Interferon γ suppresses glucocorticoid augmentation of macrophage clearance of apoptotic cells

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One of beneficial effects of glucocorticoids (GC) in inflammation may be the augmentation of macrophages' capacity for phagocytosis of apoptotic cells, a process that has a central role in resolution of inflammation. Here we define the phenotype of GC-treated monocyte-derived macrophages, comparing to IFN-γ-treated and IL-4-treated monocyte-derived macrophages and combinatorial treatment. Our data indicate that the cytokine microenvironment at an inflammatory site will critically determine monocyte functional capacity following treatment with GC. In particular, whilst GC exert dominant regulatory effects over IFN-γ in terms of cell surface receptor repertoire and morphology, the acquisition of a macrophage capacity for clearance of apoptotic cells is prevented by combined treatment. In terms of mechanism, GC augmentation of phagocytosis was reversed even when monocytes were pre-incubated with GC for the first 24 h of culture, a period that is critical for induction of a highly phagocytic macrophage phenotype. These findings have important implications for the effectiveness of GC in promoting acquisition of a pro-phagocytic macrophage phenotype in inflammatory diseases associated with high levels of IFN-γ

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1 Introduction

Macrophages play a central role in the immune system, initiating and co-ordinating both innate immunity and the adaptive immune response [1, 2]. Macrophage production of matrix, enzymes and the cytokines that regulate fibroblast migration and proliferation modulates the resolution phase of inflammation [3, 4]. In addition, the efficient clearance of extravasated leukocytes that have been induced to undergo apoptosis prevents further injury through the release of toxic or immunostimulatory intracellular contents [5]. Importantly, phagocytic clearance of apoptotic leukocytes by macrophages induces release of anti-inflammatory cytokines that promote resolution of inflammation [6] and triggers changes in macrophage behavior that promote tolerogenic responses. However, in situations where prolonged or chronic inflammatory responses occur, leukocytes may not be efficiently cleared by macrophages and consequently undergo secondary necrosis, releasing cytotoxic granule contents and further exacerbating the inflammatory response, potentially leading to the development of autoimmunity [7]. For example, the defective phagocytosis of apoptotic cells that is observed in C1q deficiency contributes to the development of systemic lupus erythematosus [8]. Thus, defining the mechanisms that regulate this important clearance process is essential for understanding the pathogenesis of many inflammatory diseases.

A number of studies indicate that micro-environmental cues, *e.g.* extracellular matrix components and the cytokine repertoire, have a critical role in determining macrophage behavior [9]. It is well established that pro- and anti-inflammatory cytokines differentially activate macrophages. LPS or pro-inflammatory cytokines such as IFN-γ and TNF-α induce "classically activated" macrophages, which can be defined by production of nitric oxide and reactive oxygen species [10, 11]. These macrophages show increased potential for the engulfment and destruction of pathogenic organisms which is reflected in the repertoire of receptors expressed; these receptors include Fc and complement receptors that enable recognition and internalization of immunoglobulin- and complement-opsonized particles [12]. Conversely, in response to cytokines with anti-in-

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The first two authors contributed equally to this manuscript.

Abbreviations: GC: Glucocorticoid **IDMEM:** Iscove's modification of Dulbecco's modified Eagles medium **MDMΦ:** Monocyte-derived macrophage

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flammatory potential such as IL-4 and IL-13, macrophage release of pro-inflammatory cytokines is inhibited, inducing an immunoregulatory phenotype. These macrophages are characterized by increased expression of specific pattern-recognition molecules, such as the mannose receptor [13, 14] and enhanced capacity for endocytosis and antigen presentation [15]. Arginase-1 expression is also enhanced and competes with NO synthases, increasing the metabolism of arginine to ornithine and urea, resulting in a decreased capacity for the production of reactive nitrogen species [16] and a reduced ability for pathogen clearance.

We have recently demonstrated that exposure of peripheral blood monocytes to glucocorticoids (GC), *e.g.* the synthetic glucocorticoid dexamethasone, results in differentiation towards a macrophage phenotype that exhibits a markedly increased phagocytic capacity for apoptotic cells; exposure to mineralocorticoids or sex steroids does not do this [17]. Prolonged exposure of monocytes to GC induced a homogeneous monocyte-derived macrophage (MDMΦ) phenotype consisting of small "rounded" cells with profound changes in their cytoskeletal organization and a loss of actin-containing podosomes [18].

We sought to define the cell surface phenotype and functional repertoires of human monocytes treated with GC and the cytokines IL-4 and IFN-γ. In this manuscript we present evidence that MDMΦ that have differentiated in the presence of dexamethasone exhibit distinct receptor expression patterns and functional status from either IFN-γ- or IL-4-activated MDMΦ. GC exert a dominant effect upon the morphology and adhesive status of MDMΦ following combination treatment with IFN-γ or IL-4. However, we demonstrate that IFN-γ profoundly inhibits the GC-induced capacity to clear apoptotic cells. Indeed, even after exposure of MDMΦ to GC for 24 h, subsequent exposure to IFN-γ inhibited the acquisition of a phagocytic phenotype, indicating that GC-induced alterations in MDMΦ function are reversible. Our data suggest that the cytokine milieu at an inflammatory site will critically determine whether GC induce an MDMΦ phenotype with high capacity to clear apoptotic cells; a phenotype that would favor the resolution of inflammation.

