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Modulation of human Th17 cell responses through complement receptor 3 (CD11 b/CD18) ligation on monocyte-derived dendritic cells

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Abstract

Objective—Apoptotic cell receptors contribute to the induction of tolerance by modulating dendritic cell function following the uptake of apoptotic cells or microparticles. Dendritic cells that have bound or ingested apoptotic cells produce only low amounts of pro-inflammatory cytokines and fail to prime effector T cell responses. Specifically, ligation of the apoptotic cell receptor CR3 (CD11 b/CD18) on human monocyte-derived dendritic cells (moDC) down-modulates proinflammatory cytokine secretion, but the consequences for human Th17 cell homeostasis and effector responses remain unknown. Here, we aimed to establish whether CD11b-ligated moDC modulate Th17 cell effector responses to assess their potential for future use in moDC-based suppressive immunotherapy.

Methods—We generated a bead-based surrogate system to target CD11b on monocyte-derived human dendritic cells and examined the effects of CD11b ligation on Th17-skewing cytokine secretion, priming, expansion and functional plasticity in DC/T cell co-culture systems at the polyclonal and monoclonal level.

Results—We show that Th17 cell expansion within the human memory CD4⁺ T cell compartment was efficiently constricted by targeting the CD11b receptor on moDC. This tolerogenic capacity was primarily dependent on cytokine skewing. Furthermore, ligation of CD11b on healthy homozygous carriers of the rs11143679 (ITGAM) variant – a strong genetic susceptibility marker for human systemic lupus erythematosus – also down-modulated the secretion of Th17-skewing cytokines.

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Potential conflict of interest

N.B. is a co-inventor on patents related to dendritic cell generation, function and use in vaccines. N.B. is a consultant/SAB member for Checkpoint Sciences, Dendreon, Merck and Dynavax. N.B. is an extramural member of the Parker Institute for Cancer Immunotherapy.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jaut.2018.05.005>.

Conclusion—Overall, our findings underline the potential of targeted CD11b ligation on human dendritic cells for the engineering of suppressive immunotherapy for Th17-related autoimmune disorders.

Keywords

Human monocyte-derived dendritic cells; Human Th17 cells; Immunotherapy; CD11b; Autoimmunity

1. Introduction

Dendritic cells (DC) are key players in the induction of immunity and the maintenance of tolerance. In their immature state under non-inflammatory, steady-state conditions, DC are proficient phagocytic cells, constantly probing soluble and particulate self and non-self antigens. Upon maturation by bacterial, viral Pathogen-Associated Molecular Patterns (PAMPs) and other stimuli, DCs upregulate costimulatory molecules, and secrete pro- or anti-inflammatory cytokines, migrate to peripheral lymphoid tissues, and efficiently present antigen to T cells [1,2]. These unique properties result in the priming of naive T cells and in the expansion of memory and/or effector T cells, their physical deletion, or functional regulation through anergy or immune deviation. The latter results from selective constriction or expansion of lineage-committed T cell populations over others.

One major source of self-antigens are apoptotic cells (AC) which arise physiologically in vast numbers in healthy and diseased individuals as a result of cell turn-over [3]. AC-derived material is captured by DC through a range of specialized apoptotic cell receptors, including the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, scavenger receptors such as LOX-1, CD36, or the complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) [4–6]. Uptake of AC-associated antigens by DC can result in the cross-presentation to CD8⁺ T cells [7], as well as in enhanced presentation to CD4⁺ T cells. Antigen presentation upon AC phagocytosis may lead to expansion of antigen-specific T cells, or their functional inactivation through diverse mechanisms, depending on the context. In particular, the type of the AC receptor triggered on phagocytosing DC can determine the functional outcome of antigen presentation. Examples of AC receptors capable of mediating immunosuppressive (or tolerogenic) immune responses include the complement receptors CR3 and CR4, while others have weak or no immunosuppressive capacities in vitro, such as $\alpha_v\beta_5$. We have shown previously that the targeted engagement of CR3 (CD11b), but not $\alpha_v\beta_5$, on human monocyte-derived dendritic cells (moDC) efficiently down-modulates the secretion of a variety of pro-inflammatory cytokines, including IL-1 β , IL-12, IL-6 and TNF α , restricts DC maturation in response to TLR signals, and diminishes the effective priming of antigen-specific T cells [8]. Thus, targeted triggering of AC receptors could potentially be harnessed to confer a tolerogenic phenotype to DC (TDC), and used to regulate pathogenic immune responses in autoimmunity, infection and cancer.

