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Complement Receptor 3 Ligation of Dendritic Cells Suppresses Their Stimulatory Capacity

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To activate T cells effectively, dendritic cells (DCs) must provide three separate signals, MHC-Ag, costimulatory molecules (such as CD80 and CD86), and proinflammatory cytokines (such as IL-12). These three signals are up-regulated in the presence of “danger signals” such as LPS or viral nucleic acids. Evidence suggests that DCs providing only the first two of these signals cannot successfully stimulate T cells. Apoptotic cells have been proposed to suppress DC immunogenicity through the ligation of apoptotic cell receptors. Complement receptor 3 (CR3) and CD36 have been suggested to be important in this process, although the mechanism by which this modulation occurs is still unclear. We demonstrate that ligation of CR3, but not CD36, directs DCs to increase surface MHC and costimulatory molecules, while suppressing inflammatory cytokine release. CR3 modulation of DCs does not require a type I IFN response, does not involve the specific regulation of the MyD88- or Toll/IL-1R domain-containing adaptor-inducing IFN-β-dependent TLR signaling pathways, and occurs even in the absence of danger signals. The functional outcome of this process is poor Ag-specific stimulation of CD4 and CD8 T cells by CR3-ligated DCs both in naive response as well as upon subsequent challenge with normal DCs. We propose that CR3 provides a “nondanger” signal that suppresses the stimulatory capacity of DCs. The Journal of Immunology, 2007, 178: 6268–6279.

Dendritic cells (DCs) are the most potent APCs. Upon stimulation with endogenous or exogenous danger signals (1), DCs undergo activation/maturaion to an immunogenic phenotype. This includes the up-regulation of costimulatory molecules, such as CD80 and CD86, as well as the secretion of proinflammatory cytokines, such as TNF-α and IL-12. In the absence of danger signals, the DCs remain in a resting/immature state, expressing low levels of costimulatory molecules and virtually no proinflammatory cytokines. It has been proposed that a T cell is tolerized after a cognate interaction with an Ag presented by a resting APC lacking costimulatory molecules (2). This is because the T cell receives “signal 1,” in the form of TCR ligation by MHC-Ag on the DC surface, in the absence of a “signal 2” such as the CD80 and CD86, which are not expressed by resting DCs. More recent evidence has refined this hypothesis to include a “signal 3,” in the form of cytokines such as IL-12 (3, 4). In this model, a DC needs to provide all three signals to induce immunity.

Materials and Methods

Mice and Abs

C57BL/6 and Rag1-/- mice were purchased from The Jackson Laboratory. IFN-α/βR-/- mice were fully backcrossed with C57BL/6 mice (National Cancer Institute (NCI)/Frederick Cancer Research and Development Center, Frederick, MD) using a speed-congenic breeding protocol based on 80 simple sequence length polymorphism markers (Biocon) by W. W. Leitner (National Institutes of Health (NIH)/NCl, Bethesda, MD) (12); IFN-αβR-/- and IFN-αβR-/- littermates were a gift from W. W. Leitner (NIH/NCl). Marilyn CD4+ anti-H-Y TCR transgenic (Tg) mice, bred on the Rag-/- background, were the gift of P. Matzinger (NIH/National Institute of Allergy and Infectious Diseases, Bethesda, MD). All mice were bred and maintained in accordance with guidelines of the Institutional Animal Care and Use Committee of The Children’s Hospital of Philadelphia, an American Association for the Accreditation of Laboratory Animal Care-accredited facility. CD8+ anti-H-Y TCR Tg mice on a Rag-/- background were purchased from Taconic Farms and housed in our facility until use.
Anti-CD80, CD86, -MHC class I (MHC I), -MHC class II (MHC II), and -CD40 Abs were purchased from BD Biosciences. IL-10R Ab 1B1.3a was a gift from Dr. E. Pearce (University of Pennsylvania, Philadelphia, PA).

