Chapter 3

Phagocytosis of apoptotic or necrotic cells differentially regulates the transcriptional expression of IL-12 family members in dendritic cells.

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Abstract

Uptake of apoptotic cells by dendritic cells (DCs) is considered to contribute to induction and maintenance of immunological tolerance. Tolerogenic DCs (tolDCs) are being sought after as cellular therapy in transplantation and autoimmunity, and can be generated in-vitro using glucocorticoids. In this study we investigated how uptake of dead cells affects the production and expression of different members of the IL-12 family by either immature DC or tolDC.

We show that compared to regular immature DCs, tolDCs display elevated levels of PS-recognising bridge molecule-receptors αvβ5 and CD36, have enhanced phagocytic abilities with accelerated uptake of apoptotic cells. We confirm that apoptotic cell uptake results in diminished production of IL-12p40 and IL-12p70 by DCs. We now show that this also results in increased expression of IL-12p35 and Ebi3. TolDCs completely lack expression of IL-12p40, yet have enhanced levels of Ebi3 and IL-12p35. Uptake by tolDCs of either apoptotic or necrotic cells does not affect the expression of Ebi3/IL-12p35, and does also not increase IL-12p40. This is distinct from culture of immature DCs with necrotic cells, which is sufficient to induce IL-12p40 secretion. Conversely, ingestion of apoptotic cells by DCs leads to increased expression of IL-12p35 and Ebi3 without affecting IL-12p40. In conclusion we have shown that uptake of apoptotic versus necrotic cells by DCs differentially regulates members of the IL-12 family. Apoptotic cells favour expression of Ebi3 and IL-12p35, and we propose that differential regulation of the IL-12 family is an additional mechanism in determining the immune response to dying cells.

Introduction

Dendritic cells (DCs) are highly specialised antigen (Ag)–presenting cells (APCs) that are uniquely capable of orchestrating a repertoire of immune responses, ranging from the initiation of Ag specific immunity, to the active induction and maintenance of specific tolerance to self Ags in the periphery[1-3].

Consequently, cellular immunotherapies have been developed with the aim to exploit the immunoregulatory functions of DCs to silence immune responses in transplantation and autoimmunity[4, 5]. Several agents have been explored for the induction of tolerogenic DCs (tolDCs), and glucocorticoids (GCs) including dexamethasone (dex) have been shown to induce the development of such a population with potent anti-inflammatory properties[6, 7].

The concept of tolerogenic DCs (tolDCs) was proposed almost 20 years ago[8, 9], and early on, diminished production of IL-12 was identified to be a hallmark feature of these cells[10, 11]. Since then, IL-12 has become part of an entire cytokine family made up of 4 related, yet distinct cytokines, namely IL-12, IL-

23, IL-27 and IL-35[12]. IL-12 and IL-23 are primarily immunogenic, playing a role in the commitment and maintenance of Th1[13, 14] and Th17[15, 16] populations respectively. IL-27 appears to play a dual role, possessing both immunogenic[17-19] and immunoregulatory properties[20-22]. The most recent member, IL-35, has been described as a potent anti-inflammatory cytokine with an important role in mediating infectious tolerance[23]. Unlike the other 3 members, IL-35 has not yet been shown to be produced by DCs, but has been described as a product of inducible regulatory T cells in mouse and man[24, 25]. Although there are several studies demonstrating that diminished IL-12 production is a critical characteristic of tolDCs, little data is available about the more recent members and their regulation[26].

Recognition and uptake of apoptotic[27] or necrotic cells[28] by DCs has been recognised as an important regulatory mechanism in immune homeostasis. Rather than merely being passively non-inflammatory, these studies imply that apoptotic cells can actively infer an immuno-regulatory state within the ingesting DCs[29, 30]. Much of this work has focused on immunogenic DCs and there have been few studies looking at the phagocytic potential of tolDC and the subsequent functional consequences for these cells[31].

In this study we investigated the phagocytic potential of tolDCs compared to DCs and analysed the regulation of the IL-12 family member subunits in both cell types upon uptake of apoptotic versus necrotic cells. We show that, compared to DCs, tolDCs are superior at ingesting specifically apoptotic material. As previously described, we confirm that apoptotic cell uptake results in diminished production of IL-12p40 and IL-12p70. We now show that this also results in the increased expression of IL-12p35 and Ebi3. We conclude that ingestion of apoptotic versus necrotic cells by DCs differentially regulates the IL-12 family members thereby playing a role in determining the immune response in such events.

Materials and Methods

Cell culture and Reagents

Human monocytes were isolated from buffy coats (ethical approval number: BTL 10.090), obtained from healthy donors using Ficoll density gradient centrifugation followed by positive selection using anti-CD14 MACS microbeads (Miltenyi Biotech). DCs were generated and cultured in RPMI 1640 supplemented with 10% FCS, 90 U/mL penicillin, and 90 µg/mL streptomycin (Life technologies), 5 ng/mL GM-CSF and 10 ng/mL IL-4 (Biosource Europe, Belgium), as described before[32]. TolDCs were generated by addition of Dex (10^{-6}M Dex) (Pharmacy, L.U.M.C., Leiden, the Netherlands) only at the start of culture (day 0). Cultures were refreshed with medium containing cytokines on day 3. For stimulation experiments immature DCs were harvested on day 6, washed and seeded accordingly, followed by addition of 200 ng/ml LPS (E.Coli 0127:B8 Sigma-Aldrich) and 100 ng/ml IFNγ (Peprotech). To quantify cellular debris in culture, images (n=15) of a number of cell culture plates were captured at day 6 and the amount of cellular fragments was counted and expressed as a percentage of viable cells for both DC and tolDC.

Flow Cytometry

Cells were harvested, washed, and stained for 30 min at 4°C in FACS buffer (PBS, 0.5% heat inactivated NHS, 1% BSA, 0.02% NaN_3) with appropriate antibodies including anti-αvβ5 (RnD systems), anti-CD93, anti-CD36 , anti-Tim-1, anti-Tim-4 (Biolegend), anti-CD91 (BD Biosciences) and anti-CD206 (clone D547). Nonconjugated antibodies were detected with PE-conjugated goat-anti-mouse Ig. Isotype matched control antibodies were used to determine the level of background staining.