Th17 cells are a distinct type of helper T cells that have been implicated in the induction and persistence of autoimmune responses [9]. The recognition of their existence has challenged the conventional concepts of static lineage-commitment into Th1 and Th2 cells, as human

Th17 cells were early shown to possess varying degrees of plasticity, especially with overlap to Th1-type CD4⁺ cells. The human Th17 prototypical phenotype is characterized by the expression of CCR6 in combination with CD161, the secretion of IL-17 (and often cosecretion of IFN γ), and the transcription factor ROR γ t with or without co-expression of Tbet [10]. Th17 cells are essential for the generation of an efficient host response to fungi and a variety of extracellular bacteria [9]. In addition to their physiologic function, they are important mediators of autoimmunity, and can have both pro- and antitumorigenic effects [11–13]. A prominent example for potentially harmful Th17 effects in malignant disease is multiple myeloma (MM) [14], where CD4⁺ Th17 cell-derived IL-17 promotes the growth of myeloma cells, colony formation and adhesion to bone marrow stromal cells (BMSC). Logically, Th17 cells have been proposed as a therapeutic target in MM as well as a range of autoimmune diseases. Models of Th17 development in vitro from naive T cells depend on the blockade of IL-4 and IFN γ , and the presence of combinations of IL-6 and TGF β and/or IL-1, IL-6, IL-21, and - at a later developmental stage - IL-23, which may confer pathogenicity in an autoimmune context and “maintain” the Th17 phenotype [15–17].

Indeed, Th17 cells display varying degrees of pathogenicity, an effect attributed in part to the effector cytokines they secrete such as IFN γ , IL-22, IL-21, IL-10 and GM-CSF [18]. While expressing Ror γ t, human Th17 cells often co-express the Th1 transcription factor Tbet and over time, secrete IFN γ . They ultimately depend on IL-23 for their maturation and pathogenicity [15,16]. Secretion of large amounts of GM-CSF has been linked to pathogenicity of Th17-mediated responses in autoimmunity, and is IL-23 dependent [19,20]. IL-22, and IL-21, while highly pleiotropic in their effects, are associated with different Th17 profiles, the former underlying a more aggressive, and the latter a more tolerogenic phenotype in Th17 cells [18]. In mouse models, IL-6 and TGF β are crucial for the development of Th17 cells and IL-17 secretion, but only Th17 cells generated in the presence of IL-23 are highly pathogenic [21].

Limiting the expansion and/or priming of Th17 cells is a major treatment strategy for autoimmunity and possibly some hematologic malignancies, with an increased occurrence of autoimmune conditions such as rheumatoid arthritis, ankylosing spondylitis, polymyositis, lupus and others [22,23].

Since the differentiation and expansion of Th17 cells is highly regulated by DC- and T-cell derived cytokines, TDC with decreased IL-1 β , IL-6, and IL-23 secretion may play a dominant role in suppression of pathogenic Th17 responses, and modulation of their activation through AC receptor targeting may be of therapeutic benefit. Along those lines, Ehrchiou et al. [24] showed loss of peripheral tolerance and increased Th17 cell numbers in mice whose antigen-presenting cells (APC) were deficient in CD11b. This suggests failure of Th17 cell-limiting immunoregulation in the absence of this receptor for apoptotic cells. Moreover, genetic studies have established homozygosity for the rs1143679 *ITGAM* variant (encoding the R77H variant of CD11b) as a major risk factor for the development of human SLE - an autoimmune disease in which pathogenic Th17 responses have been implicated [25–27]. Altogether, these data imply an important regulatory role for CD11b in the etiology or development of human disease and suggest that CR3 targeting may be exploited to ameliorate autoimmune manifestations.

In this study we assessed the impact of CD11b-ligated moDC on human Th17 cell responses. To this end, we developed a novel surrogate system for specific CD11b ligation and applied it to investigate the down-modulation of cytokines essential to Th17 proliferation, maintenance and pathogenicity (IL-23) and examined the effects of CD11b-ligated moDC on the expansion of the human CD4⁺ memory T cells, the bulk of IL-17 secreting cells. We demonstrate that CD11b targeting on DC potently suppresses their secretion of Th17 inducing cytokines, resulting in decreased expansion of Th17 cells, both in normal donors and in lupus-prone rs1143679 ITGAM variant carriers.

