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TNFα blockade impairs dendritic cell survival and function in rheumatoid arthritis

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Keywords: Anti-TNFα, rheumatoid arthritis, dendritic cells, infliximab, soluble TNF-Receptor

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Abstract

Objectives: TNFα blockade is an effective therapy for rheumatoid arthritis (RA). Immunomodulatory effects of TNFα antagonists are thought to contribute to their therapeutic action. Here, we investigated whether anti-TNFα therapeutics exerted their immunoregulatory effects through modulation of dendritic cell (DC) function.

Methods: Two complementary approaches were taken: In the first ‘in vitro’ approach monocyte-derived DC from healthy donors were matured with LPS and treated with TNFα antagonists in vitro for 48 hours. In the second ‘ex vivo’ approach monocyte-derived DC were generated from RA patients before and 8-12 weeks into anti-TNFα treatment. DC were analysed for survival, phenotype, cytokine production and T cell stimulatory capacity.

Results: TNFα blockade during DC maturation in vitro induced approximately 40 % of DC to undergo apoptosis. Importantly, the surviving DC displayed a semi-mature phenotype with reduced levels of HLA-DR, CD80, CD83, CD86 and CCR7, and their production of IL-10 was enhanced as compared to DC matured without TNFα antagonists. Furthermore, anti-TNFα-treated DC were poor stimulators of T cell proliferation and polarised T-cell development towards a higher IL-10/lower IFN-γ cytokine profile. Similarly, DC derived from RA patients after anti-TNFα treatment showed impaired upregulation of CD80 and CD86 upon LPS activation and displayed poor T cell stimulatory activity.

Conclusions: Our data show that TNFα blockade has profound effects on DC function with downstream, potentially immunoregulatory, effects on T-cells. These data provide an interesting new insight into the potential mechanism by which anti-TNFα drugs contribute to the restoration of immunoregulation in RA patients.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic synovitis, causing cartilage and bone destruction [1]. Although the precise aetiology of RA is unknown, recent genetic data clearly implicate T-cells in its pathogenesis [2, 3]. Additionally, tumor necrosis factor-α (TNFα) has been identified as a key mediator of synovial inflammation [4]. Therapeutic blockade of TNFα with neutralising antibodies (e.g. infliximab) or soluble TNFα receptor Ig constructs (e.g. etanercept) provides significant beneficial clinical effects in approximately 60% of RA patients [5].

Anti-TNFα therapeutics target inflammation at multiple levels (reviewed in [6]). For instance, they inhibit the induction of pro-inflammatory IL-1 and IL-6 [7, 8]; downregulate cartilage-destroying matrix metalloproteinases [9]; reduce recruitment of inflammatory cells to the synovium [10-12], and inhibit angiogenesis [13]. Immunomodulatory effects of TNFα antagonists have also been reported but remain largely unexplained. For instance, anti-TNFα therapeutics can reverse T-cell anergy [14], restore regulatory T-cell function in RA patients [15, 16] and reduce Th17 responses in the skin of psoriasis patients [17].

Dendritic cells (DC) are professional antigen presenting cells that regulate T-cell responses. DC have been implicated in RA pathogenesis. Myeloid DC with high T-cell stimulatory capacity are enriched in synovial tissue of RA patients [18-20]. It is thought that presentation of arthritogenic antigens to T-cells by these DC contributes to perpetuation of the inflammatory autoimmune response [21, 22]. TNFα plays a central role in DC biology. It is a classical DC maturation factor, enhancing the expression of MHC II and co-stimulatory molecules (e.g. CD80, CD86) [23]. TNFα also promotes...
DC survival [24, 25]. The maturation status and survival of DC are both important factors that determine the balance between immunity and tolerance. Whereas immature DC induce T-cell tolerance, mature DC activate T-cells and induce immunity. Enhancement of DC survival can break immune tolerance, resulting in autoimmune disease [26, 27]. Conversely, ablation of DC abrogates T-cell priming to antigens and inhibits autoimmunity [28, 29].