Induction of apoptosis and necrosis

Jurkat T cells were cultured in complete RPMI medium. Cells were washed with PBS and exposed to ultraviolet (UV)–C light (TUV lamp, 254 nm; Philips Electronic Instruments, Eindhoven, The Netherlands) at a dose of 50 J/m^2. After UV irradiation, cells were cultured for 3 hours in serum-free RPMI for the generation of apoptotic cells. Necrosis was induced by incubating Jurkat cells at 56°C in a water bath for 1 hour. Both apoptosis and necrosis were confirmed by double-staining with fluorescein isothiocyanate (FITC)–labeled annexin V and Propidium Iodide (PI)(VPS Diagnostics, Hoeven, The Netherlands) according to established methods[33]. Cells were only used if a purity of >70% was achieved i.e. apoptotic (>70% AnnV+, PI-), necrotic cells (>70% AnnV+, PI+). Neutrophils were isolated as previously described[34] and cultured overnight in serum free RPMI supplemented with 5ng/ml GM-CSF, to facilitate the generation of early apoptotic neutrophils by spontaneous apoptosis. For DC apoptosis, cells were plated at 2.5 x 10^6/ml in a 6 well plate and irradiated with a UV transilluminator with a peak intensity of 9000 mW/cm^2 at the filter surface and a peak emission of 312 nm for 4 mins, followed by culture at 37°C for 2 hours. Necrosis was induced by incubation at 56°C for 1hr. For some experiments, prior to the induction of apoptosis, Jurkat cells or neutrophils were fluorescently labeled with carboxyfluorescein diacetate succinamidyl ester (CFSE; Molecular Probes). In short, cells were suspended in PBS at 20 × 10^6 cells/mL and incubated for 15 minutes at 37°C with 5 μM CFSE. The reaction was quenched by washing the cells in medium containing 10% FCS before resuspending at 5 × 10^6 cells/mL in serum-free RPMI. The cells were subsequently used for apoptosis or necrosis induction as decribed above.

Phagocytosis assay

CFSE labeled apoptotic/necrotic cells (1 × 10^5) were cocultured with DCs, at a 1:1 ratio for 1 hour at 37°C or 4°C in 100 μL RPMI. DCs were stained with an APC-conjugated mAb against HLA-DR. Uptake was analysed by flow cytometry. The percentage of HLA-DR-positive cells that stained positive for CFSE was used as a measure for the percentage of DCs, that ingested (37°C) and/or bound (4°C) apoptotic cells. Cells were analysed using FACSCalibur and CellQuest software or FlowJo.Phagocytosis was further investigated by fluorescent microscopy using appropriate filter settings. Images were captured at indicated time points, and were analysed using ImageJ software version 1.33 (NIH Image, Bethesda, MD). For this purpose, CFSE-labelled apoptotic cells (1 × 10^5) and PKH-26 (4μM, Sigma-Aldrich) labelled DCs were cocultured at a 1:1 ratio in 24 well culture plate for 24 hours at 37°C.

Viable and apoptotic/necrotic DC cultures

Immature DCs were harvested at day 6 and plated in a 12 well plate at a density of 0.5 x 10^6/well. Apoptotic/necrotic DCs were harvested and added to viable DCs at a density of 2.5 x 10^6/well for 24 hours. Where indicated cells were then stimulated with IFNγ and LPS and left in culture for a further 24 hours.

mRNA isolation, cDNA synthesis, and RT-PCR

mRNA was isolated from DCs using an Rneasy kit according to the manufacturer's instructions (Qiagen). DNA was digested using the on-column RNase-free DNase set. cDNA was synthesised using a reverse transcription system kit (Promega) following the manufacturer's guidelines. Specific primers for human *IL27B*, *IL27A, IL12A,*

IL12B, IL10, IDO1, TGFβ and *GAPDH* (Table I) were designed using the computer software Oligo explorer and synthesised at Biolegio. Primer specificity was tested by homology search with the human genome (basic local alignment search tool or BLAST; National Center for Biotechnology Information) and later confirmed by electrophoresis through 2% agarose gels containing ethidium bromide followed by visualisation under UV light. *GAPDH* was used as an endogenous reference gene. For each sample, the relative abundance of target mRNA was calculated from the obtained Ct values for the target gene and expressed relative to the endogenous reference gene *GAPDH*.

Table I: Real Time PCR Oligonucleotide sequences

Gene	NCBI ID	Protein ID	Forward Sequence (5`-3`)	Reverse Sequence (5`-3`)
IL12B	3593	IL-12p40	CAGCAGCTTCTTCATCAGGG	GAGTACTCCAGGTGTCAGG
IL12A	3592	IL-12p35	CCAGAGTCCCGGGAAAGTC	ACCAGGGTAGCCACAAGG
IL27B	10148	Ebi3	TGGATCCGTTACAAGCGTC	AGTTCCCCGTAGTCTGTG
IL27A	246778	IL-27p28	GAGGGAGTTCACAGTCAGC	GCAGGAGGTACAGGTTCAC
IL10	3586	IL-10	GCGCTGTCATCGATTTCTTCC	GTAGATGCCTTTCTCTTGGAGCTTA
IDO1	3620	IDO	TCTGGTGTATGAAGGGTTCTG	GGGATTTGACTCTAATGAGCAC
TGFB1	7040	TGF-β1	CTGCCCCTACATTTGGAGC	AGCGCACGATCATGTTGGAC
GAPDH	2597	GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG

Cytokine production

DCs were plated at $1x10^6$/ml and treated accordingly. Cell culture supernatants were harvested after 48hr and frozen at −80°C until analysis. Subsequently they were tested for the presence of IL-12p70 and IL-12p40 (Biolegend) and IL-10 (Sanquin, Amsterdam, the Netherlands) by ELISA according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed by sample t test using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered statistically significant when P values were less than 0.05.

Results

TolDCs display reduced levels of cellular debris in culture and express elevated levels of αvβ5 and CD36.