2. Materials and methods

2.1. moDC generation, culture and activation

Human moDC were generated according to our published and patented protocol [28]. Briefly, PBMC were obtained through centrifugation over Ficoll from healthy human donors supplied by the New York Blood Center or the Feinstein Institute for Medical Research. PBMC were then plated at 4×10^6 cells/10 mL/dish on polystyrene tissue culture-coated flasks in RPMI with 5% PHS AB (Genentech), and 5% HEPES for 1–2 h at 37°, 5% CO₂. The cell fraction adherent to plastic was then washed three times with RPMI, before incubation with media substituted with 300 IU/mL rhIL-4 (R&D Systems), and 100 IU/mL rhGM-CSF for 5 days. Cells were fed with equal amounts of IL-4 and GM-CSF on days 2, and 4. On day 5, immature moDC were harvested, and washed in RPMI twice. moDC generated in this way represent more than 95% of live cells in the culture. Cells were then matured or not matured with lipopolysaccharide (LPS) (100 ng/mL) and muramyl dipeptide (MDP) (10 µg/mL), or peptidoglycan (PGN) (10 µg/mL) for 16 h, washed twice in RPMI and used for downstream experiments.

2.2. Generation of a bead-based ApoS-system for CD11b ligation on moDC

Dynabeads® Pan Mouse IgG (Invitrogen) were incubated with anti-CD11b (I-domain specific clone ICRF 44, Biolegend) or IgG (Biolegend) for 30 min at room temperature. Immature moDC were then incubated with antibody-coated beads for 30 min at 4 °C at a 5:1 bead to cell ratio and subsequently further incubated at 37 °C under light rotation before plating in 96 well plates at 100,000 cells/well and stimulation with TLR agonists as described. In some experiments, beads were ligated with $\alpha_v\beta_5$ (Millipore) as an additional control.

2.3. Assessment of cytokine secretion profile from moDC

The concentration of inflammatory cytokines was measured using cytometric bead array [CBA, (BD Biosciences)] in supernatants of moDC in the absence or presence of CD11b-ligation, and with or without TLR stimulation for 16 h. IL-23 concentration was assessed by ELISA (Invitrogen) as described above.

2.4. T cell enrichment and culture

PBMC were isolated as described. CD4⁺ memory T cells were enriched from healthy human donor PBMC in 2% PHS/1.5mM EDTA/ Dulbecco's PBS through cell sorting with dextran-coated magnetic particles using bispecific tetrameric antibody complexes (EasySep™,

Stemcell) to CD8, CD14, CD16, CD19, CD20, CD36, CD45RA, CD56, CD123, TCR γ S according to the manufacturers' instructions. In selected experiments, naive CD4⁺ T cells were isolated (EasySepTM, Stemcell) from either human peripheral or cord blood-derived PBMC. All T cell cultures were performed in RPMI supplemented with 1 mM HEPES (Life Technologies), 2mM glutamine (Sigma), streptomycin (100 IU/mL)/Penicillin 100 μ g/mL, and 5% PHS AB (Gibco) at T 37 °C, and CO₂ 5%. For co-culture studies T cells were cultured with moDCs for a duration of 12–14 days. IL-2 (30 IU/mL) was added on day 3 to the culture system to maintain cultures.

2.5. Intracellular cytokine staining

To evaluate cytokine production, T cells were re-stimulated with PMA/Ionomycin for a total of 6h. After the initial 1.5 h of stimulation, 10 μ g/mL brefeldin A was added. 4.5 h later, cells were surface stained for CD4, CD3, fixed in 4% PFA for 10 min at room T, washed in PBS and permeabilized in 0.1% saponin buffer, then stained for intracellular cytokines. Intracellular cytokine staining (ICS) was performed for assessment of the frequency and/or the number of IL-17 A, IFN γ (Fig. 1) and IL-4 producing cells in all cases. In some experiments where the modulation of Th17 effector cytokines from Th17 clones was being evaluated, cells were also stained for GM-CSF, IL-22, IL-21 and IL-10. Intracellular staining for Ror γ t was also performed on resting T cells in some experiments.

2.6. Transwell experiments

24-well transwell tissue culture plates with inserts of a pore size of 0.4 μ m (Costar transwell permeable support) were incubated with media overnight. Live moDC (n = 60,000) were either cultured in the insert (no DC-T cell contact) or co-cultured with T cells (n = 600,000) in the tissue culture chamber of the system (Fig. 2a i,ii). In a third condition, supernatant harvested from 200,000 PGN-stimulated moDC after 16 h was introduced into the insert (Fig. 2a iii). T cells were not stimulated in any contact-dependent form in conditions without moDC-T cell contact.

2.7. Th17 cell cloning

Baseline-frequency of Th17 cells in healthy donor PBMC was assessed by surface staining for CCR6 and CD161. PBMC were stimulated with Cytostim (Miltenyi Biotec) overnight and IL-17 secreting cells isolated through a IL-17 secretion capture assay (Miltenyi Biotec), and subsequently cloned through limiting dilution with allogeneic, irradiated (35 Gy) feeder PBMC in the presence of 150 IU/mL IL-2 and phytohemagglutinin-L (Sigma). Clonal expansion was assessed by ICS for IL-17 A, IL-4, and IFN γ secretion, and in some experiments in addition for GM-CSF, IL-22, IL-21 and IL-10. Irradiated feeder PBMC used for expansion die off in a few days, and expanding T cell clones comprise more than 99% of live cells. On this population, monoclonality was confirmed by TCR V β staining (IOtest Beta Mark TCR, Beckman Coulter) (Supp Fig 1).