2 Results

2.1 Dexamethasone-augmented MDMΦ phagocytosis of apoptotic cells is inhibited in the presence of IFN-γ

We examined the effects of IFN-γ or IL-4, in combination with dexamethasone, on the phagocytosis of apoptotic neutrophils by MDMΦ. Dexamethasone, or dexamethasone in combination with IL-4, strongly increased the percentage of MDMΦ that phagocytosed apoptotic neutrophils compared with phagocytosis by untreated MDMΦ (Fig. 1). We did not observe a difference in the percentage of MDMΦ that phagocytosed apoptotic cells when comparing IFN-γ-treated and control MDMΦ. However, in the presence of IFN-γ, dexamethasone failed to augment this phagocytosis (Fig. 1).

We assessed the mean fluorescence of phagocytic MDMΦ populations to provide further information about the effects of these treatments on the number of apoptotic cells phagocytosed per MDMΦ (expressed as mean fluorescence of phagocytic cells after subtraction of the mean fluorescence of non-phagocytic cells). Dexamethasone-treated MDMΦ showed increased fluorescence when compared with untreated MDMΦ (the mean±SE was 655±84 compared with 407±111). Phagocytic MDMΦ from IL-4- or IFN-γ-treated populations also showed slightly higher mean fluorescences (510±163 and 650±142, respectively). Interestingly, phagocytic MDMΦ treated with dexamethasone plus IL-4 or dexamethasone plus IFN-γ had lower mean fluorescences of 455±101 and 456±106, respectively.

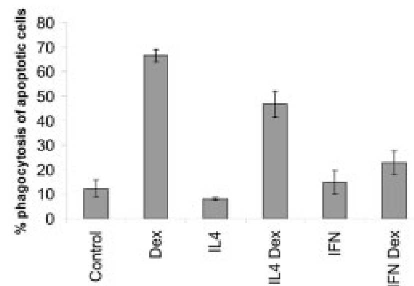

Fig. 1. Dexamethasone-induced phagocytosis of apoptotic neutrophils is reduced in the presence of IFN-γ. The phagocytic ability of MDMΦ cultured in IDMEM containing 10% autologous serum alone (control) or with combinations of dexamethasone, IL-4 or IFN-γ as described in Sect. 4.2 was determined on day 5 by flow cytometric quantification of the percentage of fluorescent MDMΦ following a 60-min incubation with CMFDA-labeled apoptotic neutrophils. Dexamethasone treatment results in a highly phagocytic population of macrophages when compared with untreated macrophages. MDMΦ treated with IFN-γ plus dexamethasone have a phagocytic ability similar to MDMΦ treated with IFN-γ alone, demonstrating that IFN-γ is dominant in determining phagocytic capacity. The results shown here represent the mean±S.E of five separate experiments.

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2.2 Suppression of pro-inflammatory cytokine release by dexamethasone

In view of the dominant effects of IFN-γ on dexamethasone-induced augmentation of phagocytic function, we next examined the release of pro-inflammatory cytokines by treated MDMφ. As would be expected, IFN-γ-treated MDMΦ strongly up-regulated production of TNF-α, IL-1 and IL-6 when compared with untreated MDMΦ or IL-4-treated MDMΦ (Fig. 2). Although production of most cytokines was effectively inhibited by dexamethasone alone, co-culture of MDMΦ in the presence of IL-4 and dexamethasone together produced further inhibitory effects on the production of IL-6.

In contrast to the dominant effects of IFN-γ on phagocytosis by dexamethasone-treated MDMΦ, reduced levels of TNF-α, IL-6 and IL-1 production were found for MDMΦ treated with dexamethasone in combination with IFN-γ, suggesting that dexamethasone remained able to suppress the inflammatory cytokine production that was induced by IFN-γ.

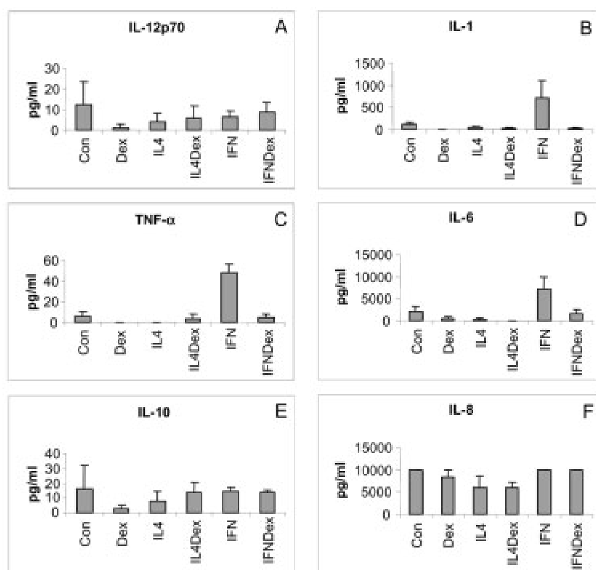

Fig. 2. Cytokine profiles of MDMΦ treated with dexamethasone ± IL-4 and IFN-γ for 5 days. Adherent monocytes were cultured for 5 days either in 10% autologous serum alone or with combinations of dexamethasone, IL-4 and IFN-γ . MDMΦ supernatants were collected after 5 days and analyzed using an inflammation cytokine bead array kit (Becton Dickinson). Cytokine levels were determined by extrapolation from standard curves using cytokine bead array analysis software. Results shown are the cytokine levels (mean±S.E) from three separate experiments using MDMΦ from three different, normal donors.