Generation of bone marrow-derived DCs (BMDCs)

We generated BMDCs as previously described (13). Briefly, we cultured bone marrow precursors from Rag1−/− mice for 6 days in complete IMDM containing 3.3 ng/ml GM-CSF (BD Biosciences). Generating DCs from Rag1−/− mouse bone marrow does not require depletion of T and B cells and Rag1−/− BMDCs behave identically as those from normal mice. To generate BMDCs from IFN-γR−/− mice and littermate controls, we depleted bone marrow precursors of lymphocytes using Thy-1.2 and B220 MACS beads (Miltenyi Biotec). BMDCs were used on days 6–7 of culture.

Ligation of BMDC receptors and TLR stimulation

Anti-CR3 biotinylated M1/70 (isotype IgG2b), anti-CD36 CRF D-2712 (isotype IgA), biotinylated anti-mouse IgA (isotype IgG2b), biotinylated mouse IgG2b isotype, and FITC-labeled streptavidin (SA-FITC) were purchased from BD Biosciences. We ligated CR3 on BMDCs using biotinylated M1/70 Ab (directed against the α-chain of CR3), or CD36 using CRF D-2712 followed by biotinylated anti-mouse IgA, or isotype control Abs. This step was followed by incubation with SA-FITC. We performed all the incubations at 4°C for 30 min. Then we allowed BMDC to rest for 3 h at 37°C before adding LPS (100 ng/ml), poly I:C (100 μg/ml) with polymyxin B (50 μg/ml), Cpg oligodeoxynucleotide 1826 (10 μg/ml), or no stimulus. Polymyxin B was necessary because sources of poly I:C tested in our laboratory contained LPS contamination. We measured IFN-γ mRNA levels 3 h after addition of stimulus; cell surface markers, cytokine expression, and APC function were measured after overnight culture.

IFN-β real-time RT-PCR

We extracted RNA using TRIzol followed by DNase digestion and purification with columns (Qiagen). We performed cDNAs using random hexamers and AMV-RT (Promega). We performed real-time PCR in triplicate in a 10-μl volume using an ABI 7900HT machine (Applied Biosystems) in 384-well plates and the SyberGreen system. We used the following forward (f) and reverse (r) primers: cyclophilin-f, 5′-GGCC TGA TGA GGC CGC C-3′; cyclophilin-r, 5′-TGT CTT TGG AAC TTT GTC TGC AA-3′; IFN-β-f, 5′-ATG GGT GGT TGC AGG C-3′; IFN-β-r, 5′-TGA CCT TTT AAA TGC AGT AGA TTC A-3′. We used the standard curve method for quantitative analysis of gene expression and normalized to the cyclophilin gene (14). We tested genomic DNA contamination in all samples performing PCR without AMV-RT.

Flow cytometry

We washed BMDCs in cold PBS, blocked FcγR with rat anti-mouse CD16/ CD32 (clone 2.4G2) mAb for 10 min, and then stained for 30 min with the following mAbs (BD Biosciences): allophycocyanin-conjugated hamster anti-mouse CD11c, PE-rat anti-mouse MHC II, CD80 and CD86, FITC hamster anti-mouse CD40, and mouse anti-mouse H2Kb. We conducted all stainings on ice. We collected cells fixed in 1% formaldehyde on either a FACSCan or FACSComp flow cytometer (BD Biosciences) and analyzed the data using FlowJo software.

Cytokine assays

We measured IL-2, IL-12 p70, TNF-α, IL-4, IFN-γ, and TGF-β from cell culture supernatants using ELISA (BD Biosciences). TGF-β was measured using acid-activated supernatants. IL-10 and IL-6 were measured using precoated ELISA plates (R&D Systems).

NF-κB activation assay

We prepared nuclear extracts from BMDCs ligated with anti-CR3 or isotype Ab and 3 h later stimulated for 0, 30, and 90 min with LPS (100 ng/ml), using NE-PER (Pierce). We assayed the extracts for activated NF-κB p65 using the EZ-Detect Transcription Factor kit (Pierce).