2.8. Statistical analysis

Data were analyzed using Student's t-test. For experiments comparing more than 2 conditions ANOVA was used with Tukey post-test for pairwise comparison. Unless stated otherwise, experiments shown depict a representative experiment out of three or more.

3. Results

3.1. Generation of a moDC-based Th17-inducing experimental system

In order to examine the effects of CD11b-ligation on DC, we first established a model system to generate Th17 cells in vitro. Antigen Presenting Cells (APC) can prime and boost Th17 cells using TLR agonists and bacterial products [29–31]. LPS stimulation through TLR4 induces the p19, p35 and p40 subunits to produce bioactive IL-12 and IL-23, whereas PGN induces IL-23 but no IL-12p70, through TLR2 and NOD2 activation [32]. Furthermore, PGN and its degradation product MDP induce NALP3-mediated activation of caspase 1 and IL-1 β secretion [33]. We co-cultured TLR-stimulated moDC, with naive cord-blood CD4⁺ T cells in the initial absence (first 3 days of co-culture), and then in the presence of 30 IU/mL of IL-2. After 2 weeks, intracellular cytokine staining (ICS) was performed to detect IL-17 and IFN γ . We also compared side by side moDC to TLR-stimulated purified primary conventional DC (cDC) or monocytes (classical CD14⁺/CD16⁻ and non classical CD16⁺) for their capacity to stimulate Th17 cells expansion (Supp Fig 2). Neither moDC, cDC or monocytes primed Th17 responses from naive CD4⁺ T cells (no blocking antibodies to IFN γ and/or IL-4 were used in our system). Instead moDC primarily induced a Th1 phenotype (Fig. 1 and Supp Fig 2) upon TLR2/4/NOD2 and TLR3 stimulations (peptidoglycan, LPS-MDP, p (I:C). IFN γ secreted during naive T cell priming in these conditions may impede differentiation of Th17 cells.

Considering that Th17 cells are mostly found in the effector-memory compartment of CD4⁺ T cells [12,34], we examined Th17 expansion from CD4⁺ memory cell populations in the presence of TLR stimulated moDC. Therapeutic intervention is also likely to target memory T cells, rationalizing this approach to modulate Th17 cell function. Co-cultures of TLR stimulated moDC and CD4⁺ memory T cells yielded high numbers of Th17 cells after 2 weeks, with percentages of IL-17 single positive cells ranging between 5 and 30% and IL-17/IFN γ -double positive cells ranging up to 50% (Fig. 1). moDCs were matured for this purpose with either TLR2 (PGN) or TLR4/NOD2 (LPS + MDP) ligands. We observed large inter-individual variance in the number of IL-17 secreting cells, however moDC activated by these agonists reliably induced Th17 expansion in more than 15 experiments, as described in the literature [34]. Expansion of memory Th17 cells during the culture was shown by CFSE labeling of the T cell starting population, as evidenced by decreased CFSE labeling in IL-17 secreting cells after 14 days (Supp Fig 3, left). Thus, PGN- and LPS + MDP-stimulated moDC very efficiently expand memory Th17 cells.

3.2. moDC-derived cytokines are the primary determinants of memory Th17 expansion

Th17 expansion may be due to the increased secretion of Th17-skewing cytokines, or the consequence of direct moDC-T cell interaction through co-stimulation. To distinguish between these possibilities, we used a transwell cell culture system (Fig. 2) containing live

moDC in one chamber and CD4⁺ memory T cells in a separate chamber (Fig. 2a). Memory T cells cultured with live PGN stimulated-moDC in the absence of direct cell-cell contact (no co-stimulation, but moDC-derived cytokines present) caused the expansion of Th17 cells to an extent comparable with conditions in which direct moDC-T cell contact was allowed, indicating that soluble factors/cytokines released by DC are sufficient to induce Th17 expansion in this system, comparable to when contact between APC and T cells is allowed. In a third condition, T cells in the bottom chamber were exposed to cytokine-containing supernatant of PGN-stimulated moDC inserted into the inlet while contact-dependent T cell stimulation was absent. In this condition, the supernatant was derived from 3 times as many stimulated DC, and accordingly further increased Th17 expansion, underlining the importance of soluble vs contact-dependent factors for Th17 expansion in the system. These data indicate that cytokines are the main drivers of memory Th17 cell expansion in this system, and that co-stimulation, at least for limited periods of time, is dispensable.