Considering the key role of TNFα in DC biology, we hypothesised that anti-TNFα therapy exerts its immunoregulatory effects in RA through modulation of DC function. Recent small studies in RA patients reported no effect of TNFα blockade on DC cytokine production [30] but a reduction in the DC maturation marker CD83 [31]. In contrast, an in vitro study of TNFα blockade during DC differentiation and maturation demonstrated no surface phenotype modulation but reduced production of IL-1β, IL-6 and several chemokines by mature DCs [32]. In the current study we have investigated the effects of TNFα blockade during DC maturation with the toll-like receptor 4 (TLR4)-ligand lipopolysaccharide (LPS). TLR4 ligands are enhanced in serum and synovial fluid of RA patients and are thought to contribute to DC activation and breakdown of tolerance in RA [33]. We have also studied the ex vivo generation of monocyte-derived DC from RA patients receiving anti-TNFα therapies. Our data suggest that, under both circumstances, TNFα blockade endows reduced T cell stimulatory properties on the DC, potentially contributing to the immunoregulatory effects of these drugs.

**Materials and Methods**

**Ethics**

Peripheral blood samples were obtained with informed consent and following approval by the Newcastle and North Tyneside Research Ethics Committee 2.

**Generation of monocyte-derived dendritic cells**

DC were generated from peripheral blood as described previously [34]. Briefly, peripheral blood mononuclear cells were isolated by density gradient centrifugation on Lymphoprep (Axis-Shadow Diagnostics, Dundee, UK). CD14+ monocytes were isolated by positive magnetic selection using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate immature DC, monocytes were cultured at 0.5x10^6 cells/ml in the presence of IL-4 and GM-CSF (50 ng/ml each, Immunotools, Friesoythe, Germany) for 6 days. All cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO₂. Medium supplemented with cytokines was refreshed on day 3.

**Treatment of dendritic cells with TNF-α antagonists**

Immature DC were washed and re-plated in fresh medium without cytokines at 0.25x10^6 cells/ml. DC were left untreated or were matured with lipopolysaccharides (LPS from e-coli, Sigma, 100 ng/ml) for 48 h in the absence or presence of a soluble TNF-Receptor p55 Ig fusion protein (10 μg/ml) [35], the therapeutic anti-TNF-α monoclonal antibody infliximab (10 μg/ml, Schering Plough, USA), or as a control human IgG (10 μg/ml, Grifols, Los Angeles, CA, USA). In some experiments dead DC were removed by the Annexin V dead cell removal kit (Miltenyi).
Apoptosis and cell phenotype analysis by flow cytometry
DC were washed and incubated with Annexin V-APC and PI in Annexin V binding buffer (BD Pharmingen, San Jose, CA, USA) for 15 minutes at room temperature. For active caspase-3 intracellular staining, DC were washed and incubated with human IgG for 15 minutes on ice, then fixed and permeabilised using Fix/Perm buffer for 20 minutes on ice, washed twice with BD Perm/Wash buffer, and blocked for 15 minutes on ice in 2 % rabbit serum. After blocking, anti active caspase-3 antibody (BD Biosciences) was added for 20 minutes at room temperature. For cell surface phenotype analysis of DC and T-cells the following mAbs were used: CD25 (clone M-A251), CD80 (clone L307.4), CD86 (clone 2331), CD83 (clone HB15e), HLA-DR (clone L243) (all from BD Pharmingen) and CCR7 (clone 150503, R&D Systems). Cells were washed and incubated with mAbs in PBS with 3% FCS, 2 mM EDTA and 0.01% sodium azide for 20 minutes in the presence of human IgG. Cell viability was assessed using Via-Probe (BD Pharmingen). Intracellular FoxP3 was detected using a FoxP3-APC staining kit (PCH101; eBioscience San Diego, CA, USA). Data were collected on a Becton Dickinson FACScan and analysed using FlowJo (Treestar, USA).

