacity to capture and process antigens during their immature state. Internalization of foreign antigen triggers maturation of DC and their migration to lymphoid organs. In the lymphoid organs, DC present the antigens to T cells and induce immune responses [1, 2]. Under steady state conditions, DC presenting self-antigens are capable of maintaining tolerance by either inducing regulatory T cells or deletion/anergy of autoreactive T cells [3, 4]. The capacity of DC to induce either an immunogenic or a tolerogenic response may provide therapeutic potential in the form of cellular therapy in various clinical settings. DC inducing immune responses can be a useful tool in cancer research, whereas DC preventing/suppressing immune responses can be an important tool in autoimmune or transplantation research [5-7].

The functional capacity of DC is strongly dependent on its activation/maturation status [8]. At least three different classes of DC activation can be distinguished, including inflammatory cytokines (like IL-1 or TNF-α), TLR ligands (like LPS) or interaction with activated T cells (like CD40-CD40L). Although several of the functional consequences are similar between these modes of activation, and especially LPS activation has been used as the prototypic way of DC maturation, it is also clear that there are major quantitative and qualitative differences. CD40 is broadly expressed on all professional antigen presenting cells as well as many other hematopoietic and non-hematopoietic cells [9]. Cross-linking of CD40 on DC results in strong activation, including high levels of IL-12 production [10, 11] and equips the activated DC with some unique features [12]. Contact with CD40L expressed on activated T cells will mostly take place in lymphoid organs, when part of the DC might have already received signals from TLR ligands and inflammatory cytokines in the periphery. However, under inflammatory conditions CD40L can also be expressed in peripheral organs [13]. The central role of CD40-CD40L in DC biology may contribute to the important consequences of either inhibiting CD40L in cases of unwanted immunity [14] or using agonistic CD40 reagents to promote immunity [15].

The important role of DC in normal homeostasis and disease pathogenesis has been demonstrated in several clinical situations and in many animal models, mostly of mouse origin. Although many experimental models of autoimmunity [16, 17] and transplantation [18, 19] are operational in rats, less information is available on rat DC. It has been demonstrated that GM-CSF is essential to obtain rat DC from bone marrow (BM) [20-22]. This cytokine has also been shown to be essential to obtain DC from human $CD14^+$ cells or mouse BM. Additional studies showed that the presence of IL-4 and Fms-like tyrosine kinase 3 ligand (Flt3L) increased the recovery of rat BM-derived DC [23-25]. It was shown that culturing BM in the presence of various combinations of GM-CSF, IL-4 or Flt3L resulted in different subtypes of DC [23-27].

In view of the central role of DC maturation in immune regulation and the pivotal role of CD40-CD40L in this process, we set out to study the response of rat DC upon activation by CD40L. To this end murine fibroblasts were transfected with a construct containing rat CD40L and the effect on rat DC was examined. Compared to LPS, CD40L was

able to induce a unique cytokine profile, which could explain the high T cell stimulatory capacity of the CD40L stimulated DC. These findings show that CD40L is a powerful stimulator of BM-derived DC.

Materials and Methods

Animals

Seven to twelve week-old male Brown Norway (BN RT1^n), Albino Oxford (AO RT1^u), Lewis (LEW RT1^l) or Dark Agouti (DA RT1^a) rats were purchased from Harlan (Horst, the Netherlands). Animals had free access to water and standard rat chow. Animal care and experimentation were performed in accordance with the local ethic committee of Leiden University Medical Center

Generation of rat CD40L expressing L-cells

To generate a cell line expressing functional rat CD40L, the gene was cloned from cDNA of rat splenocytes using a PCR based method. Briefly, primers were designed based on the published sequences of rat CD40L (GI 4102613), spanning the whole gene including start and stop codon and introducing an Xho1 and Not1 site at the 5'and 3' end respectively (table 1). This allowed directional cloning into the expression vector pME-hygro, as also used for the generation of human CD40L transfectants [28]. After amplification using a proofreading DNA polymerase, full length clones were obtained and used for sequencing. A clone showing 100% identity with the published sequence was used for transfections. Murine L-cell fibroblasts were transfected by electroporation using standard techniques and hygromycine was used for selection.