T cell stimulation assay

We prepared CD4+ and CD8+ responders from spleens of spleen-free H-2b TCR Tg Cd4 and Cd8 mice by RBC lysis. Because both mice are on the Rag1−/− background, it was not necessary to purify single lymphocyte populations. We cocultured 106 splenocytes and titrated numbers of BMDCs in the ratios indicated in 96-well round-bottom plates in complete IMDM medium with 10% FBS for 72 h, after which we collected supernatants for cytokine analysis. For restimulation experiments, we collected T cells at the 72 h time point, washed them, and replated them in 96-well round-bottom plates at a density of 50,000 cells/well. The cells were rested for 24 h, after which they were restimulated with male BMDCs in the ratios indicated. Supernatants were harvested at 7 and 24 h after restimulation for detection of IL-2 and IFN-γ, respectively.

Microscopy

We examined BMDCs under an Axioplan-2 microscope with a ×63 oil objective and we captured and analyzed digital images including three-dimensional reconstructions using Slidebook software. In experiments evaluating the need for SA cross-linking, a directly FITC-conjugated anti-CR3 M1/70 Ab was used to ligate BMDCs and compare with BMDCs ligated with biotinylated M1/70 followed by SA-FITC.

Statistical analyses

The statistical significance of DC populations analyzed by flow cytometry was determined using the probability binning method of Roederer et al. (15), with a very conservative cutoff value of T(x) = 50. This is a novel method that allows testing the hypothesis that two or more flow cytometry data distributions are different, by applying a variant of the $\chi^2$ statistic to comparing univariate distributions. Furthermore, this algorithm can rank distributions as most similar or dissimilar. For the Ag-presentation assays, two-tailed Student’s t tests were performed and values of $p < 0.05$ were considered significant. For cytokine production assays, the results were normalized to the isotype control samples and analyzed using ANOVA for repeated measures followed by multiple pairwise comparisons to detect significant differences between treatments. Differences between means were considered significant if the $p < 0.05$ using the two-tailed t test.

Results

CR3 ligation up-regulates signals 1 and 2

The regulation of signal 1 (MHC-Ag) and signal 2 (CD40, CD80, and CD86) on APCs is important for determining the nature of an immune response. To investigate the role of receptors triggered by apoptotic cells in the regulation of signals 1 and 2, we ligated CR3 and/or CD36 on BMDCs with specific biotinylated Abs and cross-linked the receptors with SA-FITC. After overnight culture, we tested the levels of MHC and costimulatory molecules on the surface of BMDCs. We found that CR3 ligation increased the expression of MHC II, CD86, and CD40, compared with isotype control Ab (Fig. 1). In contrast, the ligation of CD36 did not change the expression of any of the surface markers tested. Because the secondary Ab used to cross-link CD36 is of the same isotype as our anti-CR3 Ab, this result allows us to rule out a possible role of FcγR cross-linking in our system. The ligation of both receptors together induced a small additional increase in expression of CD86, CD40, and MHC II, compared with that induced by CR3 alone (Fig. 1). These results indicate that CR3 induces the up-regulation of signals 1 and 2 and may prepare DCs to efficiently present Ag to T cells. This function is not generally shared by receptors bound by apoptotic cells, since the ligation of CD36 did not have this effect.

Ligation of CR3 leads to its internalization, but not degradation, while ligation of CD36 results in both internalization and loss of Ab/receptor complex

The differential effects of ligation of CR3 vs CD36 on BMDCs could be due to differential expression and/or Ab-induced turnover. BMDCs highly express CR3, also known as CD11b/CD18 or Mac-1, an important marker of myeloid DCs. DC expression of CD36 is debated (16, 17). We determined constitutive expression levels of these surface receptors on our BMDCs by immunostaining and flow cytometry. In our hands, BMDCs express high levels of CR3 and CD36 (Fig. 2A). Thus, the differential effects of CR3 and CD36 on BMDC expression of signals 1 and 2 are not due to differential expression of these receptors on the cell surface.