2.3 MDMΦ cultured in dexamethasone exhibit distinct surface receptor profiles

We next compared cell surface receptor profiles of MDMΦ cultured with dexamethasone, IFN-γ or IL-4 alone, or with combinations of treatments. As would be expected, the high affinity receptor for immunoglobulin, CD64 (FcγRI), was strongly up-regulated by IFN-γ-treated MDMΦ when compared with untreated MDMΦ (Fig. 3A) and was also augmented when MDMΦ were exposed to IFN-γ in combination with dexamethasone. In contrast, CD64 was down-regulated by dexamethasone when used alone (35% reduction in levels of expression) and markedly down-regulated by IL-4 (70% reduction in expression).

Dexamethasone-treated MDMΦ expressed slightly reduced levels of HLA-DR when compared with untreated cells; MDMΦ exposed to IL-4 or IFN-γ showed increased expression compared with untreated cells (Fig. 3B). Interestingly, up-regulation of expression of HLA-DR was not observed when MDMΦ were treated with IFN-γ or IL-4 in combination with dexamethasone, suggesting a suppressive effect of dexamethasone

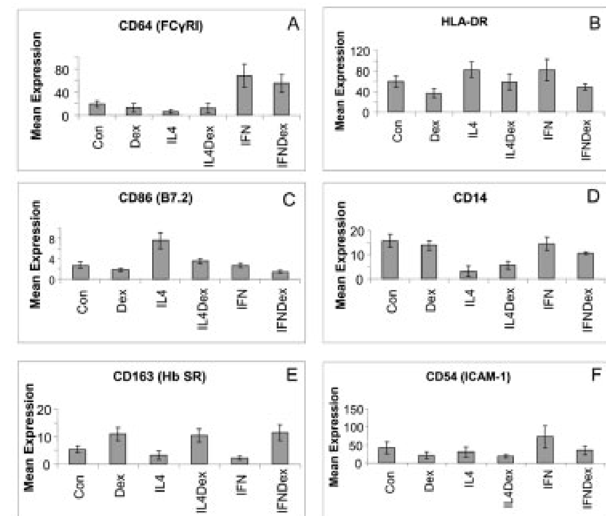

Fig. 3. Effects of dexamethasone, IL-4 and IFN-γ on macrophage cell surface receptor expression. Adherent monocytes were cultured for 5 days either in 10% autologous serum or with combinations of dexamethasone, IL-4 and IFN-γ. The surface phenotype was assessed by indirect immunofluorescence and flow cytometry using control IgG1 mAb, CD64 mAb (A), HLA-DR mAb (B), CD86 mAb (C), CD14 mAb (D), CD163 mAb (E) and ICAM-1 mAb (F). Results, corrected for the mean fluorescence intensity recorded for the IgG1 control, are expressed as the average mean fluorescence (±S.E.) recorded for each antibody from a minimum of three separate donors.

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(Fig. 3B). IL-4-treated MDMΦ expressed approximately 3-times the levels of CD86 when compared with IFN-γ-treated or dexamethasone-treated MDMΦ (Fig. 3C). Expression of the adhesion molecule ICAM-1 on MDMΦ was increased following IFN-γ treatment, consistent with published data [19]. In contrast, both IL-4 and dexamethasone reduced ICAM-1 expression and IFN-γ-induced up-regulation of ICAM-1 was suppressed following combined IFN-γ and dexamethasone treatment (Fig. 3F).

MDMΦ expression of CD14 was not affected by dexamethasone treatment alone. In contrast, IL-4-treated macrophages exhibit markedly reduced CD14 expression, consistent with the induction of an immature "dendritic-like" cell phenotype (Fig. 3D). Reduced expression of CD14 expression was also seen following treatment with IL-4 and dexamethasone in combination, suggesting that the effects of IL-4 predominate (Fig. 3D). The divergent effects of IL-4 and dexamethasone were also found when expression of the mannose receptor was examined. Dexamethasone and IFN-γ both down-regulated expression of the mannose receptor, whereas IL-4 up-regulated expression (mean fluorescence intensities: control=58.7, IL-4=85.8, dexamethasone=18.4, IFN-γ=4.7) (*n*=2). Expression of the GC-responsive hemoglobin scavenger receptor CD163 [20] was down-regulated following culture in the presence of IL-4 or IFN-γ alone, but increased in dexamethasone-treated MDMΦ (Fig. 3E). Increased CD163 expression levels were also observed following combined treatment with dexamethasone and IFN-γ, again suggesting a dominant effect of dexamethasone.