Generation of rat dendritic cells

DC were prepared as described by Grauer *et. al.* [23] with some modifications. In brief, BM was isolated from tibia and femur from BN, AO, DA or LEW rats by flushing the bones with medium. Red blood cells were lysed with lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$. The remaining BM cells were passed through a 80 µm sieve. Cells were cultured in 6 wells plates (Costar, Cambridge, MA) in 3 ml of RPMI+ medium containing RPMI 1640 (Invitrogen, Breda the Netherlands) 10% heat-inactivated FCS (BioWhittaker, Vervier, Belgium), penicillin/streptomycin (Gibco), Fungizone (Gibco), β-Mercapto-ethanol (50 µM, Merck, Darmstadt, Germany*)*, L-Glutamine (2mM, Gibco) in the presence of rat GM-CSF (2 ng/ml, Invitrogen), rat IL-4 (5 ng/ml, Invitrogen) and human Flt3L (50 ng/ml, kindly provided by Amgen), at a density of $1.5x10^6$ cells per well. At day 2 and 4 all medium was removed and replaced by fresh medium containing cytokines (see above). At day 7, non-adherent and semi-adherent cells were harvested and activated with LPS (500 ng/ml, Salmonella Typhosa, Sigma, St. Louis, MO, USA) or rat CD40L for 24 hours. CD40L-activation was performed with irradiated (80Gy) rat CD40L transfected L-cells (L-rCD40L) in a DC:L-cell ratio 2:1. Non-transfected L-cells (L-orient) were used as control cells. Cells were plated in 6 well plates as described above.

Generation of monocyte-derived human DC

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy blood donors by Ficoll density gradient centrifugation. Cells were positively selected by CD14 MACS microbeads (Miltenyi Biotech, GmBH Berisch Gladbach, Germany), and cultured in RPMI+ medium, human GM-CSF (5 ng/ml, Leucomax®, Novartis Pharma BV, Arnhem, the Netherlands) and human IL-4 (10 ng/ml, Peprotech, RockyHill, USA) at a density of $1.5x10^6$ cells/well in a 6 well plate (Costar).

Flow cytometric analysis

DC were stained with monoclonal antibodies against CD163 (ED2), αβ-TCR (R73), κ-light chain (HIS8), MHC class II (OX6) (all kindly provided by Dr. E. de Heer, LUMC, Leiden the Netherlands), CD40 (Santa Cruz Biotechnology, Heidelberg, Germany), CD80, CD86 (BD, Breda, the Netherlands), OX62 (Serotec, Dusseldorf, Germany) and CD11b/c (OX42, kindly provided by Dr. P. Kuppen, LUMC Leiden, the Netherlands) in Facs-buffer (1% BSA, 1% heat inactivated normal human serum and 0.02% sodium azide in PBS). Binding was visualized using phycoerythrin (PE)-conjugated goat anti-mouse Ig (Dako, Glostrum, Denmark) or goat anti-rabbit Ig (Southern Biotechnology, Birmingham, Alabama, USA).
L-cells were stained for CD40L expression by incubating human CD40-Ig (12 µg/ml) in Facs-buffer, followed by a mouse anti-human IgG (ATCC Hb43, 5 µg/ml American Type Culture Collection, Halifax, GA). Binding was visualized using PE-conjugated goat anti-mouse Ig (Dako, Glostrum, Denmark). The cells were assessed for fluorescence using a FACS Calibur (BD, Mountain View, CA). Data analysis was performed using WinMDI 2.8 software.

Cytokine analysis by ELISA

Cytokines were measured in supernatants of the activated cells. Rat IL-12p40 (Invitrogen), rat IL-10 (R&D Abingdon, UK), rat IFN-γ (BD, Breda, the Netherlands) and human IL-10 (Sanquin, Amsterdam, the Netherlands) were measured following the instructions provided by the supplier.

Allogeneic Mixed Lymphocyte Reaction

Splenocytes were derived from LEW spleen using a 70µm cell strainer (BD). For T cell isolation, splenocytes were incubated with anti-κ light chain (Clone HIS8) and anti-MHC class II (OX6) in a buffer containing 2% heat inactivated FCS and 2.5 mM EDTA. After incubation, cells were washed and incubated with BioMag® goat anti-mouse IgG particles (Polysciences Inc, Warrington, PA, USA). Cells binding to the beads were removed using a magnet (BD) and the negative population was used as responder cells. Stimulator cells were irradiated (40 Gy) and added in graded doses to $1x10^5$ allogeneic T cells in a 96-well U-bottom tissue culture plates (Costar) in RPMI+ medium in a final volume of 0.2 ml/well. Cell proliferation was quantified by incubating the cells during the last 16 hours of day 5 with 0.5 µCi (37kBq) of [methyl-^{3}H] thymidine (NEN™ Life Science Products, Inc., Boston, MA, USA). Results are presented as the mean cpm ±SD obtained from triplicate cultures.

Statistical analysis

Statistical significant differences were determined in Graphpad Prism® using the Mann-Whitney test. Differences were considered significant when $p<0.05$.

Results

Generation and phenotypic analysis of bone marrow derived rat dendritic cells

Rat bone marrow cells were cultured in the presence of GM-CSF and IL-4 with or without the addition of Flt3L. Under the conditions where Flt3L was added to the BM cultures, significantly higher numbers of viable cells were recovered at day 7 of culture (**Fig. 1A**), demonstrating the need of Flt3L to obtain high numbers of DC. Cell cultures were characterised with large clusters of cell aggregates as previously described [23] (**Fig. 1B**).