3.3. CD11b-ligation on moDC down-modulates the secretion of Th17- skewing cytokines

We previously demonstrated that CD11b ligation inhibited the secretion of IL-1 β and IL-6 (as well as IL-12 and TNF α) by moDC upon TLR4 ligation [8]. Here, we examined whether CD11b could also affect Th17 differentiation, expansion, and function. We reported previously that biotinylated human red blood cells linked to streptavidin conjugated anti-CD11b antibody, efficiently down-modulated both IL1 β and IL-6 in LPS-stimulated DC [8]. We created a surrogate system to mimic apoptotic cells that specifically ligated CD11b. Pan Mouse IgG-Dynabeads were coupled to anti-CD11b or IgG isotype control antibodies and then incubated for 16 h with moDC, to induce CD11b crosslinking and downstream signaling. Secretion of inflammatory cytokines by moDC was measured upon TLR triggering to monitor the effect of specific CD11b triggering, as compared to isotype-matched antibody-coated beads. CD11b-ligation potently inhibited the secretion of the Th17-inducing cytokines IL1 β , IL-6 and IL-23, upon TLR2 (Peptidoglycan) or TLR4/NOD2 (LPS + MDP) stimulation (Fig. 3) - signals that are of high relevance to Th17 expansion and differentiation. Thus, specific apoptotic cell receptor triggering and more specifically CD11b/CD18, very efficiently suppresses the secretion of Th17-promoting cytokines by moDC upon TLR engagement.

3.4. CD11b-ligated moDC contract human Th17 cell populations

We next investigated the effect of CD11b ligation on Th17 differentiation and expansion by moDC, the main objective of the present study. moDCs were co-cultured with allogeneic CD4⁺ memory cells for 12–14 days after CD11b ligation. CD11b-ligated moDC (TDC) that were subsequently stimulated with PGN, induced the expansion of significantly fewer Th17 cells than PGN-matured control moDC with IgG control ligation (Fig. 4), *i.e.*, we observed an average 20% reduction in the total number of IL17⁺ secreting and an average 35% reduction in IL-17 single positive cells across 10 experiments ($p < 0.01$, *t*-test) (Fig. 4) after 14 days. Reductions in IL-17 secreting cells were observed after 12–14 days of co-culture for most donors but could also be observed after only one week in some donors. This results was highly reproducible across 10 experiments with several DC and T cells donors. Memory Th17 cells strongly proliferated in the presence of PGN-matured moDCs, whereas the relative expansion of Th17 cells was significantly diminished in the CD11b-ligated

conditions (Supp Fig 3). These data demonstrate that CD11b ligation can suppress Th17 memory expansion, an effect likely mediated in part by decreased secretion of Th17-promoting cytokines (IL-1 β , IL-6, IL-23) by TLR-stimulated moDC, as these cytokines enhance proliferation or cytokine secretion of Th17 cells.

3.5. CD11b-ligated moDC do not modulate the Th17 effector phenotype at the single cell level

Next, we examined the possibility that CD11b-ligated moDC might modulate the effector phenotype of human Th17 cells by limiting the secretion of effector cytokines such as IL-17, GM-CSF, IFN γ and IL-22 from Th17 cells, while upregulating IL-10 and IL-21, which can modulate their pathogenicity. To determine the potential effects of CD11b-ligation on moDC on Th17 plasticity at the single-cell level, we generated and expanded clones from pre-enriched human Th17 cells. PBMC were stimulated with Cytostim (a human superantigen-analog), and Th17 cells enriched by a cytokine-secretion capture assay for IL-17 and cloned through limiting dilution. More than 20 Th17 clones were obtained from 4 donors. Clonality was confirmed by V β staining (Supp Fig 1). Virtually all of these clones co-secreted IFN γ with IL-17.

Th17 cell clones were then co-cultured with DC that were ligated with anti-CD11b expressing beads and PGN. Intracellular staining for IL-17 and IFN γ - and in some experiments - GM-CSF, IL-4, IL-22, IL-21, was performed on day 14 of co-culture. In contrast with polyclonal T cell populations, IL-17 secretion of T cell clones was unchanged (Fig. 5), as was the secretion of GM-CSF, IFN γ , IL-4, IL-22 and IL-21 (Supp Fig 4). This indicates that CD11b ligation on moDC does not affect the phenotype and cytokine secretion of monoclonal differentiated memory Th17 cells, in contrast to the constriction of proliferation/expansion observed in heterogenous cells populations.