DC cytokine production
DC were stimulated at 2x10^5 DC/ml with CD40-ligand transfected J558L mouse cells (kindly provided by Peter Lane, Birmingham University, UK) at a 1:1 ratio. After 24 h, IL-10 and TNF-α in supernatants were quantified by sandwich ELISA (BD Pharmingen).

DC-T-cell cultures
T-cells were isolated from peripheral blood using CD3-negative selection with the RosetteSep T-cell enrichment cocktail (Stem Cell Technologies, Canada). DC were co-cultured with allogeneic CD3^+ T cells at a 1:10 ratio, unless otherwise stated. Cytokines in supernatants were analysed by ELISA and T-cell proliferation was assessed by measuring ^3H-thymidine incorporation in the last 16 h of culture. In restimulation experiments T-cells primed by DC for 6 days were rested for 5 days with 0.1 ng/ml rIL-2 (kind gift from Prof. John Robinson), washed and restimulated with CD3/CD28 expander beads (1:1, Dynal). No residual DC were observed in the T-cell cultures prior to restimulation. After 72 h, supernatants were harvested. Cytokines in supernatants (IL-10, IL-4, IFNγ, IL-17) were quantified by sandwich ELISA (BD Pharmingen).

Statistics
Mann Whitney, 2-way ANOVA with Bonferroni posttests and t-tests were performed using GraphPad software (San Diego, CA, USA). A p-value <0.05 was considered statistically significant with a 95% confidence interval.

Results
TNFα blockade during DC maturation results in apoptosis
Because DC produce TNFα and other survival factors in response to LPS, the effect of TNFα blockade on DC apoptosis after LPS activation was investigated. Two TNFα antagonists were used: A soluble TNF-Receptor p55 Ig fusion protein (TNFR) and the chimeric anti-TNFα monoclonal antibody infliximab. Both TNF antagonists were used at 10 μg/ml because i) this concentration is within the range of anti-TNF serum
concentrations found in patients undergoing treatment [6] and ii) in vitro experiments with a TNFR1-Fas transfected cell line showed that this concentration effectively blocked cell death induced by LPS-activated DC supernatants (data not shown).

Removal of GM-CSF/IL-4 from immature DC cultures led to high levels of apoptosis and cell death within 48 h, as visualised by the high percentage of Annexin V<sup>pos</sup>/PI<sup>neg</sup> (early apoptotic), Annexin V<sup>pos</sup>/PI<sup>pos</sup> (late apoptotic and dead cells) and active caspase-3<sup>pos</sup> cells (apoptotic cells, Figure 1A and B). LPS activation of immature DC rescued a large number of cells from apoptosis, but the addition of TNFR or infliximab abrogated this ‘rescue’. Immature DC could also be rescued from apoptosis by addition of soluble rTNFα at concentrations produced by DC upon LPS activation (around 10 ng/ml, data not shown). Control IgG did not affect the level of apoptosis, indicating that the effect of the TNF-α antagonists was not mediated through Fc-gamma receptor binding. Apoptosis of LPS-activated DC treated with TNFR (LPS-TNFR DC) or infliximab (LPS-inflix DC) increased during the 72 hour culture period (Figure 1C). These data show that TNFα blockade abrogates the enhanced survival of DC following maturation in response to LPS.

**TNFα blockade affects phenotype and cytokine production of LPS-activated DC**

To assess modulation of DC maturation by TNFα antagonists, the expression of CD80 and CD86 (co-stimulatory molecules), CD83 (DC maturation marker), HLA-DR (antigen-presenting molecule) and CCR7 (chemokine receptor) was measured. Dead cells were excluded from the analysis. CD80, CD86 and CD83 expression were significantly reduced by treatment of DC with TNFα antagonists, whereas HLA-DR expression was not significantly inhibited (Figure 2A). There was a trend towards reduced CCR7 expression after anti-TNF treatment (p=0.057). DC clustering, normally observed after LPS activation of DC in vitro, did not occur in the presence of TNFα antagonists (Figure 2B).