We studied the phenotype of the cells cultured with Flt3L at day 7 by FACS. The cells were found to have a characteristic immature BM-derived DC phenotype: expressing MHC class II and low levels of the co-stimulatory molecules CD80, CD86 and CD40 (**Fig. 2**). In addition the cells expressed NKR-P1A, CD11b/c and OX62, a marker known to be present on most, but not all, rat DC [29]. In contrast, the macrophage marker (CD163) and T cell markers such as CD4 and TCR were not detectable.

Generation of murine fibroblasts expressing rat CD40L

To be able to study the effect of CD40-mediated activation of rat DC, we utilized a system of transfected cells, as successfully used for human DC [30]. Initial experiments indicated that human CD40L is not efficient for the crosslinking of rat CD40 (data not shown). Therefore, full length rat CD40L was cloned into an expression vector (see materials and methods) and transfected into murine L-cell fibroblasts. After selection with hygromycin, stably transfected cells were selected, showing interaction with human CD40-Ig in a small percentage of transfected cells (5%). This population was enriched

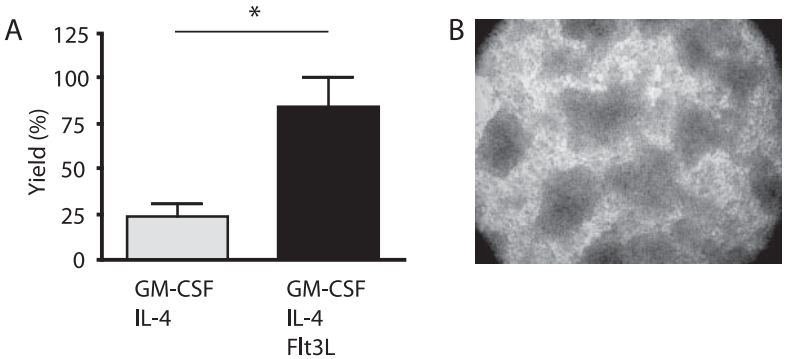

Figure 1. Generation of rat bone marrow derived dendritic cells.
A) Cell yield generated at day 7 of culture in the presence or absence of Flt3L. The input of bone marrow cells at day 0 was set on 100%. Results are the mean ± SD of 5 experiments ($p<0.05$). B) Cell aggregates present on day 5 of culture.

by cell sorting, resulting in a population with homogeneous and strong expression of CD40L (**Fig. 3A**). To confirm the rat origin of the CD40L molecule, we performed RT-PCR with species specific primers. Both in L-rCD40L (rat) and L-hCD40L (human), PCR products of the expected size were specifically amplified (**Fig. 3B**).

Stimulatory capacity of CD40L-DC is stronger than LPS-DC

To study the ability of the generated DC to stimulate allogeneic T cells, immature and LPS- or CD40L-matured DC were used as stimulators in a primary allogeneic mixed lymphocyte reaction (MLR). Increasing numbers of irradiated DC were added to $1x10^5$ allogeneic splenocytes. Both LPS-stimulated DC (LPS-DC) and CD40L-stimulated DC (CD40L-DC) induced a higher proliferation of the responder cells compared to immature DC (Fig. 4A). Remarkably, CD40L-DC appeared to induce a much stronger splenocyte proliferation, whereas LPS-DC and CD40L-DC induced similar amounts of IFN-γ by the responder cells (**Fig. 4B**). Additional experiments with CFSE labelled responder splenocytes showed that B cells were a major proliferating cell population when cultured together with CD40L-DC (data not shown).

Since CD40L is an important stimulator of B cell proliferation, carry over of L-CD40L used for DC maturation might be an explanation. Indeed direct activation of splenocytes with L-CD40L resulted in proliferation but no IFN-γ (**Fig. 5A**). Therefore we purified allogeneic T cells from spleen and used these as responder cells. Purified T cells did not respond directly to L-CD40L stimulation (**Fig. 5B**). In contrast, allogeneic T cells stimulated by CD40L-DC showed both proliferation and IFN-γ production.