3.6. moDC generated from carriers of the rs1143679 ITGAM variant are amenable to the tolerogenic effects of CD11b-ligation

To test the relevance of our findings in a biologic context relevant to human autoimmune disease, we chose to recapitulate some of our experiments on cells obtained from carriers of the human rs1143679 *ITGAM* variant, a strong genetic susceptibility marker for human SLE [26,35–37]. The rs1143679 *ITGAM* variant displays an arginine to histidine mutation in position 77, which impairs cell adhesion, phagocytosis, and prevents inflammatory cytokine regulation, thus enhancing IL-6 secretion by macrophages [38]. moDCs from genotyped healthy human volunteers - either homozygous for rs1143679 or noncarriers ('AA' and 'GG', respectively, according to the accepted nomenclature for the risk allele 'A' and the non-risk allele 'G') - provided by the Feinstein Institute, NY, were generated and stimulated with LPS/MDP or PGN for 16 h. Upon TLR stimulation, rs1143679 carriers showed highly increased production of IL-1 β , IL-6, and TNF α in moDC supernatants when compared to non-carriers (Fig. 6a). Next, we ligated CD11b on moDC from both groups using our surrogate system prior to TLR stimulation as described and assessed cytokine production in supernatants after 16 h. Down-modulation of the inflammatory cytokines IL-6, IL-1 β , and TNF α was observed in both groups (Fig. 6b). We next tested whether this would translate into decreased Th17 expansion, as observed in non-carrier donors. CD11b-ligated moDC

were co-cultured with CD4⁺ memory T cells for 14 days and the frequency of Th17-producing cells was measured after restimulation with PMA/Ionomycin. As expected, CD11b-ligated moDC from carriers, as well as non-carriers, significantly reduced the number of IL-17 secreting cells (Fig. 6c and d). Thus, moDC ITGAM variant carriers display enhanced cytokine secretion upon TLR stimulation, but they are as efficiently suppressed, by CD11b ligation as non-carriers, which results in significantly reduced expansion of Th17 memory cells. Although *ITGAM* represents only one of several disease susceptibility loci for SLE, the strong DC hyper-responsiveness observed in healthy rs1143679 carriers highlights its significance in a more genetically permissive background, and the potential of CD11b targeting to dampen detrimental inflammation in SLE.

4. Discussion

The overall objective of this work was to assess the tolerogenic effects of CD11b ligation on human moDC and their capacity to modulate Th17 cell responses as a potential application of moDC-based suppressive immunotherapy for Th17-mediated human autoimmune diseases.

In the course of our attempts to establish a Th17-inducing experimental system, we confirmed the findings of van Beelen et al. [34] who succeeded in inducing the proliferation of human Th17 cells through co-culture of LPS + MDP or PGN-matured moDC with memory T cells. In accordance with their findings, moDC were unable to prime Th17 cells from naive CD4⁺ T cells, possibly because high levels of IFN γ -secreting cells were generated during the culture, which may impede Th17 differentiation. Differentiation of Th17 cells from naive CD4⁺ T cells usually requires neutralization of IFN γ and IL-4.

Our results indicate that potent immunomodulation through CD11b targeting of DC can be achieved, and that it leads to a strong Th17-dampening effect on human CD4⁺ IL-17 secreting memory cells. This effect was likely mediated through inhibition of the cytokine-dependent proliferation of Th17 cells, as CD11b ligated moDC secrete dramatically less IL-1 β , IL-6, IL23, and transwell experiments suggest that moDC derived cytokines are the main driver of Th17 expansion. Our results contrast with results by Ling et al. in murine DC, where *Itgam*^{-/-} mice secreted decreased levels of inflammatory cytokines upon stimulation by LPS alone [43]. On the contrary, Ehrichtiou et al. found increased IL-6 production by *Itgam*^{-/-} APC and enhanced differentiation of naive CD4 T cells into Th17 cells upon antigen challenge [24]. Our triggering of CD11b through antibody ligation recapitulates this DC phenotype in human cells, upon stimulation by LPS-MDP or PGN. In this system, co-stimulation was not required for the expansion of memory Th17 cells - a finding in concordance with known properties of CD4⁺ memory T cells which may proliferate, to a certain extent, through cytokine-mediated effects in the absence of co-stimulation. Cytokines secreted by moDC most likely regulate differential Th17 proliferation vs other T cell populations.

Thus, decreased IL-1 β , IL-6 and IL-23 likely decrease expansion of Th17 cells, whereas CD11b ligation had no effect on monoclonal Th17 cultures, despite blunted IL-1 β , IL-6 and IL-23 secretion by moDC. Th17 clones are highly differentiated cells, and their overall

cytokine secretion profile was not affected by moDC suppression. However, it is possible that CD11b targeting can also modulate the pathogenicity of developing Th17 cells, as developmental plasticity is closely linked with cell cycling and chromatin modifications, and IL-1, IL-6 and IL-23 have crucial roles in Th17 differentiation from naive T cells.