We have previously shown that the generation of DCs in the presence of glucocorticoids led to the induction of a potent tolDC population with the ability to suppress allogenic T cell proliferation and cytokine secretion[6, 32]. When generating these tolDC, we observed significantly reduced yields (Fig.1A). Despite this, we noted that the cultures were notably cleaner compared to untreated DCs (Fig.1B). Quantification showed that, compared to control DC, tolDC had significantly less cellular debris after 6 days in culture (Fig.1C).

Cell surface FACS analysis demonstrated that both immature DC and immature tolDC expressed several molecules implicated directly or as an accessory bridging molecule, in the uptake of apoptotic cells, including αvβ5, CD93, CD36, CD91, CD206 and the PS receptor Tim-3, but not Tim-1 (Fig.1D). Several receptors had a tendency to be elevated, and expression of the integrin αvβ5 and the thrombospondin 1 receptor CD36 was consistently and significantly higher in tolDC compared to DC across a number of different donors (Fig.1E), together suggesting that tolDCs may have an enhanced phagocytic capacity compared to DCs.

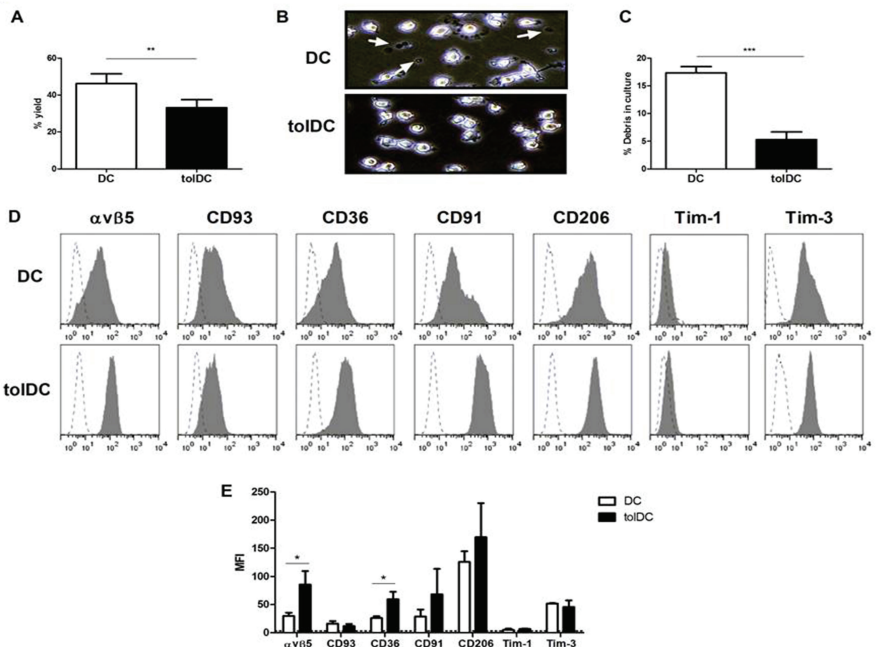

Figure 1:tolDC cultures display signs of enhanced phagocytosis. Monocytes were isolated from PBMCs and DCs were generated by culturing in RPMI/ 10% FCS supplemented with GM-CSF and IL-4. TolDCs were generated by addition of Dex (10^{-6}M Dex) at the start of culture (day 0). (A) Indicates lower harvest yield of immature tolDCs after 6 days of culture, data shown is mean ± SEM (n=7; **p= 0,0066).(B) Viable immature DC or tolDC were imaged on day 6 of culture. Arrows in DC indicate the presence of dying cell debris which is not presence in the cultures of tolDCs. (C) Indicates quantification of cell debris in culture as a percentage of viable cells, data shown is mean ± SEM (n=3; ***p=0.0003). (D) Representative histograms of cell surface FACS analysis on DC and tolDC at day 6 of culture, stained for a number of different known receptors involved in apoptotic cell clearance. Histograms shown were gated on PI negative cells, dashed lines represent isotype control mAb staining. Results shown are from a representative experiment of 3 independent experiments performed (E) Demonstrates mean ± SEM (n=3 ; * $p \leq 0,03$) of those 3 experiments.

TolDC display enhanced and accelerated phagocytosis of apoptotic cells.
To compare the phagocytic ability of immature DCs and immature tolDCs we used a phagocytosis assay where DCs were incubated with viable, early apoptotic or necrotic CFSE labelled Jurkat cells[33]. Incubation of DCs with apoptotic or necrotic cells was followed by a temperature-dependent uptake (Fig.2A). Although DCs were already efficient at taking up both apoptotic and necrotic material, tolDC displayed a significantly enhanced ability to phagocytose apoptotic cells (Fig.2B). Interestingly no difference was observed in necrotic cell uptake between DC and tolDC. Additional experiments demonstrated that, compared to DCs, tolDCs were also more efficient at ingesting apoptotic primary neutrophils (Fig.2C). To confirm our findings that tolDC possess enhanced phagocytic abilities we performed microscopic imaging over time. Representative pictures (Fig.2D) at 2 and 24 hours show a time-dependent clearance of apoptotic cells by viable DCs and tolDCs. Quantification demonstrated that tolDCs were more efficient in clearance of apoptotic cells (50% in 8 hours) compared to DC (50% in 15 hours) (Fig.2E). This accelerated uptake was not evident for the clearance of necrotic cells, whereby both DC and tolDC had a similar clearance (50% in 13-15 hours respectively) (Fig.2E).