2.4 Effects of dexamethasone and cytokines on MDMΦ morphology

Our analysis suggests that suppression of dexamethasone-augmented phagocytosis by IFN-γ is not reflected by surface phenotype alterations, with dexamethasone exerting a dominant effect. Morphological examination demonstrated that a homogeneous population of smaller, less-well-spread MDMΦ was induced by GC when compared with the heterogeneous untreated MDMΦ population (Fig. 4). When cultured in the presence of IL-4 alone, MDMΦ exhibited a highly spread, polarized morphology with pronounced cellular processes and evidence of the formation of homotypic cell aggregates (Fig. 4). A heterogeneous MDMΦ population comprising multinucleated "giant" cells together with smaller macrophages was observed in the presence of IFN-γ (Fig. 4). Culture of MDMΦ in the presence of IFN-γ plus dexamethasone, or IL-4 plus dexamethasone, yielded a population of smaller "rounded" cells similar to those seen with dexamethasone alone. Together these data suggest that dexamethasone exerts dominant effects upon MDMϕ morphology.

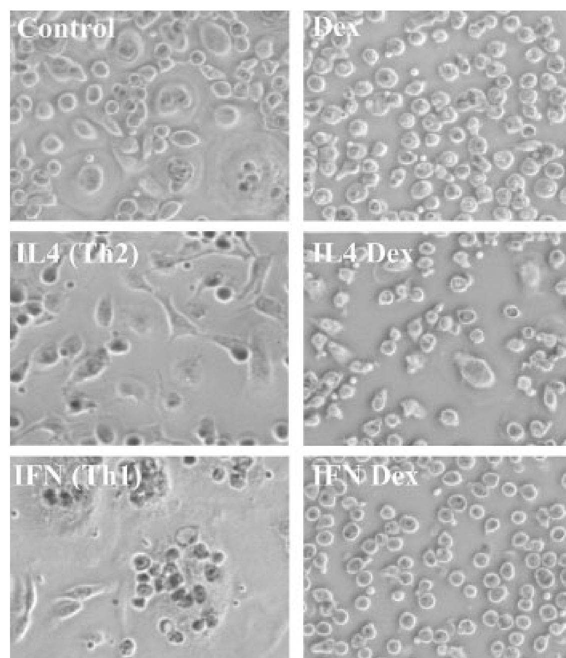

Fig. 4. Dexamethasone is dominant over IFN-γ and IL-4 cytokines in inducing cellular morphology. Adherent monocytes were cultured for 5 days on glass coverslips either in 10% autologous serum or with combinations of dexamethasone, IL-4 and IFN-γ. Representative photomicrographs depict cellular morphology using phase-contrast microscopy with a ×63 objective. Control MDMΦ represent a heterogeneous population of cells containing giant, multinucleated cells as well as smaller mononuclear cells. Dexamethasone-differentiated MDMΦ form a homogeneous population of small, rounded cells. Combined treatment with either IL-4 or IFN-γ plus dexamethasone also yields a homogeneous population of small rounded cells, similar to the dexamethasone-treated population, implying that dexamethasone is dominant over both cytokines in inducing cellular morphological changes.

2.5 Dexamethasone treatment alters adhesion structures in MDMΦ

We next examined the characteristic "podosome-like" adhesion signaling complexes consisting of a punctate actin foci surrounded by a ring of paxillin, vinculin and other cytoskeletal proteins [21] that are present in MDMΦ. Untreated or IFN-γ-treated MDMΦ contain abundant podosomes, whereas these structures were absent from dexamethasone-treated MDMΦ (Fig. 5). IFN-γ-treatment appears to drive differentiation towards

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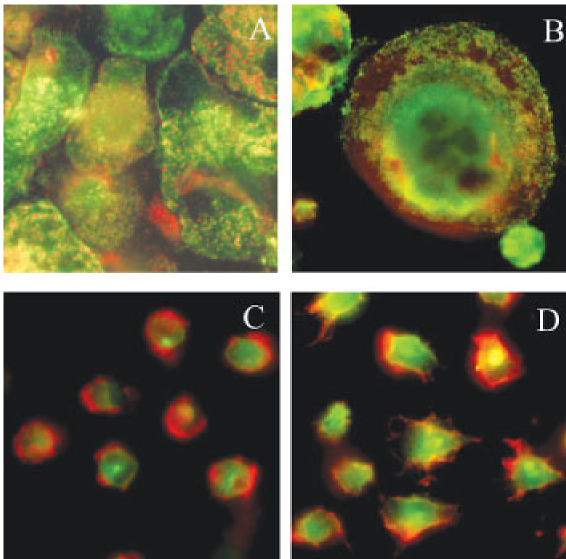