Our findings suggest the potential usefulness of CD11b ligation for therapeutic manipulation of pathogenic Th17 cell responses in the setting of autoimmunity, either through re-injection of ex vivo generated autologous moDC, or through in vivo approaches facilitating CD11b ligation on circulating or tissue-resident dendritic cells. moDC can easily be generated in vitro and have been given to patients for different indications with a very good safety profile [39]. Furthermore, CD11b targeting provides a convenient way to modulate DC function. As has been demonstrated with DEC-205 [40,41] targeting of CD11b and antigen by antibody-protein fusion may also allow antigen delivery and presentation by targeted DC, leading to antigen-specific modulation of Th17 function. Other AC receptors than CD11b may be amenable to targeting, as immunomodulation of DC is likely mediated through multiple receptors. However, we showed previously that $\alpha_v\beta_5$ targeting had no effect on secretion of Th17-inducing cytokines [8].

We demonstrate that carriers of the ITGAM variant rs1143679 showed highly enhanced cytokine secretion of IL-1, IL-6 and IL-23 by moDC, which may contribute to the increased risk of SLE displayed by the carriers. This observation strengthens our conclusion that CD11b signaling strongly regulates moDC function, as it was demonstrated for macrophages [38]. Despite increased cytokine secretion, ITGAM variant carriers were as susceptible as non-variant donors to the immunosuppressive action of CD11b-targeted moDC. These findings contrast with those of another report [42] which showed that the tolerogenic effects of a synthetic low molecular ligand of CR3 - Leukadherin 1 (LA1) - on macrophages was attenuated or abrogated in homozygous ITGAM carriers. These discrepancies could be explained by the different cell type investigated, the strong binding affinity of our antibody/surrogate system, or by different CR3 binding sites and recruitment. The mechanisms of CD11b-mediated suppression are yet to be fully uncovered, but its potency, as evidenced in this work, may provide an opportunity for limiting harmful autoimmune Th17 responses in human autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Peptidoglycan and LPS-MDP matured human moDC promote Th17 expansion from CD4⁺ memory T cells, but do not prime Th17 cells from naïve T cells.

A) PGN (upper panels) and LPS-MDP-matured (lower panels) moDC were co-cultured with allogeneic naïve CD4⁺ T cells (left) or CD4⁺ memory T cells (right) for 14 days. ICS was performed for IFN γ and IL-17 upon re-stimulation of T cells with PMA/Ionomycin. While moDC successfully primed naïve T cells into Th1 lineage commitment, there was no induction of Th17-secreting cells. Significant Th17 populations emerge, however, when CD4⁺ memory T cells are used as the starting population for co-culture. B) Summary of 15 independent experiments with 26 donors displaying mean and range of IL-17 secreting cells in unstimulated, PGN- and LPS/MDP-stimulated co-culture conditions with CD4⁺ memory T cells. In a few experiments larger Th17 expansions were observed, contributing to inter-experiment variance. moDC (monocyte-derived dendritic cells); PGN (peptidoglycan); MDP (Muralmydipeptide).

Fig. 2. moDC-derived cytokines are essential for Th17 expansion from CD4⁺ memory T cells while co-stimulation is dispensable.

A *Trans*-well cell culture system: i) DC and Tm are co-cultured in the lower chamber of the system allowing unrestricted cell-cell contact and exposure to DC-derived cytokines. ii) DC are separated from Tm through a *trans*-well mesh preventing cell migration and subsequent DC-T cell contact while cytokines can pass. iii) Supernatant harvested from PGN-stimulated moDC is introduced into the upper chamber of the culture system. No DC are present. B) and C) Lack of DC-T cell contact (co-stimulation) does not significantly diminish the expansion of IL-17 or IFNγ producing CD4⁺ T cells in the system. The addition of cytokine-containing supernatant from large numbers of PGN-matured moDC (derived from more than trice as many moDC compared to ii) in the upper compartment causes a significant boost in Th17 cell expansion without co-stimulation. Data shown includes 9 observations from 9 different moDC donors for conditions i) and ii), and 6 observations from 6 moDC donors for condition iii). Three independent experiments were performed. Panel C displays results from one representative experiment. Bars represent mean ± SEM. **P < 0.005, ns = not significant. moDC (monocyte-derived dendritic cells); PGN (peptidoglycan); Tm (memory T cells); sme (same); diff (different); cpt (compartment); sup (supernatant).