Cross-linking of many surface receptors leads to their internalization and subsequent degradation as a means of terminating the signal. We ligated BMDCs with either anti-CD36 Ab or anti-CR3
Ab, followed by SA-FITC, and analyzed the BMDCs by flow cytometry 18 h after initial ligation. FITC signal remained detectable on both CR3- and CD36-ligated BMDCs after 18 h of receptor ligation, although the CD36 signal appeared diminished from its original level (Fig. 2B). To assess whether this was due to loss of the receptor/Ab complex as a result of ligation, we compared the FITC signal from cells 18 h after ligation to the FITC signal from cells immediately stained and fixed with the same Ab/SA-FITC combination. Because cell transfer or temperature change might also affect expression levels, we further compared these cells with BMDCs that were mock ligated without Ab and then cultured for 18 h. These mock-ligated BMDCs were then stained and fixed with the same Ab/SA-FITC combination described above. We did not find differences in CR3 or CD36 levels between BMDCs immediately stained and fixed and BMDCs that had undergone mock ligation, demonstrating that cell transfer and temperature change did not affect expression levels. Instead, ligation of CD36, but not CR3, led to decreased signal intensity when compared with mock controls (Fig. 2C). To determine whether CD36 and/or CR3 were internalized, we examined these same BMDCs by microscopy. Staining with anti-CR3 or anti-CD36 Abs after mock ligation revealed a surface-staining pattern with a rim signal around the cell membrane (Fig. 2D and F). In contrast, in both CR3- and CD36-ligated cells, we detected FITC signal in a cytoplasmic, granular pattern, demonstrating internalization of both molecules upon Ab binding (Fig. 2E and G). Taken together, the microscopy and flow cytometry data suggest that internalization of CD36, but not internalization of CR3, is accompanied within 18 h by loss of the receptor/Ab complex. This loss of CD36 may terminate its signal and prevent a full response to our Ab treatment. In contrast, the level of CR3/Ab complex does not decrease. Its continued presence, even if localized intracellularly, may allow it to provide a continuous signal and therefore measurable effects on BMDC activation.

CR3 ligation does not up-regulate signal 3

In our initial experiments, we showed that CR3, but not CD36, up-regulates signals 1 and 2 on BMDCs (Fig. 1). To determine whether CR3 is also able to induce DC expression of signal 3, we tested the production of proinflammatory cytokines in BMDCs ligated with CR3 and/or CD36 after overnight culture. We found virtually no expression of IL-12 p70, TNF-α, or IL-6 in BMDCs upon ligation of CR3 or CD36, while very high levels of these cytokines were seen using a powerful proinflammatory signal such as LPS (Table I). Simultaneous ligation of both CR3 and CD36 also did not yield any change in production of these cytokines.
These results indicate that CR3 induces up-regulation of signals 1 and 2, but not signal 3, and may thus prepare DCs to present Ag to T cells in a nonstimulatory fashion. To determine whether this phenotype is accompanied by induction of anti-inflammatory cytokines, we measured the expression of IL-10. Neither CR3, nor CD36, nor the combination of the two stimuli induced our BMDCs to produce IL-10, in contrast to the IL-10 production seen in response to LPS (Table I).