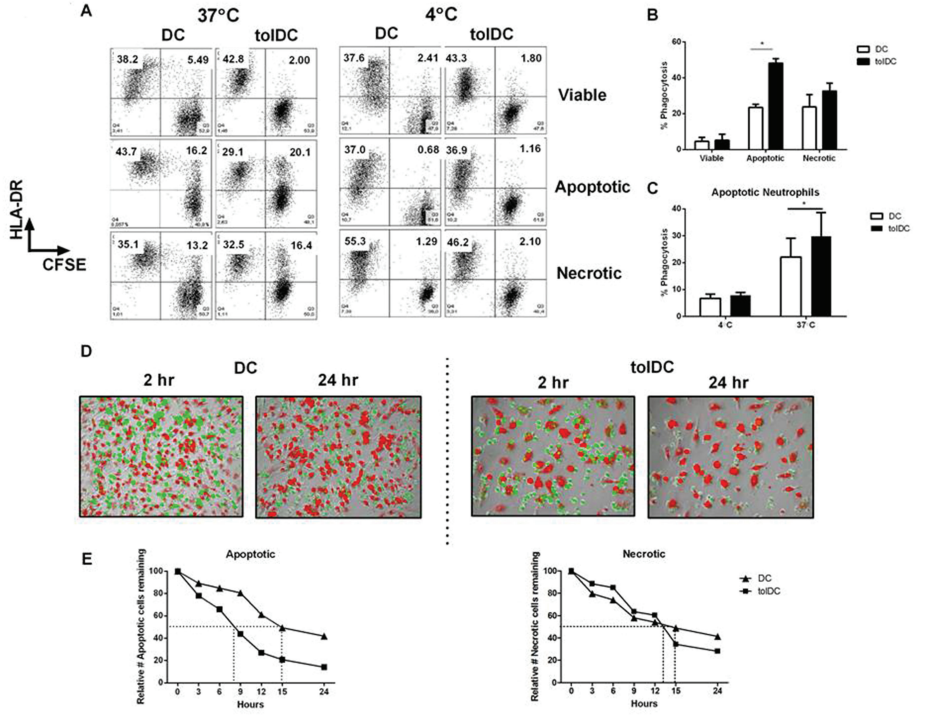

Figure 2: Increased and accelerated uptake of apoptotic cells by tolDC compared to DC. Viable immature DC or tolDCs were incubated with CFSE labelled viable, apoptotic and necrotic Jurkats at a ratio of 1:1 for 1hr at (A) 37°C or 4°C. DCs were detected using APC labelled HLA-

DR antibody and percentage phagocytosis was determined by dividing the number of double positive (HLA-DR APC+, CFSE+) DCs by the sum of single (APC+) and double positive (APC+, CFSE+) DCs. Data shown in (A) is representative of 4 independent experiments. Data shown in (B) is the mean ± SEM (n=4; **p=0.0019 using paired t test). (C) Viable immature DC or tolDCs were incubated with CFSE labelled neutrophils, which had undergone spontaneous apoptosis, at a ratio of 1:1 for 1hr at 37°C or 4°C. DCs were detected using APC labelled HLA-DR antibody and percentage phagocytosis was determined by dividing the number of double positive (HLA-DR APC+, CFSE+) DCs by the sum of single (APC+) and double positive (APC+, CFSE+) DCs. Data shown in (C) is the mean ± SD (n=3; *p≤ 0.05 using paired t test). (D) Viable PKH labelled DC or tolDC were incubated with CFSE labelled apoptotic Jurkat cells at 37ºC in a ratio of 1:1 and followed in time for 24 hours using Cell IQ® live cell imaging. Representative pictures were taken of both DC and tolDC at 2 and 24 hours. Images captured were analysed (E) by counting the number of remaining unbound and uningested apoptotic or necrotic Jurkat cells at indicated time points and expressed relative to dying cells counted at t=0. Dashed lines in (E) indicate $t^{1/2}$(the point at which 50% of the dying cells were cleared by DC and tolDC respectively).

Apoptotic cells profoundly inhibit IL-12p40 and IL-12p70 production by activated DCs.

To further explore the immune regulatory effects of apoptotic cell uptake by viable DCs, we focused on the regulation of IL-12 production. In order to mimic the experimental conditions during tolDC generation and removal of cellular debris (Fig.1C), we generated apoptotic and necrotic DCs/tolDCs by UV irradiation and heating respectively. Apoptotic cells were characterised as Annexin V positive and PI negative (AnnV+, PI-), while necrotic cells were double positive (AnnV+, PI+) (Fig.3A).Viable immature DCs alone produced low amounts of IL-12p40 and this production was significantly inhibited when these cells were exposed to apoptotic cells. In contrast, addition of necrotic cells induced a significant increase in IL-12p40 production (Fig.3B). Under these experimental conditions, production of IL-12p70 could not be detected (data not shown). As a control, no IL-12p40 was released from apoptotic or necrotic DCs alone, in the absence of viable immature DCs. None of the conditions described resulted in a detectable production of IL-12p40 by immature tolDCs (Fig.3B).

Activation of DCs using a combination of LPS and IFNγ induced a strong IL-12p40 (Fig.3C) and IL-12p70 (Fig.3D) production. Activation in the presence of apoptotic cells led to a significant inhibition of both IL-12p40 and IL-12p70 production, while this inhibition was not observed for necrotic cells. Also under these activation conditions, mature tolDCs were completely hampered in their capacity to produce either IL-12p40 or IL-12p70. IL-10 has previously been shown to be upregulated in APCs upon uptake of apoptotic material. We measured production of IL-10, but did not see any significant differences upon uptake of apoptotic or necrotic cells (Fig.3E) Activation of DCs with a combination of IFNγ+LPS resulted in increased production of IL-10 in both viable DC and tolDC.

Activation in the presence of apoptotic or necrotic cells did not significantly alter IL-10 production by mature DCs or mature tolDCs under any conditions analysed (Fig.3F). In summary uptake of apoptotic cells by mature DCs significantly inhibits production of IL-12, while IL-10 remains unaffected.