Interestingly, upon CD40 ligation LPS-TNFR DC and LPS-inflix DC produced significantly higher levels of IL-10 than control DC (LPS-IgG DC), while TNFα levels were similar (Figure 2C). These data show that TNFα blockade during LPS maturation results in a DC population with a semi-mature phenotype and substantially enhanced IL-10 production.

**TNFα antagonists reduce T-cell stimulatory capacity of DC**

We next determined whether the partial maturation of DC treated with TNFα antagonists affected their ability to stimulate allogeneic CD3<sup>+</sup> T-cells. DC treated with TNFR or infliximab had a significantly reduced capacity to induce T-cell proliferation and IFNγ production (Figure 3A and B). The reduced T-cell stimulatory capacity of LPS-TNFR and LPS-inflix DC was not simply due to lower levels of live DC in these populations, because removal of apoptotic DC did not enhance T-cell proliferation (Figure 3C). However, from these experiments it cannot be excluded that apoptotic DC may have influenced the maturation of the surviving DC. Nevertheless, together these data show that blocking TNF during maturation diminishes the immunostimulatory capacity of DC.

**Anti-TNFα treated DC modulate cytokine production by T-cells**

To assess whether anti-TNFα treatment of DC affects the ‘quality’ of T-cell responses, T-cells expanded by the various DC populations were analysed further. Firstly, the
expansion of regulatory T-cells was determined by measuring percentages of CD25<sup>hi</sup>/Foxp3<sup>pos</sup> T-cells. FoxP3 was measured 11 days after T cell activation to ensure that transient activation-induced FoxP3 expression in non-Treg cells had disappeared [36]. Although T-cells primed by anti-TNFα-treated DC (Figure 4A) contained a higher percentage of Tregs than T-cells primed by immature DC, the percentages of Tregs were similar to the percentages found in T-cell populations primed by mature DC (LPS DC or LPS-IgG DC). The data indicate that neutralisation of TNFα during DC maturation does not obviously alter the ability of these DC to expand Tregs.

Secondly, the polarising activity of anti-TNFα treated DC on T-cell cytokine production was investigated. Resting T-cells were recovered from primary DC-T-cell cultures and re-stimulated with CD3/CD28 beads. T-cells primed by anti-TNFα DC produced significantly higher levels of IL-10, IL-4, and IL-17 but lower levels of IFNγ than T-cells primed by mature LPS-DC (Figure 4B). These data indicate that anti-TNFα treatment of DC affects the ‘quality’ of T-cell responses by skewing their cytokine profile, overall favouring anti-inflammatory cytokines.

**Phenotype and function of DC derived from RA patients before and after anti-TNFα therapy**

To investigate the effects of TNFα blockade on DC from RA patients treated with anti-TNF drugs, monocyte-derived DC were generated from RA patients *ex vivo*, before and after 8-12 weeks of anti-TNFα therapy (Adalimumab or Etanercept, see Table 1). No anti-TNFα was added to *in vitro* cultures. LPS-induced upregulation of CD80 and CD86 was lower during anti-TNFα therapy in most patients (Figure 5A and B): CD80 was lower in 3 out of 5 cases and CD86 was lower in 4 out of 4 cases. Furthermore, in 4 out of 5 cases the T-cell stimulatory capacity of DC during anti-TNFα therapy was reduced (Figure 5C) shown by a decreased ability to initiate T-cell proliferation. Comparing the cytokine profiles of primed T-cells revealed considerable heterogeneity. However, there was a trend towards enhanced IL-10 and reduced IL-4, IFNγ and IL-17 production by T-cells primed by mature DC derived during anti-TNFα therapy (Figure 5D). These data suggest a reduced T-cell stimulatory capacity of DC derived from RA patients on anti-TNFα drugs. However, no association was found between the clinical response to anti-TNFα therapy and functional modulation of DC.