CR3 ligation decreases the ability of DCs to stimulate a primary T cell response

We evaluated the functional outcome of the changes in costimulatory molecules and cytokines elicited by CR3 ligation by testing the capacity of CR3-ligated BMDCs to stimulate naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells in vitro. For these studies, BMDCs presented H-Y Ag to either MHC I- or MHC II-restricted anti-H-Y TCR Tg T cells. H-Y is an Ag produced and presented by the MHC I molecules of all male cells. Cell death in culture allows for exogenous acquisition of H-Y, and therefore presentation on the MHC II of male BMDCs. Female BMDCs produce no H-Y and therefore do not present this Ag on either MHC I or MHC II in culture. We found that ligation of CR3 decreased the ability of male BMDCs to elicit an IFN-γ response from both CD4 and CD8 anti-H-Y TCR Tg T cells (Fig. 3A). In CD4 T cells, CR3 ligation reduced IFN-γ production by ~64%. In CD8 T cells, the inhibition of T cell stimulation was even more dramatic, with IFN-γ production reduced to levels similar to those induced by female BMDC in an Ag-nonspecific fashion. Despite these differences in IFN-γ production, neither CD4 nor CD8 cells showed differences in proliferation when exposed to CR3- or isotype-ligated DCs as measured by both absolute cell counts as well as thymidine incorporation (data not shown). To determine whether the decrease in IFN-γ

Table I. Absolute cytokine production by CR3- and CD36-ligated DCs

<table>
<thead>
<tr>
<th></th>
<th>TNF-α</th>
<th>IL-12 p70</th>
<th>IL-6</th>
<th>IL-10</th>
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<tr>
<td>Isotype</td>
<td>ND</td>
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<tr>
<td>CD36</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>CR3</td>
<td>ND</td>
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<tr>
<td>Both</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Isotype plus LPS</td>
<td>5,345 ± 2,116</td>
<td>285 ± 80</td>
<td>42,370 ± 3,270</td>
<td>80 ± 8.7</td>
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</tbody>
</table>
production was due to a shift in cytokine polarization, we measured IL-4 production and found that no IL-4 was detectable by ELISA in these cultures (data not shown). These results indicate that the altered phenotype induced in BMDCs by CR3 ligation impairs DC stimulatory function, leading to poor Ag presentation to naive T cells.

T cells exposed to CR3-ligated DCs show a blunted response to secondary challenge with Ag

Because CR3 ligation of DCs suppressed their ability to induce primary CD4 and CD8 T cell responses, we investigated whether CR3-ligated BMDCs could induce long-term tolerance to Ag in T cells. We cocultured CD4 and CD8 anti-H-Y TCR Tg T cells with male BMDCs ligated with isotype or CR3 Abs, or with isotype-ligated female BMDCs as a control. After 72 h of coculture, we harvested, washed, and replated the T cells in fresh medium to allow them to rest for 24 h. Challenge of these T cells with untreated, fully stimulatory male BMDCs showed that T cells initially exposed to CR3-ligated BMDCs generated a quantitatively lower secondary response compared with T cells initially exposed to isotype-ligated BMDCs as in A for 72 h were washed and rested for 24 h. These cells were then exposed to male BMDCs. IL-2 was measured in the supernatants after 7 h. C. IFN-γ measured from the supernatants of cells treated in the same fashion as B after 24 h of culture. All plots show representative experiments of four experiments. * A value of p < 0.05 for T cells initially exposed to isotype-ligated male BMDCs compared with CR3-ligated male BMDCs as calculated by two-tailed Student t test.