Fig. 5. Dexamethasone is dominant over IFN-γ and IL-4 cytokines in determining the adhesion status of the macrophages. Adherent monocytes were cultured for 5 days on glass coverslips either in 10% autologous serum or with combinations of dexamethasone, IL-4 and IFN-γ. Fixed and permeabilized cells were stained for F-actin using rhodamine phalloidan and paxillin (paxillin mAb and FITC-conjugated anti-mouse-IgG), to visualize cytoskeletal organization. In the representative photomicrographs shown, podosomes are characterized by a foci of actin (red) which is surrounded by a ring of paxillin (green). Untreated control MDMΦ contain abundant podosomes (A). Podosomes are also present in IFN-γ-treated MDMΦ (B). By contrast, MDMΦ differentiated in the presence of dexamethasone alone or dexamethasone plus IFN-γ (C and D, respectively) do not contain podosomes. These data demonstrate the dominance of dexamethasone over IFN-γ in determining the adherence status of the macrophages.

a multinucleated phenotype and the podosomes in these "giant" cells were particularly striking, where a cortical ring of podosomes were formed, similar to the resorption zone described for osteoclasts (Fig. 5B) [22]. Interestingly, IL-4 induces a polarized morphology with podosomes localized at the leading edge of the cell (data not shown). Podosomes were absent from MDMΦ cultured in the presence of dexamethasone (Fig. 5C) or IFN-γ plus dexamethasone (Fig. 5D), in keeping with morphological observations. Podosomes were also absent from MDMΦ treated with IL-4 plus dexamethasone (data not shown). These data confirm the dominant effect of dexamethasone in terms of both morphology and cytoskeletal organization in MDMΦ.

2.6 The mechanism of IFN-γ suppression of phagocytosis

In view of the finding that the first 24 h of culture was critical for GC-mediated augmentation of MDMΦ phagocytosis [18], we sought to investigate whether IFN-γ could over-ride the effects of dexamethasone after this initial 24-h culture period. MDMΦ were exposed to either dexamethasone or IFN-γ for the first 24 h of culture, followed by culture for 4 days in medium alone, dexamethasone or IFN-γ. MDMΦ phagocytosis of apoptotic cells was augmented when monocytes were cultured in medium for 24 h followed by the addition of dexamethasone although less than when dexamethasone was present during the first 24 h (Fig. 6A). Addition of IFN-γ alone failed to alter the phagocytic capacity of MDMΦ when compared to untreated control MDMΦ. However, if monocytes were incubated with IFN-γ for 24 h prior to the addition of dexamethasone, augmentation of MDMΦ phagocytosis of apoptotic cells was not observed. Surprisingly, if monocytes were incubated with dexamethasone for the first 24 h of culture followed by addition of IFN-γ, the phagocytic capacity was also not augmented.

In view of the "switch off" of phagocytosis following IFN-γ treatment 24 h after dexamethasone treatment of monocytes, we next sought to test whether engagement of IFN-γ signaling in GC-treated MDMΦ would also inhibit phagocytosis. MDMΦ were treated with dexamethasone at day 0 and then IFN-γ was added on day 1, day 3 or day 6 (on day 6, it was added either 1 h or 3 h prior to the phagocytosis assay) (Fig. 6B). The results show that treatment with IFN-γ for a few hours prior to phagocytosis did not inhibit phagocytic ability when compared with MDMΦ treated with dexamethasone alone. Indeed, the suppressive effect of IFN-γ upon GC-augmented phagocytosis was less pronounced the longer the macrophages were exposed to dexamethasone before IFN-γ was added. These results demonstrate that the IFN-γ-mediated suppression of augmented phagocytosis most likely induces changes in MDMΦ phenotype rather than being a direct consequence of engagement of IFN-γ-mediated signaling pathways.

3 Discussion

One of the most important observations in our study is that IFN-γ strongly suppressed the dexamethasone-induced augmentation of apoptotic cell phagocytosis by macrophages (Fig. 1, 6A and 6B). Indeed, IFN-γ retained the ability to inhibit GC-augmented phagocytosis even when monocytes had been pre-treated with dexamethasone for 24 h. Exposure of MDMΦ to IFN-γ at later time points following dexamethasone treatment failed to

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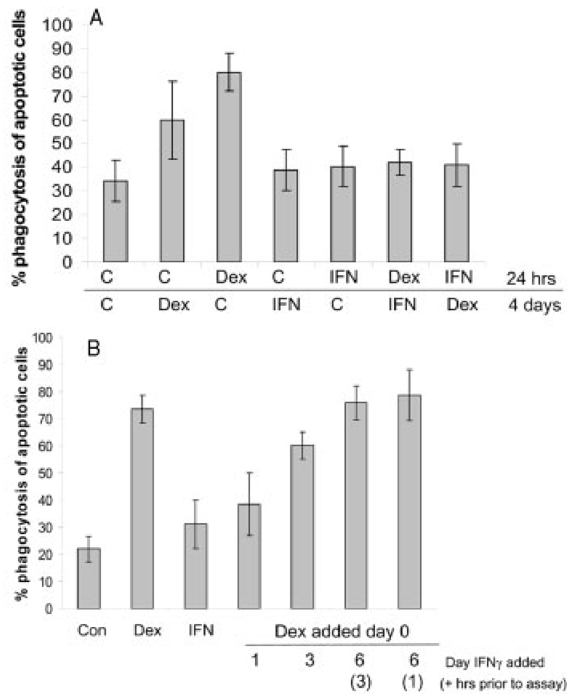