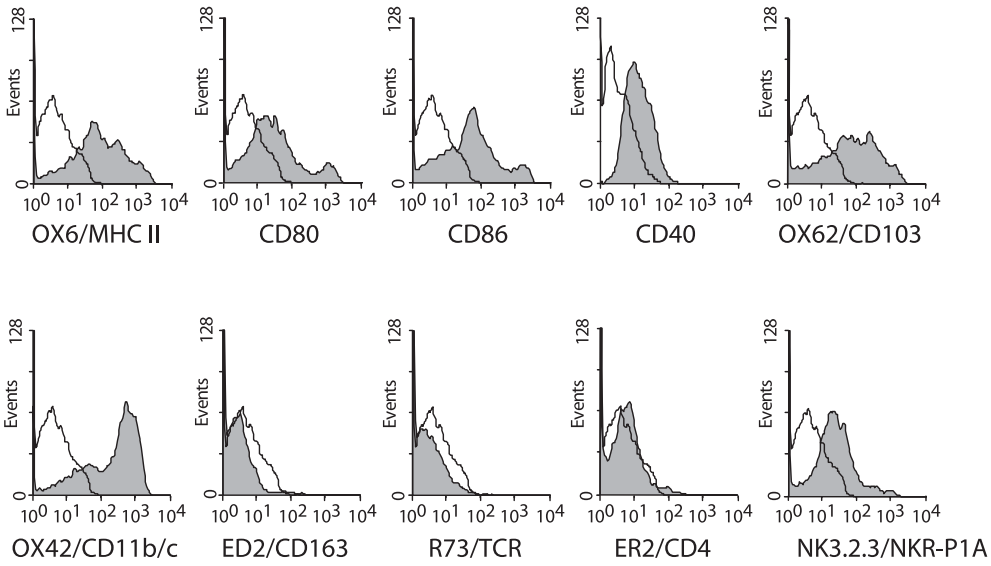

Figure 2. Phenotype of bone marrow derived dendritic cells.
Day 7 cells were stained with various monoclonal antibodies directed against surface molecules and analysed by flow cytometry. Specific staining is represented by filled histograms, open histograms represent basal fluorescence level after staining with only GaM-PE. Results shown are representative for 3-5 experiments.

Based on these findings we compared the stimulatory capacity of LPS-DC and CD40L-DC on purified responder T cells. Both LPS-DC and CD40L-DC induced increased proliferation compared to immature DC, showing highest proliferation on day 5 of culture, without significant difference between the mode of activation (**Fig. 6A**). Importantly, under these conditions where proliferative responses were similar, CD40L-DC induced a much stronger IFN-γ production by responder T cells (**Fig. 6B**).

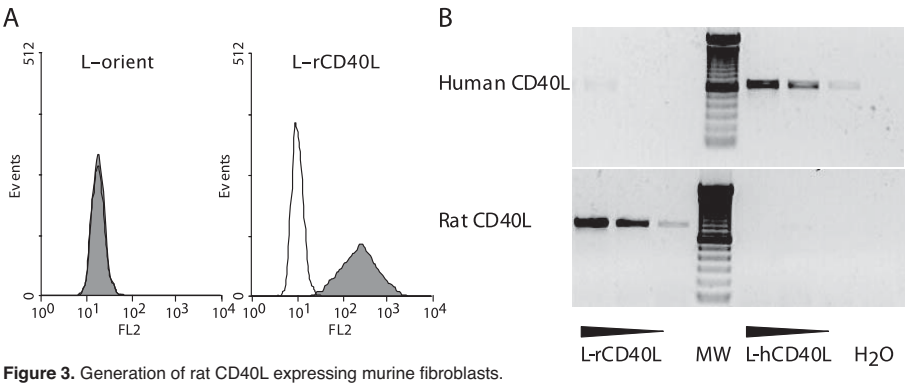

Figure 3. Generation of rat CD40L expressing murine fibroblasts.
A) Untransfected (L-orient) and stable transfected rat CD40L (L-rCD40L) cells were stained with a human CD40-Ig fusion protein and binding was visualized by flow cytometry. Specific staining is represented by filled histograms, open histograms represent basal fluorescence level after staining with only GaM-PE. B) Serial dilutions of cDNA from L- human CD40L or L- rat CD40L were amplified using specific human or rat CD40L primers. A 100 bp ladder was used to determine the sizes of the products.