**Table 1.** Rheumatoid arthritis patients on anti-TNFα treatment

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Sex</th>
<th>Disease Duration (years)</th>
<th>anti-TNF Drug</th>
<th>MTX</th>
<th>Pred.</th>
<th>RhF</th>
<th>anti-CCP</th>
<th>Pre-treatment DAS</th>
<th>Post-treatment DAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFRA02</td>
<td>40</td>
<td>F</td>
<td>2</td>
<td>Adalimumab</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>5.27</td>
<td>2.77</td>
</tr>
<tr>
<td>TNFRA04</td>
<td>57</td>
<td>M</td>
<td>4</td>
<td>Etanercept</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>7.17</td>
<td>2.12</td>
</tr>
<tr>
<td>TNFRA05</td>
<td>76</td>
<td>F</td>
<td>1</td>
<td>Etanercept</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>7.46</td>
<td>4.43</td>
</tr>
<tr>
<td>TNFRA07</td>
<td>49</td>
<td>F</td>
<td>2.5</td>
<td>Etanercept</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7.42</td>
<td>6.77</td>
</tr>
<tr>
<td>TNFRA08</td>
<td>40</td>
<td>F</td>
<td>4</td>
<td>Etanercept</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7.08</td>
<td>6.34</td>
</tr>
</tbody>
</table>

MTX = methotrexate therapy; DAS; Pred. = prednisone therapy; RhF = rheumatoid factor; anti-CCP = anti-cyclic citrullinated peptide antibodies; DAS = disease activity score; F = female; M = male; N.D. = not determined
Discussion

Our experiments demonstrate a profound effect of TNFα blockade on the generation and maturation of DC from their myeloid precursors. Our *in vitro* data clearly indicate that blockade of TNFα during final DC maturation results in enhanced DC apoptosis and induction of semi-mature DC, with reduced co-stimulatory molecule expression and T-cell stimulatory capacity. Importantly, T-cells activated by these semi-mature DC produced enhanced levels of anti-inflammatory IL-10 and IL-4 and lower levels of pro-inflammatory IFNγ. It is perhaps surprising that IL-17 levels are modestly but significantly increased in these experiments but this could be a downstream consequence of reduced IFNγ levels [37, 38]. When we compared monocytes derived from RA patients receiving TNFα blockade to cells harvested pre-treatment from the same patients we found that, even without adding anti-TNF drugs *in vitro*, they also generated DC with reduced co-stimulatory molecule expression and T-cell stimulatory capacity. These effects on DC generation were most likely caused by anti-TNFα treatment per se, as RA patients receiving and responding to methotrexate alone did not show impaired DC generation or function (data not shown). The question of whether anti-TNF modulated DC function through neutralisation of autocrine TNFα and/or via reverse signalling was not addressed here, but both mechanisms may be important [6, 39].

The difference in IL-17 and IL-4 production between T-cells in our *in vitro* and *ex vivo* experiments was subtle. Furthermore, these are fundamentally different experiments from the perspective of anti-TNFα exposure. In the *in vitro* experiments monocytes develop normally and are only exposed to TNFα blockade during the final stages of DC maturation. The *ex vivo* experiments are complementary because monocytes develop in the presence of TNFα blockade, which is absent during DC development and maturation. Despite this we can detect an ‘imprinted’ effect of *in vivo* anti-TNF exposure in the *ex vivo* cultures, with the overall finding of reduced T cell stimulatory capacity. Thus, our data suggest an important effect of TNFα on both DC precursors and DC.

Although TNFα blockade was originally viewed as a simple but potent anti-inflammatory therapy, studies on patients receiving treatment have shown enhancement of sub-optimal regulatory T-cell function [15] and the generation of a novel subset of regulatory T-cells in association with therapy [16]. Our findings suggest an additional mechanism by which anti-TNFα therapy enhances immunoregulation, via the generation of ‘semi-mature’ DC that secrete high levels of IL-10 and with the capacity to generate T-cells with enhanced anti-inflammatory cytokine secretion. Furthermore, by inducing apoptosis in DC during maturation, anti-TNFα therapeutics may also act by reducing the strength of the autoimmune response.