Fig. 6. IFN-γ reverses and blocks dexamethasone augmentation of MDMΦ phagocytosis of apoptotic cells. (A) Adherent monocytes were cultured for the first 24 h in IDMEM containing 10% autologous serum alone, dexamethasone or IFN-γ. Cells were washed and then treated for the remaining 4 days in IDMEM containing 10% autologous serum, dexamethasone or IFN-γ to give a checkerboard of combination treatments (24 h / 4 days). The phagocytic ability of the MDMΦ populations was then determined by flow cytometry as described in Sect. 4.5. Results are shown as the mean percentage of macrophage phagocytosis ± S.E. from at least three separate experiments. Augmentation of MDMΦ phagocytic ability was observed following exposure to dexamethasone for the first 24 h only, or for the final 4 days of culture when compared with untreated MDMΦ. In contrast, exposure to dexamethasone followed by IFN-γ, or to IFN-γ prior to dexamethasone, resulted in a significant down-regulation in MDMΦ phagocytic ability, demonstrating that IFN-γ can both reverse and block the dexamethasone-augmented phagocytosis of apoptotic neutrophils. (B) Adherent monocytes were cultured in IDMEM containing 10% autologous serum and dexamethasone, with the subsequent addition of IFN-γ on day 1, or day 3, or 1 h or 3 h prior to the phagocytosis assay on day 6. The phagocytic ability of the MDMΦ populations was then determined by flow cytometry. Results are shown as mean percentage of macrophage phagocytosis ± S.E. from at least three separate experiments. MDMΦ that were cultured in dexamethasone and exposed to IFN-γ on day 1 showed a suppression in phagocytic ability compared with MDMΦ treated only with dexamethasone. The addition of IFN-γ on day 3 led to only a slight reduction in phagocytic ability and MDMΦ exposed to IFN-γ for 1 h or 3 h only showed no inhibition in phagocytic ability.

reverse the augmentation of phagocytosis, suggesting that the inhibition was not due to a direct effect of IFN-γ-mediated signaling. Previous studies have also suggested that the cytokine environment may regulate macrophage phagocytosis of apoptotic cells. When human MDMϕ were given a 4-h exposure of IFN-γ after 4 days of culture, they displayed augmented phagocytosis of apoptotic cells [23]. In contrast, phagocytosis of apoptotic cells by rat bone-marrow-derived macrophages was inhibited by treatment for 48 h with IFN-γ [24]. Our data suggest that although IFN-γ-treated MDMΦ show some augmentation of phagocytosis of apoptotic cells, acquisition of a pro-phagocytic macrophage phenotype in response to GC is lost in the presence of this cytokine.

One important implication of this work is that GC may fail to stimulate macrophage phagocytosis in a Th1 environment. Thus, one of the potential beneficial effects of GC-treatment, *i.e.* facilitation of apoptotic cell clearance, may be inhibited by IFN-γ. Interestingly, a Th1 cytokine environment has been suggested to contribute to corticosteroid resistance in diseases like rheumatoid arthritis [25]. This may also impact on the use of IFN-γ to boost host defense against infection via macrophage activation following severe injury. Many trials of IFN-γ therapy have failed to show a clear improvement in patient outcome [26]. Whether IFN-γ inhibits the ability of endogenous GC to regulate macrophage phagocytosis is not known. Interestingly, expression of 11β hydroxysterone dehydrogenase is rapidly induced during monocyte differentiation [27] concomitantly with acquisition of capacity for phagocytosis of apoptotic cells [28].

We have previously suggested that the altered adhesion observed in dexamethasone-treated MDMΦ may be linked to augmentation of phagocytic capacity. On the basis of morphological appearance, we would have predicted that co-incubation with either dexamethasone plus IFN-γ or dexamethasone plus IL-4 would give rise to a highly phagocytic phenotype. However, our data provide the first evidence for dissociation between morphology/adhesion status and phagocytic capacity. Thus, although cells treated with IFN-γ plus dexamethasone exhibit a small and rounded cell morphology without distinct podosome adhesion structures, the augmented phagocytic capacity of dexamethasone-treated macrophages is not shared by MDMΦ treated with IFN-γ plus dexamethasone.

For other characteristics of MDMΦ that we examined, dexamethasone exerted dominant effects over IFN-γ, inhibiting IFN-γ-driven pro-inflammatory cytokine production and morphological appearance. Expression of CD163 was up-regulated by dexamethasone even in the presence of IFN-γ and the IFN-γ-dependent up-

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regulation of ICAM-1 and HLA-DR was attenuated by dexamethasone. Our phenotype analysis also revealed that the expression levels of the mannose receptor and CD14, both previously implicated as phagocytic receptors [29, 30], show no correlation with the increased phagocytic ability of dexamethasone-treated MDMΦ. The mannose receptor was down-regulated following GC treatment and CD14 showed no change in expression when compared with untreated MDMΦ, potentially excluding the involvement of these receptors in the augmented phagocytosis seen following GC treatment of MDMΦ.