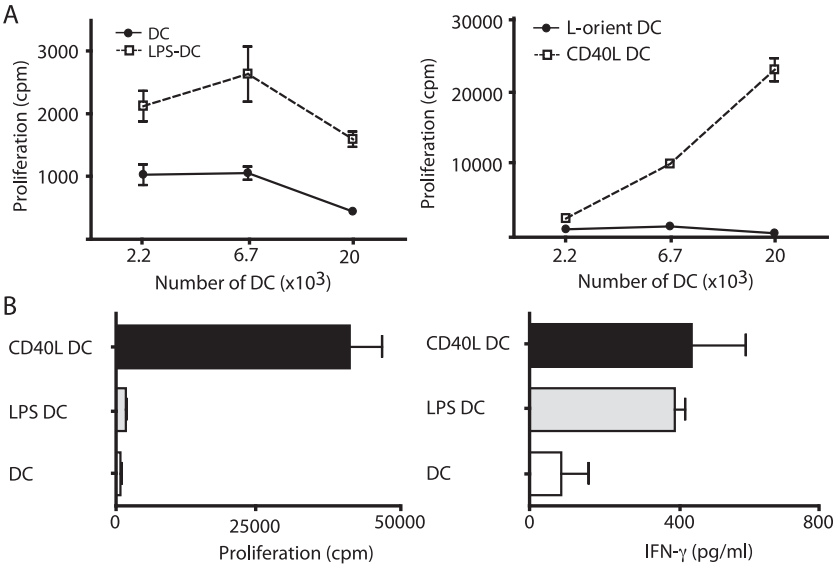

Figure 4. Stimulatory capacity of LPS or CD40L-activated DC of BN origin on allogeneic splenocytes isolated from LEW rats.
A) Splenocytes ($1x10^5$) were cultured with various numbers of irradiated (40 Gy) BN DC for 5 days. Solid line represents the immature DC, dotted line the stimulated DC showing the results as a mean ± SD of triplicate cultures. This figure is representative of 3 experiments. B) The bars represent $2x10^4$ immature DC (white), LPS stimulated DC (gray) or CD40L stimulated DC (black). Cells were pulsed at day 5 of culture with ^{3}H-thymidine during the last 16 hr and IFN-γ production was measured in the supernatant. Results shown are the mean ± SD of three independent experiments.

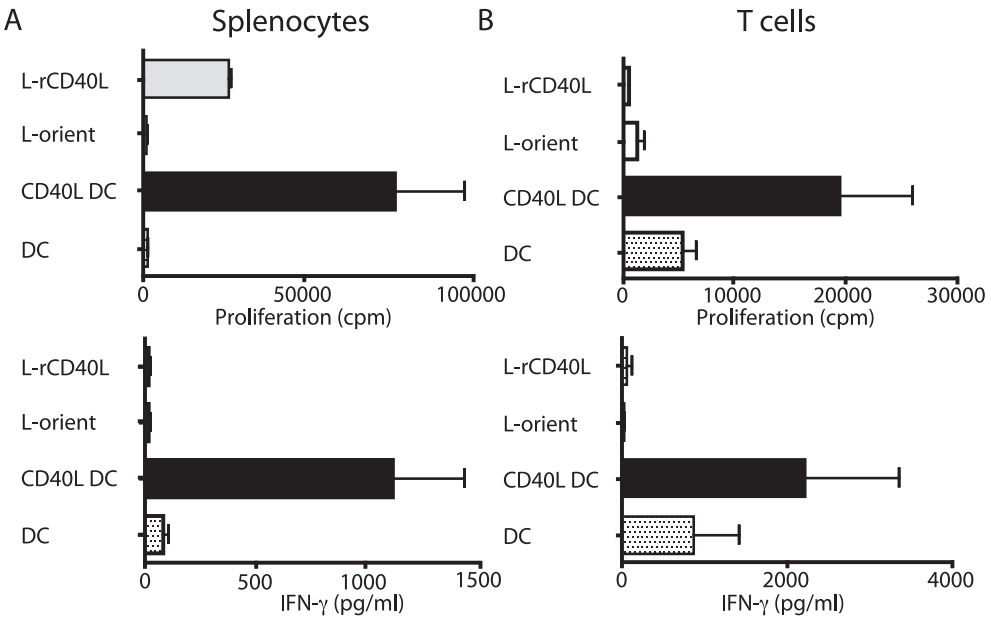

Figure 5. Comparison of the allogeneic response of splenocytes and purified T cells.
Allogeneic splenocytes (A) or T cells (B) were purified from LEW spleen, and $1x10^5$ cells were cultured in the presence of $2x10^4$ DC of BN origin, showing immature DC (dotted bar), CD40L-DC (black bar), $0.5x10^4$ L-orient (white bar) or L-rCD40L cells (gray bar) for 5 days. Cells were pulsed with ^{3}H-thymidine for the last 16 hrs and harvested. IFN-γ production was measured in the supernatant. Results are the mean ± SD of triplicate cultures, representative of three independent experiments.

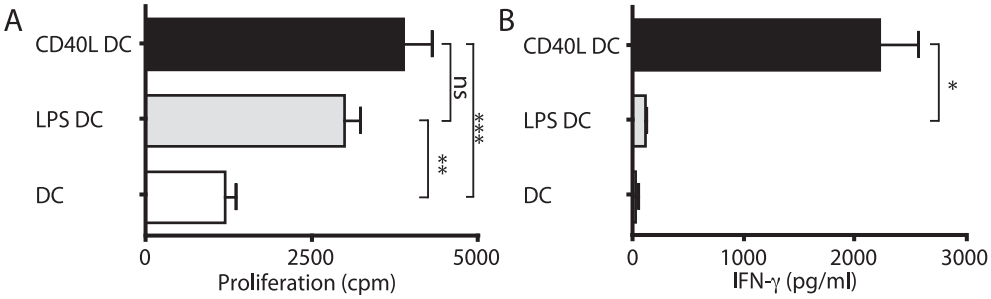

Figure 6. T cell stimulatory capacity of LPS or CD40L-activated DC.
Allogeneic T cells were purified from LEW rat. T cells ($1x10^5$) were cultured in the presence of $2x10^4$ DC of BN origin, showing immature DC (white bar), LPS-DC (gray bar) or rat CD40L-DC (black bar) for 5 days. A) Cells were pulsed with ^{3}H-thymidine for the last 16 hrs and harvested. B) IFN-γ production was measured in the supernatant. Results are expressed as the mean ± SD of three independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.0005$).