Differences between our data and limited previous reports also attest to the critical importance of experimental conditions (e.g. type of anti-TNFα used and time point of anti-TNFα exposure) when interpreting similar phenomena. Van Lieshout et al [32] cultured monocytes in the presence of a monovalent PEGylated form of p55 TNFr (essentially a monomeric form of our sTNFr). In contrast to our data, even after maturation with LPS they could not demonstrate a difference in surface phenotype compared to conventionally matured DC, although chemokine, IL-1β and IL-6 production were reduced when DC from RA patients were matured in the presence of this agent. Another study showed that the addition of infliximab at the start of the monocyte-derived DC culture period resulted in immature DC with reduced CD1a and CD86 expression as well as reduced T-cell stimulatory capacity. These effects were less
marked following DC maturation with LPS [40]. Zaba et al [17] used immunohistochemistry and real-time PCR of psoriatic skin to demonstrate reduced IL-23, IL-17 and IFNγ levels following etanercept therapy, as well as reduced CD83 and DC-LAMP expression by skin DC – data more consistent with our ex vivo findings, although it is unclear whether these effects were caused by reduced infiltration of inflammatory cells and/or immunomodulation. They also generated immature DC in vitro in the presence of etanercept, showing reduced CD86 and HLA-DR expression and reduced T-cell stimulatory capacity. A further study showed the pro-apoptotic effects of etanercept on dermal DC in psoriatic plaques [41], again consistent with our observations on DC survival.

TNFα is a pleiotropic cytokine with acute and chronic effects on many cell types. Consequently TNFα blockade, which is widely used to treat active RA, psoriasis and inflammatory bowel disease, can no longer be viewed as a purely anti-inflammatory therapy [42]. It has pro-apoptotic effects on various cells and has also been shown to have immunoregulatory consequences in vivo. To these effects can now be added an important modulation of DC survival, phenotype and function. RA is accepted by many as an autoimmune disease initiated by inappropriate T-cell responses against self antigens and several novel approaches are being developed to target T-cells [43]. However, T-cells are instructed in their activities by DC, which therefore have an even more pivotal role in the development and control of autoimmunity and thus provide a more tempting target. Clinical studies are starting to hint at the potential of TNFα blockade to induce remission in the early stages of RA [44] and evolving data, including those presented here, suggest mechanism(s) for these therapeutically revolutionary observations.

In conclusion we have adopted two complementary approaches to study the effect of TNFα blockade on DC survival, phenotype and function. Whereas DC are more likely to die when TNFα is blocked during their maturation, those that survive exhibit a semi-mature phenotype and poor T cell stimulatory capacity. In line with this, DC derived from RA patients during anti-TNFα therapy have a similar phenotype. Incubation of T-cells with anti-TNFα-treated DC skews the T-cell cytokine profile towards higher IL-10/lower IFNγ production, potentially contributing to the previously demonstrated immunoregulatory effects of TNFα blockade.

Acknowledgement
We would like to thank Tom Wooldridge for the collection of blood samples from RA patients. Mr. Wooldridge is funded by Newcastle’s UK NIHR Biomedical Research Centre for Ageing and Age Related Disease.

Competing interests: None.

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Ethics: Peripheral blood samples were obtained with informed consent and following approval by the Newcastle and North Tyneside Research Ethics Committee 2.
Figure Legends

Figure 1. TNFα blockade during DC maturation results in apoptosis. Immature monocyte-derived DC were extensively washed to remove cytokines and re-cultured in the absence or presence of LPS (LPS DC), LPS and human IgG (LPS-IgG DC), LPS and TNFR (LPS-TNFR DC) or LPS and infliximab (LPS-inflix DC) for 48 h (A, B) or for various time periods as indicated (C). Apoptosis was measured using Annexin V/PI staining (A) or activated caspase-3 intracellular staining (B, C). Data are representative of at least 3 independent experiments.