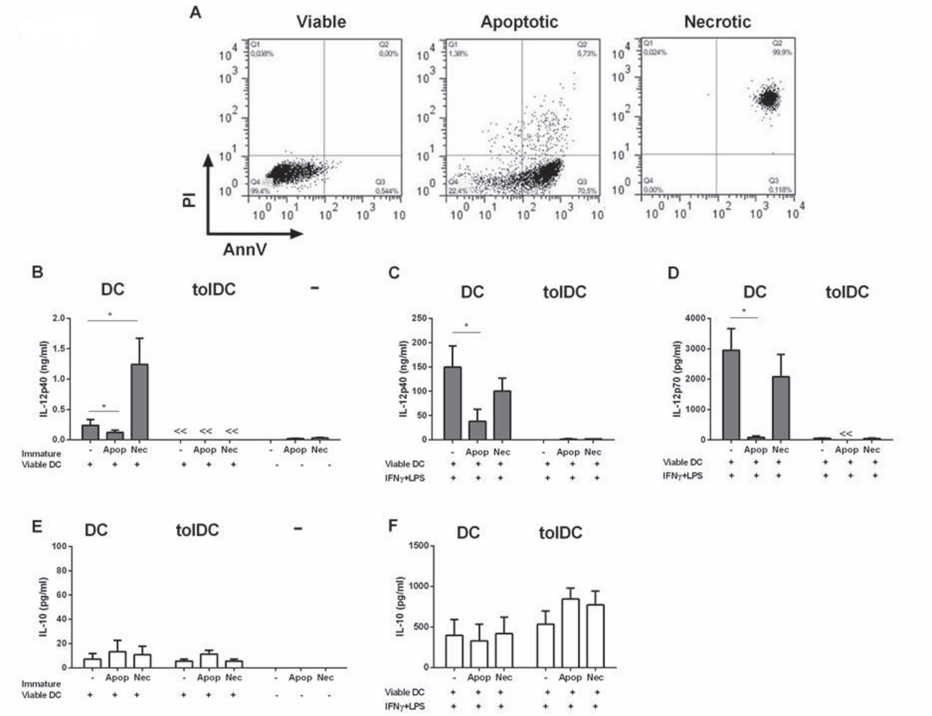

Figure 3: Stimulation of DCs with IFNγ and LPS in the presence of apoptotic DCs profoundly inhibits IL-12 production. (A) Viable, (UV-irradiated) Apoptotic or (heat-treated) Necrotic DCs were generated and defined by Annexin V/PI positivity. Viable immature DCs and tolDCs were harvested at day 6 and cultured for 48hr alone, with apoptotic DC, or with necrotic DC. As a control apoptotic and necrotic DCs were additionally cultured in the absence of viable cells to account for production of cytokines by dying cells alone. After culture the supernatants were harvested and an ELISA was performed for (B) IL-12p40 and (E) IL-10. Alternatively viable DC and tolDC were cultured alone, with apoptotic DC, or with necrotic DC for 24 hours and then stimulated with a combination of IFNγ and LPS for a further 24 hours. After that the supernatants were harvested and analysed for (C) IL-12p40, (D) IL-12p70, (F) IL-10. Data shown is mean ± SEM (n=4) *p=<0.03, DC alone versus DC with apoptotic/necrotic DC using paired t-test.

Apoptotic cell uptake does not alter transcriptional expression of IDO, TGF-β or IL-10.

Three key soluble mediators that have been shown to be upregulated upon uptake of apoptotic cells are IDO, TGF-β and IL-10. We observed by ELISA that IL-10 remained unaffected by uptake of either apoptotic or necrotic cells even upon

subsequent activation with IFNγ+LPS (Fig.3).
We further challenged immature DC and immature tolDCs with apoptotic or necrotic autologous cells and found that the ingestion of dying cells did also not alter the expression of either *IDO* or *TGFβ* (Fig.4A, B). We questioned whether stimulation of DCs and tolDCs upon ingestion of apoptotic or necrotic material may alter the expression of *IDO* or *TGFβ* but stimulation in the presence of dying cells did not significantly alter the expression of any molecule assessed in mature DCs or mature tolDCs (Fig. 4A, B).

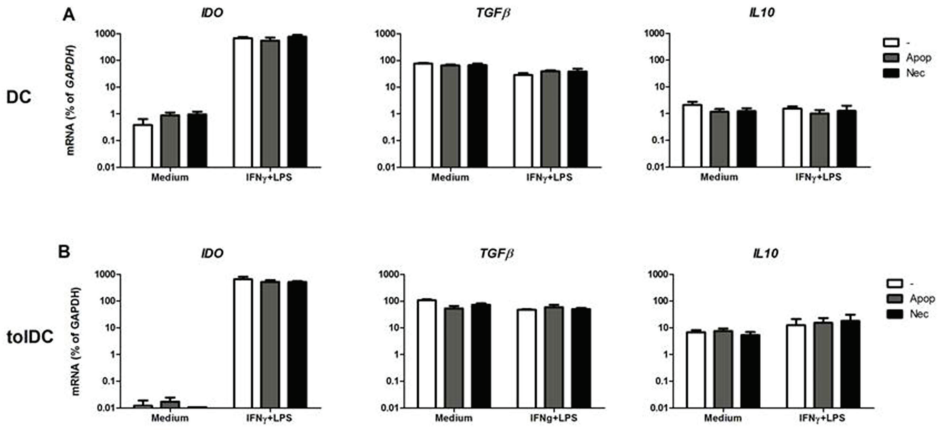

Figure 4: Apoptotic cell uptake does not alter transcriptional expression of IDO, TGF-β or IL-10. Viable, (UV-irradiated) Apoptotic or (heat-treated) Necrotic DC and tolDCs were generated as described. Viable immature DC and tolDC were harvested at day 6 and cultured alone, with autologous apoptotic, or with autologous necrotic DC/tolDC for 48 hours after which mRNA was isolated followed by cDNA synthesis. Alternatively cells were additionally stimulated for 6 hours with IFNγ+LPS after the initial 48 hour incubation. The transcript levels were determined by RT-PCR in (A) DC (B) tolDC for *IDO, TGFβ* and *IL10*. Data shown is mean ± SD (n=2-3). Relative mRNA expression indicates expression of the mRNA of interest relative to the expression of GAPDH.

TolDCs have decreased transcriptional expression of IL-12p40, yet have enhanced levels of both Ebi3 and IL-12p35.

We have shown that tolDCs are completely impeded in their ability to produce IL-12p40 and IL-12p70. Considering that IL-12p70 is a heterodimer of IL-12p40 and IL-12p35, and belongs to the larger IL-12 family, we sought to investigate the expression levels of the different subunits of the IL-12 family in DCs and tolDCs. We demonstrate, in line with our protein data on IL-12p40 and IL-12p70, that immature tolDCs have strongly diminished transcriptional expression of *IL12B* (Fig.5A). Strikingly however, these cells have an increased expression of *IL12A*, along with its alternate pairing subunit, *IL27B* (Fig.5A). *IL27B* can also heterodimerise with *IL27A* to form IL-27, however no significant difference was

noted between immature DCs and immature tolDCs in terms of *IL27A* expression. In view of the enhanced phagocytic abilities of tolDCs (Fig.2C), we questioned whether uptake of dying cells could affect the elevated *IL27B* and *IL12A* levels. To investigate this, we further challenged viable immature tolDCs with apoptotic or necrotic tolDCs or apoptotic or necrotic immunogenic DCs and analysed expression of *IL12B, IL12A, IL27B* and *IL27A*. However addition of apoptotic or necrotic cells to immature tolDCs did not alter the expression of the different IL-12 family subunits (Fig.5B).