CD40L induces high levels of IL-12 and no IL-10 in contrast to LPS

To explain the superior IFN-γ production of T cells by CD40L-DC, we compared DC maturation induced by LPS and CD40L. In our rat DC cultures, both LPS and CD40L activation only resulted in a limited enhancement of CD86 and MHC class II expression, which was not different between the two modes of DC activation (**Fig.7**). As the balance between IL-12 and IL-10 has been proposed to be important for the IFN-γ production by T cells [31], we investigated the cytokine production by activated DC. Non-activated DC did not produce IL-12 or IL-10, confirming the immature status of the DC. Both LPS and CD40L induced the production of IL-12, but the production induced by CD40L was significantly higher (**Fig. 8A**). Strikingly, when the production of IL-10 was measured in the same supernatants, LPS-DC produced significantly more IL-10 (**Fig. 8A**). Experiments that focused on the effect of CD40L, showed that in 5 out of 8 experiments the IL-10 production was even non-detectable in supernatants of CD40L activated DC, despite the fact that these cells were efficiently activated as shown by their IL-12 production (data not shown).

To rule out the possibility that activation using L-rat CD40L is not suitable for induction of IL-10 production, we made use of the fact that rat CD40L is able to bind to human CD40 (Fig. 3A). In line with previous data using L-human CD40L, also L-rCD40L was able to induce IL-10 production by human monocyte-derived DC at a level comparable to LPS activation (**Fig. 8B**).

To exclude that this specific regulation of IL-12 and IL-10 was strain specific, cytokine production was also investigated from LPS- or CD40L-DC, generated from Lewis, Dark Agouti and Albino Oxford rats. Although the absolute levels of cytokines produced by activated DC of different rat strains were different, they all showed a similar pattern of regulation. LPS induced the production of IL-12 and high levels of IL-10. In contrast, DC produced especially high levels of IL-12 upon CD40L activation, with a limited production of IL-10 (**Fig. 8C**).

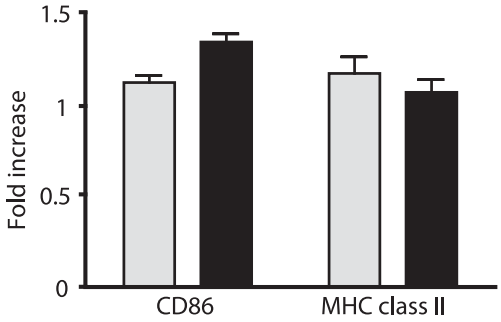

Figure 7 LPS or CD40L stimulation of rat DC results in limited enhancement of CD86 and MHC class II expression.
Day 7 immature DC were stimulated with 500 ng/ml LPS (gray) or CD40L (black) for 24 hr. Expression levels of CD86 and MHC class II were analysed by flow cytometry. Relative increase of expression levels on stimulated DC in relation to immature DC was calculated. Results are shown as the mean ± SD of three independent experiments.