Figure 2. TNFα blockade affects phenotype and cytokine production of LPS-activated DC. Immature monocyte-derived DC were extensively washed to remove cytokines and re-cultured in the absence or presence of LPS (LPS DC), LPS and human IgG (LPS-IgG DC), LPS and TNFR (LPS-TNFR DC) or LPS and Infliximab (LPS-Inflix DC) for 48 h. (A). Phenotype of DC was determined by flow cytometry. Debris and dead cells were excluded on the basis of the forward scatter, side scatter and viability staining using Via-probe. Data from independent experiments are shown and were calculated as the fold change compared to LPS-DC. Horizontal lines represent median values. (B) Morphology of DC populations as shown by phase contrast microscopy (100X). (C) DC were washed to remove TNFα antagonists and stimulated with CD40L-expressing cells for 24 h. IL-10 and TNFα levels in supernatants were measured by ELISA and expressed as the percentage of cytokine production by LPS DC. LPS DC produced 642 ± 476 pg/ml IL-10 and 769 ± 610 pg/ml TNFα. Data of 4 independent experiments are shown. Horizontal lines represent median values. *p<0.05 and **p<0.01 as determined by Mann Whitney test.

Figure 3. DC matured in the presence of TNFα antagonists have low T-cell stimulatory capacity. Immature DC or DC activated with LPS (LPS DC), LPS and human IgG (LPS-IgG DC), LPS and TNFR (LPS-TNFR DC) or LPS and Infliximab (LPS-Inflix DC) for 48 h were washed and co-cultured with allogeneic CD3+ T-cells (1x10^5) for 6 days. Proliferation was measured by 3H-Thymidine incorporation (A, C) and IFNγ production in supernatants (using 10x10^3 DC) was determined by ELISA. (B). In some experiments dead cells were removed from the DC populations prior to co-culture with T-cells (20x10^3 DC: 1x10^5 T cells) using the Annexin V dead cell removal kit (C). Data are representative of at least 3 independent experiments and are shown as mean +/- SEM of triplicate cultures. (A) *p<0.05 indicate significant differences between T cell proliferation induced by LPS-IgG DC and LPS-TNFR DC and between T cell proliferation induced by LPS-IgG DC and LPS-Inflix DC as determined by 2-way ANOVA with Bonferroni posttests. (B, C) *p<0.05 as determined by t-test. NS: not significant.

Figure 4. DC matured in the presence of TNFα antagonists do not preferentially expand regulatory T-cells but skew T-cell cytokine profiles. DC activated with LPS (LPS DC), LPS and TNFR (LPS-TNFR DC) or LPS and infliximab (LPS-Inflix DC) for 48 h were washed and co-cultured with allogeneic CD3+ T-cells at a 1:10 ratio. After 6 days T-cells were recovered and rested for another 5 days with 0.1 ng/ml rIL-2. (A) Flow cytometry analysis of CD25 and FoxP3 expression. Debris and dead cells were excluded on the basis of the forward scatter, side scatter and viability staining using Via-probe. Percentage of FoxP3+ cells is indicated in the Figure. Data are representative.
of at least 3 independent experiments. (B) T-cells primed by DC populations were washed and restimulated with CD3/CD28 expander beads. After 72 h supernatants were harvested and cytokine levels determined by ELISA. Results on cytokine production by T-cells primed by anti-TNF-treated DC (T_{LPS-TNFR DC} or T_{LPS-Inflix DC}) are presented as a percentage of cytokine production by T-cells primed by mature DC (T_{LPS DC}). T_{LPS DC} produced 361 ± 267 pg/ml IL-10, 253 ± 248 pg/ml IL-4, 20.4 ± 6.6 ng/ml IFNγ and 869 ± 699 pg/ml IL-17. Horizontal lines represent median values. p-values were determined by Mann Whitney test and indicate significant differences to T_{LPS DC}.