Fig. 3. CD11b-ligation of human moDC down-modulates the secretion of Th17-skewing cytokines.

moDC were incubated with beads, anti-CD11b conjugated beads or isotype-conjugated beads, then stimulated with LPS + MDP (upper row, grey) or PGN (lower row, black). Cytokine secretion was assessed by CBA (IL-1b and IL-6) or ELISA (IL-23) in culture supernatants at 16 h. CD11b-ligated conditions show significant down-modulation of cytokine secretion as compared to control-IgG-ligated controls for IL-1b (left), IL-6 (center), and IL-23 (right panels). Bars represent mean $\pm$ SEM in percentages of cytokine secretion levels relative to the control condition. * $P < 0.05$, **** $P < 0.0001$. For IL-1b and IL-6 data depict results of 5 independent experiments with a total of 11 (IL-1b) and 12 (IL-6) donors. For IL-23, three independent experiments with 3 donors are shown. DC-CD11b (monocyte-derived dendritic cells with Dynabeads® Pan Mouse IgG, CD11b-labeled); DC-IgG (monocyte-derived dendritic cells with Dynabeads® Pan Mouse IgG, control-IgG-labeled); PGN (peptidoglycan); MDP (Muramyl dipeptide).

Fig. 4. CD11b-ligated human moDC constrict CD4⁺ Th17 cell responses.

A) CD11b-ligated moDC and control IgG-moDC were matured with PGN and cocultured with CD4⁺ Tm. On day 14, cells were re-stimulated with PMA/Ionomycin and ICS for IL-17 and IFNγ was performed. Plots show CD3/CD4-positive T cells after co-culture with CD11b-ligated, PGN-matured moDC (left), and control-IgG PGN-matured moDC (right).

B) CD11b-ligated moDC significantly dampen the expansion of total IL-17 secreting CD4⁺ T cells (left), single-positive (IL-17⁺/IFNγ⁻) Th17 cells (middle), but not doublepositive Th17 cells (right) compared to IgG- ligated control-moDC. Data shown represent 10 donors from 7 independent experiments. Bars represent mean ± SEM. *P < 0.05, **P < 0.005, ns = not significant. moDC (monocyte-derived dendritic cells); PGN (peptidoglycan).

Fig. 5. CD11b-ligated moDC do not modulate Th17 cell plasticity at the monoclonal level.

A) and **B)** PGN matured, CD11b and IgG control-ligated moDC were cocultured with human Th17 cell clones for 14 days, then re-stimulated with PMA/Ionomycin and ICS-stained for IL-17 and IFN γ . All clones were IL-17/IFN γ co-secreters, and had previously been selected from allogeneic human donors based on IL-17 secretion in response to super-antigen stimulation in a cytokine-capture assay, and monoclonally expanded in a limiting dilution approach with irradiated feeder PBMC, PHA-L and IL-2. No significant differences in IL-17 and IFN γ production for each respective Th17 clone between CD11b ligated and control IgG-ligated co-culture conditions were observed indicating lack of CD11b-moDC-mediated phenotypic modulation at the single cell level. Compiled data from a total of four moDC donors and 2 allogeneic Th17 clones (one per 2 independent experiments) are shown. Bars represent mean $\pm$ SEM. NS=not significant. moDC (monocyte-derived dendritic cells); PGN (peptidoglycan); PBMC (peripheral blood mononuclear cells); PHA-L (leucoagglutinin).

Fig. 6. moDC from ITGAM carriers secrete more inflammatory cytokines than non-carriers and are susceptible to CD11b-mediated modulation.

A) moDC from 3 healthy carriers of the ITGAM variant (AA) or from 3 healthy non-carriers (GG) were separately pooled and stimulated with LPS-MDP, or incubated in pooled autologous plasma to measure spontaneous cytokine secretion. IL-6 secretion was assessed in supernatants at 16 h. ITGAM variant carriers secreted more IL-6 than non-carriers. B) ITGAM variant (AA) or healthy non carrier (GG) pools were pre-incubated with CD11b targeting, isotype control (IgG), or beads alone (Beads) and stimulated with LPS-MDP for 16hrs. IL-6 levels are efficiently down-modulated upon CD11b-ligation as compared to control beads, both for ITGAM variant and noncarrier donors. C) and D) CD11b-ligated or control bead-ligated moDC from ITGAM variant or non-carrier donors were stimulated by LPS + MDP (C) or PGN (D) and co-cultured with allogeneic CD4⁺ memory T cells. The number of IL-17 secreting cells was calculated as the fraction of cells secreting IL-17 multiplied by the total number of T cells by ICS after 14 days and normalized to the control beads condition. CD11b ligation equally suppresses expansion of IL-17 secreting CD4⁺ T cells by carrier or non-carrier moDC. Bars represent mean $\pm$ SEM of triplicates. *P < 0.05, **P < 0.01.