Our data support the suggestion that monocytes treated with the GC differentiate to a phenotype distinct from the classically activated IFN-γ- treated MDMΦ or an "alternatively" activated IL-4-treated MDMΦ. We found that the mannose receptor, a well-defined marker of alternative activation [13], was expressed at higher levels on IL-4-treated MDMΦ, but was reduced on MDMΦ cultured in dexamethasone. Several other receptors exhibit differential expression on IL-4- and GC-treated MDMΦ, including HLA-DR, CD86 and CD163. MDMΦ that have differentiated in GC or IL-4 exhibit distinct phenotypes, the latter inducing a polarized appearance with many cellular processes that is very different from the "rounded" appearance of dexamethasone-treated cells. Most importantly in terms of capacity for clearance of apoptotic cells, IL-4 fails to induce a MDMΦ phenotype that is capable of efficient phagocytosis of apoptotic cells. IL-4-treated monocytes would have the potential for antigen presentation and immunomodulation (high HLA-DR and CD86), whereas dexamethasone-treated MDMΦ exhibit "anti-inflammatory" characteristics with reduced capacity for immunostimulation because of down-regulation of HLA-DR and CD86.

Although both IL-4 and GC may be involved in countering pro-inflammatory factors, their impact on macrophage function would have distinct consequences in the outcome of an inflammatory response. For example, discordant regulation of the capacity of macrophages for antigen presentation and apoptotic cell clearance may be critical for "safe" disposal of apoptotic and necrotic cells without the potential for "cross-presentation" of autoantigen and the induction of an autoimmune response. Together, these data suggest that the functional repertoire of IL-4- or dexamethasone-treated MDMϕ is distinct. GC promote a "tolerogenic" phenotype similar to that reported to be induced by IL-10 [31, 32]. Interestingly, like GC, IL-10 up-regulates expression of CD163, although the mechanism appears to be distinct [33]. Preliminary experiments indicate that treatment of monocytes with anti-IL-10 antibodies fails to block the development of the phenotype we observe following treatment with GC (S. J. Heasman, unpublished observations). However, IL-10-cultured MDMΦ show some augmentation of phagocytic capacity for apoptotic cells (data not shown), suggesting that there may be parallels in the mechanism of action of GC and IL-10.

In summary, the role that particular cytokines, steroids and lipid mediators play individually, and in concert, will critically determine the balance between pathogen clearance, resolution of inflammation, tolerance induction and wound healing. In particular, data presented here suggest that defining the interplay between GC and IFN-γ in the regulation of macrophage function may unveil novel therapeutic targets for treatment of inflammatory disease.

4 Materials and methods

4.1 Antibodies and other reagents

All chemicals were from Sigma (Poole, Dorset, GB) unless otherwise stated. Antibodies were used at saturating concentrations as determined by titration in indirect immunoassays and flow cytometry as follows: HLA-DR (clone WR18, IgG2a, used at 1/100, Serotec, Oxford, GB), CD14 (clone UCHM1, IgG2a, provided by Dr. Peter Beverley, Edward Jenner Institute for Vaccine Research, Compton, GB), C54 (clone 15.2, provided by Dr. Nancy Hogg, Cancer Research UK, London, GB), CD64 (clone 10.1, IgG1, used at 1/100, provided by Dr.Nancy Hogg), CD86 (clone BU63, IgG1, used at 1/50, Caltag, B-D Biosciences, GB), CD163 (clone Bermac, IgG1, used at 1/35, Dako, Oxford, GB), mannose receptor (clone 19.2, IgG1, Serotec), and IgG1 control (MOPC, mouse IgG1 plasmacytoma, obtained from ECACC, GB).

4.2 Cell isolation and culture

Mononuclear cells (MNC) and polymorphonuclear cells were isolated from human blood by dextran sedimentation and centrifugation over discontinuous Percoll™ (Amersham Pharmacia Biotech, Buckingham, GB) gradients as previously described [34]. The MNC (typically 15–20% monocytes by morphological analysis of cytocentrifuge preparations or flow cytometric determination on the basis of laser scatter properties and CD14 reactivity) were resuspended at $4{\times}10^6$ /ml in Iscove's modification of Dulbecco's modified Eagles medium (IDMEM; Life Technologies, Paisley, GB) and enriched for monocytes by selective adherence to 48-well (0.5 ml/well) or 6-well (4.0 ml/well) tissue culture plates for 1 h at 37°C in 5% CO_2. Adherent monocytes were washed three times and then allowed to differentiate for 5 days in IDMEM containing penicillin/streptomycin and 10% autologous serum prepared by recalcification of platelet-rich plasma. Washed adherent MDMΦ consisted of >90%

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CD14-positive cells at day 5 as assessed by flow cytometry. In our hands GC did not induce monocyte apoptosis as reported by Schmidt et al. [35]. Our unpublished data suggest that autologous serum over-rides the pro-apoptotic effects of GC on monocytes. As detailed in the text, dexamethasone was added to a final concentration of up to 1 μm, whereas recombinant IL-4 and recombinant human IFN-γ (both obtained from R&D Systems, Minneapolis, USA) were used at 10 ng/ml.