Figure 5. DC derived from RA patients after anti-TNFα therapy have lower T-cell stimulatory capacity. Monocyte-derived DC were generated from the peripheral blood of RA patients before (pre) and after taking anti-TNFα antagonists for 8 to 12 weeks (post). DC were left immature or were matured with LPS for 24 h. (A, B) Expression of co-stimulatory molecules CD80 and CD86 pre and post anti-TNFα therapy. (A) LPS-induced upregulation of CD80 and CD86 expression was calculated by dividing the MFI (median fluorescence intensity) of CD80 or CD86 on LPS-matured DC by the MFI of CD80 or CD86 on immature DC, respectively. Results of 5 (CD80) and 4 (CD86) independent RA patients are shown. NS: p-values >0.05 as determined by Mann Whitney test, indicating non-significant differences between LPS-induced CD80 and CD86 expression on DC pre and post anti-TNF-therapy. (B) The CD80 and CD86 flow cytometry plots shown are from different RA patients. (C, D) T-cell stimulatory and polarising capacities of mature DC pre and post anti-TNFα therapy. Mature DC were co-cultured with allogeneic CD3⁺ T-cells at a 1:10 ratio. T-cells from the same donor were used for DC derived from RA patients pre and post anti-TNFα therapy. (C) After 3, 5 and 7 days proliferation was measured by adding ³H-Thymidine for 16 hours. Data of 5 RA patients are presented and are shown as mean +/- SEM of triplicate cultures. *p<0.05 as determined by 2-way ANOVA with Bonferroni posttests, indicating significant differences between T cell stimulatory capacity of DC pre and post anti-TNF therapy. (D) T-cells were cultured for 10 days, washed and restimulated with CD3/CD28 expander beads. After 72 h supernatants were harvested and cytokine levels determined by ELISA. Results on cytokine production by T-cells primed by mature DC post anti-TNFα therapy are presented as a percentage of cytokine production by T-cells primed by mature DC pre anti-TNFα therapy. The absolute levels of cytokines produced by T-cells primed by mature DC pre anti-TNFα therapy were: 2067 ± 1026 pg/ml IL-10, 17.9 ± 18.1 ng/ml IL-4, 5.8 ± 3.2 ng/ml IFNγ and 453 ± 474 pg/ml IL-17. Horizontal lines represent median values. NS: p-values >0.05 as determined by Mann Whitney test indicating non-significant differences between T cell cytokine production pre and post anti-TNF-therapy.
References


Figure 1

A

![Graph showing Annexin V and Propidium Iodide levels for different DC types](image)

B

![Histogram showing No. of cells for different DC types](image)

C

![Line graph showing % Apoptotic cells over time for different DC types](image)
Figure 2

A

CD80

CD83

CD86

CCR7

HLA-DR

B

Immature DC  LPS DC  LPS-IgG DC  LPS-TNFR DC  LPS-Inflix DC

C

% IL-10 production of LPS DC

% TNF production of LPS DC

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Figure 3

A

- Open circle: LPS DC
- Triangle: LPS-IgG DC
- Solid line and circle: LPS-TNFR DC
- Star: LPS-inflix DC
- Cross: Immature DC

Proliferation (cpm x 10^-3)

Number of DC x 10^-3

B

- Immature DC
- LPS DC
- LPS-IgG DC
- LPS-TNFR DC
- LPS-inflix DC

IFN-γ (pg/ml)

C

- Solid line: Total cells
- Hatched: Live cells

Proliferation (cpm x 10^-3)

LPS-IgG DC
LPS-inflix DC

NS
Figure 4

A

CD25

FoxP3

Immature DC  LPS DC  LPS-IgG DC  LPS-TNFR DC  LPS-Inflix DC

B

cytokine production (% of T<sub>LPS DC</sub>)

IL-10  IL-4  IFN<sub>γ</sub>  IL-17

p = 0.0071  p = 0.0466  p = 0.0071  p = 0.0206
Figure 5

A

LPS induced upregulation of CD80/86 expression

Day

anti-TNF therapy

pre post pre post

CD80

CD86

B

Pre Post

CD80

CD86

C

RA02 RA04 RA05

RA07 RA08

D

NS NS NS NS NS

% cytokine production

of pre anti-TNF therapy