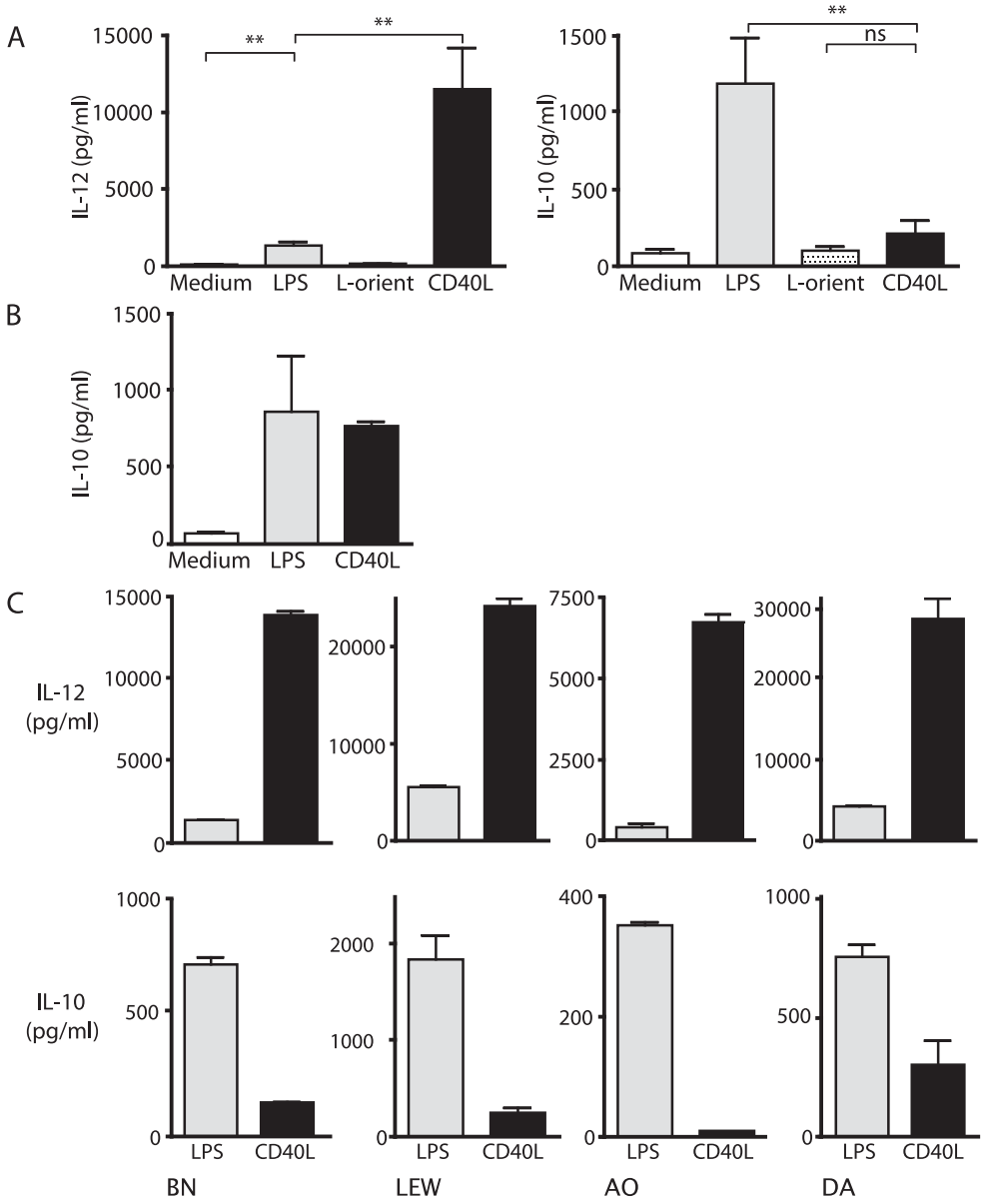

Figure 8. Cytokine profile of LPS or CD40L stimulated rat DC.
A) IL-12 and IL-10 production by BN derived DC cultured in the presence of medium (white), 500 ng/ml LPS (gray), L-orient (dotted) or L-rCD40L (black) was measured in supernatant 24 hr after stimulation using ELISA. Results are the mean ± SD of 3 independent experiments (**p<0.01). B) Cytokine profile of rat CD40L or LPS stimulated monocytes-derived human DC. After 24 hr stimulation of DC by either rat CD40L or 200 ng/ml LPS IL-10 production was measured in supernatant by ELISA. Results are the mean ± SD of three independent experiments. C) Cytokine profile from BN, LEW, AO or DA derived DC in the presence of 500 ng/ml LPS (gray) or L-rCD40L (black) in a 2:1 ratio. Results are the mean ± SD of duplicate cultures representative of three independent experiments. Data depicted for IL-10 and IL-12 production are derived from the same experiment.

Discussion

Since CD40 triggering plays an important role in the induction of immune responses in human and mouse models, in the present study we investigated the response of rat DC upon stimulation with CD40L. Previously it has been demonstrated that various culture conditions result in different rat DC types. Phenotypically DC generated in the presence of GM-CSF, IL-4 and Flt3-L, showed a typical expression profile of NKR-P1A, CD11b/c and OX62, which has been described before [12]. Upon stimulation with CD40L these DC produced high levels of IL-12 and little IL-10 and showed a strong capacity to stimulate allogeneic T cells associated with high levels of IFN-γ.

To investigate the stimulatory capacity of LPS-DC versus CD40L-DC both responder splenocytes and T cells were used in this study. Studies on the stimulatory capacity of rat DC have been performed both with allogeneic splenocytes and with purified T cells as responder cells [23, 24, 26, 32, 33]. Although in many of these cases this may not change the interpretation of the experiments, we observed that CD40L-activated DC induced a strong proliferative response of splenic B cells. A co-transfer of L-CD40L together with CD40L-activated DC into the MLR could explain this, as we found that L-CD40L could directly activate splenic B cell proliferation. In addition, also a direct effect of DC on B cells could contribute to the observed B cell response [34]. This indicates that it is important to use purified responder cells of interest, in our study T cells, to obtain reliable results.