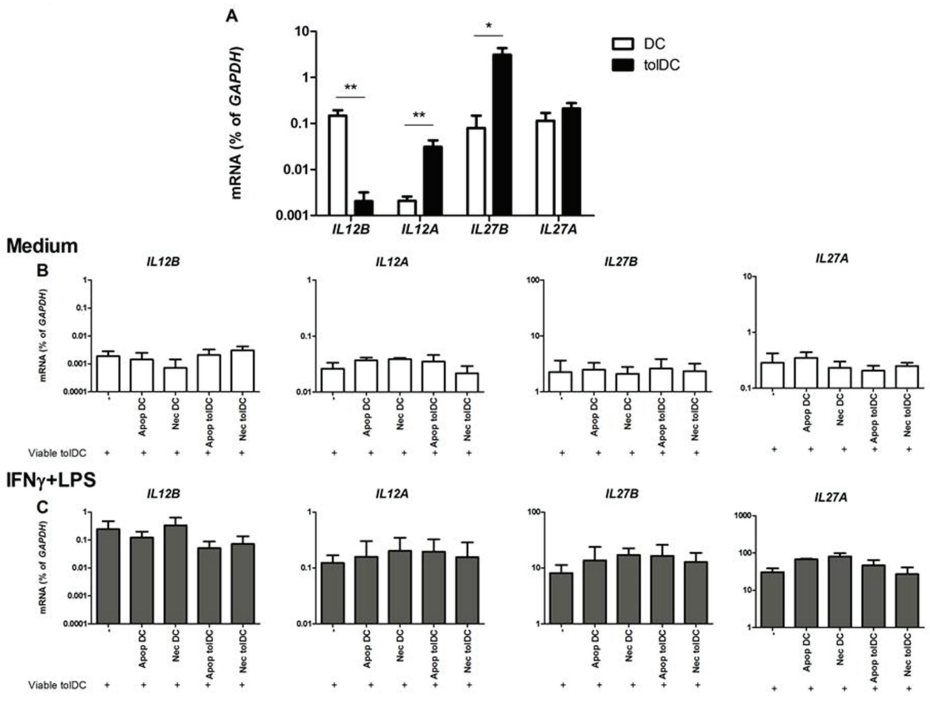

Figure 5: tolDCs maintain expression of IL-12p35 and Ebi3 in the absence of IL-12p40. Immature DCs and tolDCs were harvested on day 6, mRNA was isolated and cDNA was generated for (A) RT-PCR analysis for expression of *IL12B, IL12A, IL27B*, and *IL27A*. Relative mRNA expression indicates expression of the mRNA of interest relative to the expression of *GAPDH*. Viable, (UV-irradiated) Apoptotic or (heat-treated) Necrotic DCs or tolDCs were generated and defined by Annexin V/PI positivity (data not shown). Viable immature tolDC were harvested at day 6 and cultured alone, with apoptotic DC/tolDC, or with necrotic DC/tolDC for 48 hours after which mRNA was isolated followed by cDNA synthesis. Alternatively cells were additionally stimulated for 6 hours with IFNγ+LPS after the initial 48 hour incubation. The transcript levels were determined by RT-PCR in (B) Medium and (C) IFNγ+LPS stimulated tolDCs for *IL12B, IL12A, IL27B* and *IL27A*. Data shown is mean ± SEM (n=4-7) **p ≤ 0.002, *p ≤ 0.03 DC versus tolDC using Mann-Whitney test.

We questioned whether stimulation of tolDCs upon ingestion of apoptotic or necrotic material may alter the stable cytokine profile of these cells. Stimulation with IFNγ +LPS led to increased expression of all IL-12 members with *IL27A* demonstrating the greatest increase. Stimulation in the presence of dying cells did not significantly alter the expression of any IL-12 family members assessed, indicating that tolDCs maintain a stable cytokine profile upon stimulation, irrespective of challenge with either apoptotic or necrotic cells (Fig.5C).

Culture of immature DC with apoptotic DCs leads to increased expression of both IL-12p35 and Ebi3.

To investigate whether the robust stability of transcriptional expression of the IL-12 family was a general characteristic of DCs, or was intrinsic for tolDCs upon ingestion of apoptotic and necrotic cells, we cultured immature DCs with apoptotic or necrotic DCs. We first established that culture of apoptotic or necrotic DCs with viable DCs more broadly altered the function of DCs, by assessing their allostimulatory capacity following uptake of apoptotic or necrotic material. We observed that ingestion of apoptotic DCs by viable cells resulted in an impaired allostimulatory ability, as assessed by $CD4^+$ T cell proliferation and IFNγ production. Conversely, ingestion of necrotic DCs by viable DCs significantly enhanced both allogenic T cell proliferation and IFNγ production (Fig.6A). In contrast to IFNγ, IL-17A production remained unaffected. Focusing on DCs, we observed microscopically that culture of DCs with apoptotic cells resulted in the formation of small clusters, whereas culture of immature DCs with necrotic cells induced a more distinct morphological change, with some of the viable cells becoming more stretched and adherent; typical features suggestive of activated DCs (Fig.6B). When analysing mRNA expression we observed that *IL12B* was not affected by co-culture with apoptotic cells, but was significantly enhanced by necrotic cells (Fig.6C). This was in line with our initial ELISA data (Fig.3B). In contrast, although still relatively low, there was an increased expression of both *IL12A* and *IL27B* when DCs were cultured in the presence of apoptotic DCs (Fig.6C). Importantly, this stimulatory effect was not observed in the presence of necrotic cells. The expression of *IL27A* was not significantly altered upon uptake of apoptotic or necrotic cells (Fig.6C).