Isolated polymorphonuclear cells (typically 95–98% neutrophils by morphological examination of cytocentrifuge preparations) were resuspended at 20×10^6 cells/ml cells in IDMEM and labeled with the fluorescent cell tracker dye carboxymethylfluorescein diacetate (CMFDA; Molecular Probes, Leiden, The Netherlands), 2 μg/ml for 15 min at 37°C in 5% CO_2. Cells were then washed and cultured in 75 mm tissue culture flasks (Nunc, Fisher Scientific, Leicestershire, GB) for 18–24 h at 4×10^6/ml in IDMEM containing 10% autologous serum to induce spontaneous apoptosis. Microscopic examination of nuclear morphology of cytocentrifuge preparations of cultured cells was used to assess levels of apoptosis and neutrophils were typically 50–60% apoptotic with 15–20% necrotic cells present, as determined by dual annexin V / propidium iodide staining and flow cytometry.

4.3 Flow cytometric determination of cytokine release

Supernatants from MDMΦ that had been cultured for 5 days were analyzed for the presence of IL-8, IL-6, IL-1β, IL-10, TNF-α and IL-12p70 using the Human Inflammation BD Cytometric Bead Assay (Becton Dickinson, Oxford, GB) as described by the manufacturer's assay protocol. Briefly, 50 μl samples of cell supernatant were incubated with capture beads to the six cytokines and a PE cytokine detection reagent for 3 h at room temperature. Samples were then washed once and 6000 events were acquired using a FACSCalibur flow cytometer (Becton Dickinson). Analysis of the median FL-2 fluorescence associated with bead populations defined by FL-3 labeling was made using Cellquest software (Becton Dickinson).

4.4 Flow cytometric analysis of surface receptor expression

MDMΦ obtained after 5 days of culture were washed in Hanks' balanced salt solution (HBSS) and incubated with 5 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS on ice for 15 min to detach the cells from the wells. The detached MDMΦ were centrifuged at 220×g and washed in Ca^{2+}/Mg^{2+}-free PBS containing 0.2% BSA and 0.1% sodium azide (flow buffer). The pelleted cells were then incubated on ice for 30 min with saturating concentrations of monoclonal antibodies to cell surface determinants. The cells were then washed twice in flow buffer and incubated for a further 30 min on ice with FITC-labeled $F(ab')_2$ fragments of goat anti-mouse-immunoglobulin (Dako; used at 1/50). Following two further washes in flow buffer, the labeled cells were analyzed using either an EPICS XL (Beckman Coulter, High Wycombe, GB) or FACSCalibur flow cytometer (Becton Dickinson) after acquiring 5000–8000 events per sample. Post-acquisition analysis was performed either using Cellquest (Becton Dickinson) or EXPO32 (Beckman Coulter).

4.5 Quantitation of phagocytosis of apoptotic cells

Monocytes were cultured for 5 days in 48-well plates as described above either in the presence of 10% autologous serum alone (control), or in the presence of dexamethasone, IL-4 or IFN-γ, or combinatorial treatments at the concentrations detailed in the text. MDMΦ were gently washed in Ca^{2+}/Mg^{2+}-free HBSS and phagocytosis of apoptotic neutrophils was then performed as described previously [36]. Briefly, MDMΦ were co-incubated with 0.5 ml of CMFDA-labeled apoptotic neutrophils at 4×10^6/ml for 1 h at 37°C in 5% CO_2 after which the neutrophils were gently aspirated and 0.25 ml of trypsin/EDTA added. Following incubation at 37°C in 5% CO_2 for 15 min and then at 4°C for 15 min, MDMΦ were detached by vigorous pipetting. Flow cytometry was then used to determine the proportion of phagocytic macrophages (gated on the basis of forward- and side-scatter properties) and the percentage of FL-1-labeled MDMΦ was determined.

4.6 Indirect immunofluorescence analysis of macrophage cytoskeleton

Monocytes were plated on sterile coverslips in 24-well plates at 4×10^6/ml. After 5 days of differentiation, adherent MDMΦ were fixed in 2 ml of 3% (w/v) para-formaldehyde at room temperature for 20 min. Coverslips were then washed three times in Ca^{2+}/Mg^{2+}-free PBS and free aldehyde groups were then quenched with 50 mM NH_4Cl/PBS at room temperature for 15 min. After three further washes in PBS, cells were permeabilized using 0.1% Triton X-100 for 4 min. Following a further three washes in PBS the coverslips were incubated for 10 min in heat-inactivated AB serum (1/10) to block non-specific antibody binding to Fc receptors. To visualize podosomes within MDMΦ, cells were then incubated on ice for 30 min with mAb specific for paxillin (BD Transduction Labs, Belgium). The cells were washed and then labeled by incubating for 30 min with an Alexa-488 goat anti-mouse-immunoglobulin antibody (1/400 in PBS; Molecular Probes). After a further three washes cells were incubated with rhodamine phalloidin (1/800 in PBS; Molecular Probes). The coverslips were then mounted onto slides using an antifadent mounting medium (Molecular Probes) and examined under oil immersion microscopy using a ×63 objective using an Axiovert S100 immunofluorescence microscope with Coolsnap LCD camera and Openlab image acquisition software.

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