In the present study we could show that the stimulatory capacity of CD40L-DC on allogeneic T cells was much higher than the activation induced by LPS-DC. This was especially true for the IFN-γ production. The cytokine production by activated T cells is influenced by a number of factors, in particular the balance between IL-12 and IL-10 produced by stimulated DC [31]. IL-12 is a potent driver of Th1 cell differentiation, whereas IL-10 blocks IL-12 secretion and impairs the ability of DC to generate Th1 responses [35-37]. The observation that CD40L-DC induce higher levels of IFN-γ production by the T cells compared to LPS-DC can thus be an effect of the cytokine profiles induced by the stimuli.

To elucidate the effect of CD40 triggering on rat DC it is important to use rat CD40L since human CD40L showed no cross-reactivity with rat CD40. Previous studies on the effect of CD40L on rat DC made use of soluble CD40L or a murine CD40L-CD8 fusion protein [23, 32]. Equal amounts of IL-12 production by the DC were detected upon stimulation by soluble CD40L or LPS, whereas IL-10 production was not shown [23]. Studies with the fusion protein showed that CD40L was able to induce more IL-12 secretion by the DC compared to LPS, however, both CD40L-DC and LPS-DC did not produce IL-10 [32]. When studying the effect of CD40L one should keep in mind that the kind of stimulator and the type of cross-linking can influence the strength of the response. It has been shown that the use of recombinant oligomeric CD40L is more effective than soluble CD40L, probably because this can engage multiple receptors and therefore induce a more effective signal [38, 39]. The L-rCD40L cells used in this study express high levels of CD40L on the membrane, potentially allowing a high level of cross-linking of CD40. We found that L-rCD40L was very efficient for the induction of IL-12 production, making the absence of IL-10 even more striking. Studies in which

murine BM-derived DC were stimulated with soluble α-CD40 demonstrated that CD40 triggering resulted in high IL-12 and low IL-10 production [40], reflecting the results found in the present study. On the other hand, human monocyte-derived DC produce both IL-12 and IL-10 after CD40 triggering and have a strong capacity to induce T cell proliferation. Interestingly, when the IL-10 production is neutralized, the T cell stimulatory capacity is enhanced [40].

Although human CD40L does not cross-react with rat CD40 (data not shown) or mouse CD40 [39], rat CD40L is able to bind human CD40. This enabled us to investigate the effect of rat CD40L on human DC. We found that the L-cells used in this study were able to induce IL-10 production by monocyte-derived human DC. Therefore, it is most likely the inability of the rat DC to produce IL-10 after CD40 triggering. Together, these results suggest that TLR4 activation of rat BM-derived DC induces signalling pathways resulting in IL-10 and IL-12 production, whereas CD40L especially favors the signalling pathway leading to IL-12 production. In addition, as both mouse and rat BM-derived DC show little production of IL-10, in contrast to human monocyte-derived DC, it is probably the progenitor cell chosen for the generation of DC that plays a role in this difference. This implies that when DC-based therapy is effective in disease models, it is important to understand the function of these in vitro generated DC. Only when this is known, the use of DC in therapeutic settings can be translated to the human situation.

CD40 signalling in monocyte-derived human DC or BM-derived mouse DC has been shown to induce p38 MAPK, ERK, JNK, Akt and NF-κB activation, which are regulated through various TNF-R associated factors (TRAF) [41, 42]. Until now, most research on the TRAF molecules has been performed in B cells [43, 44] and less is known about the role of TRAFs in DC. So far, TRAF6 has been shown to be important in controlling the IL-12p40 production by DC after CD40 activation [42]. In B cells TRAF3 has an inhibitory role in CD40-induced JNK activation and antibody secretion. Furthermore, it was shown that *Traf3*$^{-/-}$ BM-derived DC were hampered in their IL-10 production after LPS stimulation, whereas the IL-12p40 production increased [45]. The ratio TRAF6 versus TRAF3 recruitment may thus be important for the cytokine profile induced by TLR or CD40 triggering. This suggests that the absence of IL-10 production by CD40L-DC might be due to a signalling defect towards TRAF3. Alternatively, it is also known that IL-10 is able to suppress IL-12 production [46, 47], indicating that the high levels of IL-12 production by CD40L-DC in the present study might be partially related to the absence of IL-10.

In conclusion, LPS and CD40L differentially regulate IL-10 production by BM-derived rat DC. The results presented here show that CD40L-DC produce higher levels of IL-12 and are able to induce high levels of IFN-γ production by the T cells compared to LPS-DC. This suggests that signalling through CD40 involves different signalling pathways and may therefore induce stronger immune responses than signalling through TLR4. Like human and murine models, CD40L also plays an important role in the rat immune response. CD40L can therefore play a key role in therapeutic settings. In cancer research, CD40L stimulated DC can be used as a potential cellular therapy to induce a strong immune response against tumours. On the other hand, in the case of auto-immunity or transplantation it is important to prevent the induction of a strong immune response, which could be achieved by blocking the CD40-CD40L interaction.

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