Discussion

In the last decade a shift in rationale has been seen in the field of apoptosis where it is becoming increasingly evident that apoptotic cells are not just immunologically inert, but actively shape the immune capacity of the ingesting cell[35]. In this study we found that apoptotic and necrotic cell uptake profoundly affects the

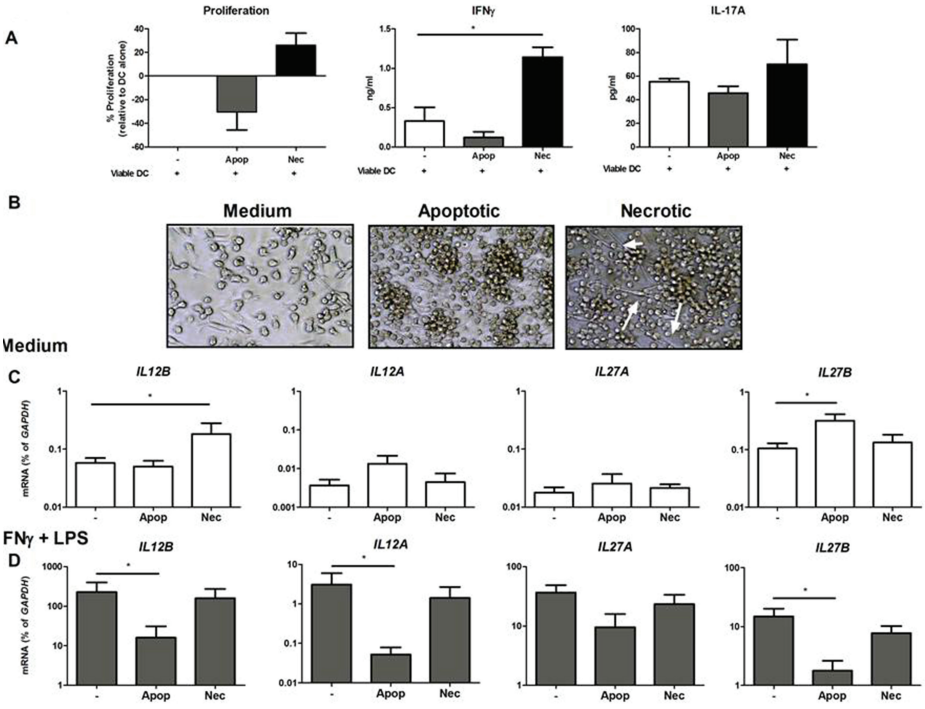

Figure 6: Culture of immature DC with apoptotic or necrotic DCs has differential effects on the transcriptional regulation of IL-12 family members. Viable (A) immature DCs were harvested at day 6 and cultured for 48hr alone, with apoptotic DC, or with necrotic DC. The cells were then harvested washed and cultured at a ratio of 1:40 with allogeneic $CD4^+$ T cells for 5 days. Proliferation was determined by the addition of ^3H for the last 18 hours and supernatants were assessed for IFNγ and IL-17A production. (B) Clusters of cells present in apoptotic conditions suggest enhanced activation in immature DCs cultured with apoptotic material. Arrows in necrotic conditions indicate the activated and adherent viable DCs observed when cultured in the presence of necrotic DCs. Cells were harvested after the 48 hour culture and mRNA was isolated followed by cDNA synthesis. Alternatively cells were additionally stimulated for 6 hours with IFNγ+LPS after the initial 48 hour incubation. RT-PCR was performed in (C) Medium and (D) IFNγ + LPS stimulated DCs for *IL12B, IL12A, IL27B and IL27A*. Relative mRNA expression indicates expression of the mRNA of interest relative to the expression of *GAPDH*. Data shown is mean ± SEM (n=3-7) *p≤0.05 DC alone versus DC with apoptotic/necrotic DC using Mann-Whitney test.

cytokine profile of the ingesting dendritic cell and that uptake of apoptotic cells by immature DCs led to increased expression of Ebi3 and IL-12p35.We observed previously that DCs generated in the presence of dexamethasone became a potent immunoregulatory population with complete absence of IL-12 and the ability to suppress allogenic T cell proliferation[6, 32]. In this study we demonstrate that these same tolDCs have enhanced expression of several receptors involved in the clearance of dying cells, including αvβ5[36], CD36[37] and CD91[30], and

have enhanced phagocytic abilities compared to non tolerogenic DCs. As a consequence, although tolDCs have reduced cell yields after culture there is a complete absence of cellular debris, indicating that tolDCs continuously ingest dying bystander cells.

An intriguing observation in our study was that the accelerated clearance of apoptotic cells by tolDC was not observed for necrotic cells. This resembles our previous observation that anti-inflammatory type-2 macrophages displayed a preference for recognition of early apoptotic cells[33]. Some molecules, most notably phosphatidylserine (PS)[33, 38], have been implicated as triggers for phagocyte recognition and subsequent removal of apoptotic cells. We showed that tolDCs do express elevated levels of PS-recognising bridge molecule-receptors, however blockade of these molecules during phagocytosis assays did not alter the % uptake of apoptotic material by tolDCs (data not shown).

Several studies have looked at the effect of phagocytosis on DC function, including IL-12 production[29, 39]. However limited data is available with regard to the other members of the IL-12 family, particularly the most recently identified, IL-35. Although IL-12 and IL-35 share IL-12p35, the common α-chain, both cytokines possess a unique spectrum of functional activities. Where IL-12 is widely acknowledged as a critical mediator in the induction and maintenance of Th1 responses[13, 14], data regarding IL-35 is more finite. Studies have shown that IL-35 is a potent immunoregulatory cytokine responsible for the induction of iTr35 a specific subset of regulatory T cells[23, 40]. Considering that DCs possess all the chains of the IL-12 family, a complex relationship exists between the opposing roles of IL-12 and IL-35 in the induction of specific T cell responses, although the definitive role of IL-35 has yet to be fully ascertained[41-43].

We questioned whether regulation of the IL-12 family more broadly could be an additional response to apoptotic versus necrotic cell uptake. We show that addition of necrotic cells induced a significant increase in IL-12p40 production. Activation of DCs with LPS and IFNγ induced a strong IL-12p40 and IL-12p70 production which was profoundly inhibited in the presence of apoptotic cells. This inhibition was not observed for necrotic cells, which not only reaffirms the consensus that apoptotic cells generally do not provoke inflammation, but also that they actively downregulate immunity[30, 44-47]. Still, there are some conflicting reports regarding this calming effect of apoptotic cells on DC activation, since some studies have shown that apoptotic cells can induce IL-12 production in DCs[48, 49]. Often the apoptotic cells used in these studies had been stimulated prior to apoptosis induction which may explain some discrepancies, or in addition the method of inducing apoptosis may also lead to the considerable variation between different studies. Many studies have been performed to elucidate the mechanisms of how necrotic cells lead to immune activation. The most extensively studied

danger signals released from necrotic cells, include heat shock proteins[50], HMGB1[51], nucleic acids and degradation products such as urate. In contrast, molecular mechanisms into how apoptotic cells may promote tolerance are far less defined. Interest in this particular aspect has been growing in the past years and a recent publication by Sekar et al, demonstrated that uptake of apoptotic tumour cells by DCs lead to Ebi3 upregulation in an SP1 dependent fashion[52]. It would be of interest to explore the role of SP1 and S1PR4 in our model using DCs as a source of apoptotic material.

In our study, transcriptional analysis confirmed a reduced expression of IL-12p40 in tolDCs. Strikingly, the expression of the other 3 members was maintained in tolDCs and specifically for IL-12p35 and Ebi3 the expression was even elevated compared to DCs. Challenge of tolDCs with apoptotic material from autologous tolDC or non-tolerogenic DCs, did not further enhance expression of IL-12p35 or Ebi3, however ingestion of necrotic cells did also not enhance the immunogenic chain IL-12p40. We have shown that tolDCs generated with dex are phenotypically very stable and retain their anti-inflammatory profile despite repeated challenge with immunogenic stimuli, including TLR agonists and CD40 ligation[32]. We show that in addition, ingestion of necrotic cells also does not break this anti-inflammatory state. We hypothesised that non tolerogenic DCs may be not be so steadfast in their cytokine expression upon encountering apoptotic versus necrotic cells. We chose DCs as a source of dying material to avoid contaminating mRNA from other cellular sources, as many cells constitutively express IL-12p35 to varying degrees. In addition the ratio (1:5) of viable to dying cells we used is in line with ratios used in other studies[27, 30, 31, 53]. We first observed that uptake of apoptotic or necrotic material by DCs altered their allostimulatory capacity, with apoptotic cell uptake decreasing both T cell proliferation and IFNγ production while necrotic cell uptake increased proliferation and IFNγ production. In line with these findings, we observed that uptake of necrotic DCs led to the upregulation of IL-12p40, which was not observed with apoptotic cell uptake. Interestingly while IL-12p40 remained unaffected, ingestion of apoptotic DCs led to the upregulation of both IL-12p35 and Ebi3 in the viable cells. Previous studies have demonstrated that DCs which had engulfed apoptotic cells had a diminished T cell stimulatory capacity which they found was not dependent on either IL-10 or TGF-β[29]. Although several factors including IDO[54] have been shown to mediate T cell inhibition by DCs post apoptotic cell uptake, we also did not observe any effect on IL-10, TGF-β or IDO in our study. It is interesting to consider the role which IL-35 may play in such circumstances. In fact, although regulatory T cell derived IL-35 has been shown to be important in immune regulation, speculation is rife as to whether DCs, particularly tolerogenic DC subsets may also contribute to IL-35 production[55]. Unfortunately reagents to

accurately determine IL-35 production are currently lacking and have somewhat hampered further understanding into this exciting cytokine. Our data, although speculative, does show a robust regulation of both IL-12p35 and Ebi3 by apoptotic cell uptake and may imply a role for IL-35 as an additional molecule in the control of the immune response to dying cells.

Although we have shown that ingestion of dying cells alone can alter the transcriptional cytokine profile within the DC, this is even more profound when the DCs are subsequently activated. In line with previous work we found that IL-12p35 is significantly inhibited in activated DCs upon uptake of apoptotic cells[56]. Surprisingly this was also true for Ebi3. Further work is needed to dissect the answer as to why apoptotic cells induce Ebi3 expression in immature DCs yet can also suppress its upregulation in mature DCs, and furthermore the full role of IL-35. Although speculative, several groups have supported the idea that uptake of apoptotic DCs by DCs is particularly proficient at promoting tolerance within the ingesting DCs[44, 57]. Although an exact mechanism remains elusive, some interesting findings have suggested that cells undergoing apoptosis are capable of producing cytokines and chemokines which can influence the response of the ingesting phagocyte[58]. One could imagine that DCs would be particularly adept at secreting potent immune mediators that may conflict, or alternatively synergise, with non-sterile or sterile signals depending on the concentration and duration of stimulus.

Many groups in the area of transplantation are striving to identify the best way to induce tolerance in allograft recipients[2, 59, 60]. A promising strategy may be to culture tolDCs of recipient origin and feed them with donor apoptotic cells. Indeed it has been shown in a murine model that targeting of DCs with donor apoptotic material can restrain indirect allorecognition[61], a major hurdle in the quest for transplant tolerance. If future therapeutic interventions will be based on this principal it is imperative to understand the full effects of apoptotic and necrotic cell uptake on DC and tolDC functions including the effect on their cytokine repertoire. It is promising that in this study tolDCs maintained a stable immunoregulatory cytokine profile irrespective of ingestion of apoptotic or necrotic cells even upon subsequent stimulation.

Taken together our results demonstrate that compared to DCs, tolDCs are superior at ingesting apoptotic material, and maintain a stable cytokine profile irrespective of apoptotic or necrotic cell uptake. Uptake of apoptotic cells by DCs results in diminished production of IL-12p40 and IL-12p70. We demonstrate that this also results in the increased expression of IL-12p35 and Ebi3. We conclude that ingestion of apoptotic versus necrotic cells by DCs results in differential regulation of the IL-12 family members thereby playing a role in determining the immune response in such events. Further understanding into the processes

governing DC phagocytosis and the effect it exerts over DC cytokines may open up many avenues towards the goal of limiting allograft rejection in transplantation and dampening down immune responses in autoimmune diseases.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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