CR3 ligation inhibits the cytokine production induced by TLR triggering

Poor T cell stimulation could be due to a poor display of Ag, to low levels of costimulatory molecules, or to an insufficient production of IL-12 by DCs (18–20). Because we observed that CR3 ligation increases the expression of signals 1 and 2 on our BMDCs (Fig. 1), we excluded the first two possibilities, and hypothesized that the decreased capacity of CR3-ligated BMDCs to stimulate T cell IFN-γ production (Fig. 3) might be due to suppression of signal 3 (cytokine production). We were unable to measure constitutive cytokine production by BMDCs, as this was below the detection limits of our ELISAs (Table I), although it was still enough to induce a good T cell response by isotype control Ab-treated BMDCs (Fig. 3). Therefore, to bring the cytokine production by BMDCs up to levels detectable by our assays and to determine whether CR3 ligation suppresses signal 3, we stimulated BMDCs, preligated with CR3, CD36, or both, with the TLR ligand LPS. We
found that CR3 ligation suppressed LPS induction of cytokines, most prominently IL-12 and TNF-α, as well as IL-10 and IL-6 (Fig. 4, A–D). CD36 ligation did not alter the expression of cytokines by BMDCs, and the ligation of both receptors did not modify the inhibitory effect induced by CR3. Trypan blue-excluding cells were counted from multiple experiments and there were no differences between any of the treatment groups indicating that the cytokine differences were not due to cell death (data not shown). These results indicate that ligation of CR3, but not CD36, indeed inhibits the expression of signal 3 by DCs. Because IL-10 levels, traditionally an anti-inflammatory cytokine, were decreased, we considered the possibility that CR3 could decrease inflammatory

FIGURE 4. Cytokine production is suppressed in BMDCs after CR3 ligation. Levels of (A) IL-12 p70, (B) TNF-α, (C) IL-10, and (D) IL-6 were decreased after ligation with anti-CR3 (■) but not anti-CD36 Abs (□), when compared with isotype control Abs (▲). Ligation of both receptors (▲) was not different from CR3 ligation alone. These effects were seen for all three stimuli used. N.D. signifies nondetectable levels. Histograms and error bars represent the averages and SEM of three to four experiments. Because of the normal variability from culture to culture typical of primary culture of DCs, the absolute cytokine production of each experiment varies widely. Therefore, we have summarized the absolute values normalized to isotype control samples. *, Values significantly different from CD36, as calculated by ANOVA and post hoc analyses. Similar results were obtained by calculating a simple Student’s t test against the value 1.0 (p < 0.05). E, BMDCs were ligated as previously described. Three hours after ligation, the BMDCs were exposed to LPS (100 ng/ml) along with anti-IL-10R Ab, 1B1.3a (1 μg/ml)(■), or no Ab (▲). IL-12p 70 and TNF-α were measured by ELISA after overnight culture. F, NF-κB p65 activation from DC nuclear extracts. Activity was measured by a transactivating NF-κB p65 chemiluminescent ELISA at 0, 30, and 90 min after stimulation with LPS. ■, Isotype-treated BMDCs; ■, CR3-ligated BMDCs. Data are representative of three experiments. G, BMDCs were ligated and then stimulated with LPS, poly I:C, or CpG as previously described. BMDCs were also left unstimulated after ligation. Acid-activated TGF-β was measured in the supernatants after overnight culture. The background OD value for the TGF-β levels in medium alone was subtracted from all samples. Error bars represent SEM of three experiments. *, A value of p < 0.05 compared with the zero value by the simple Student’s t test, or compared with the isotype control, where appropriate.
cytokines through increased IL-10 consumption (21). Therefore, we repeated the LPS stimulation of ligated BMDCs in the presence of blocking IL-10R Ab 1B1.3a (1/9262 g/ml). Although total TNF-α and IL-12 production was increased by IL-10R blockade, as expected, CR3 ligation was able to suppress TNF-α and IL-12 by similar proportions in the presence and absence of the blocking Ab (Fig. 4E). These results imply that the suppressive effect of CR3 on the production of inflammatory cytokines is not dependent on increased IL-10 consumption.

The inhibition of cytokine production by CR3 ligation in BMDCs is not the result of the blockade of a specific TLR-signaling pathway. TLRs signal through two major pathways: the MyD88-dependent pathway and/or the MyD88-independent TRIF-dependent pathway (22). To determine whether the inhibition of signal 3 by CR3 ligation is due to the targeting of a specific signaling pathway downstream of a TLR, we measured the effect of CR3 or CD36 ligation on the activation induced by TLR ligands triggering either or both pathways. We described above the results obtained in BMDCs stimulated by LPS, a stimulator of TLR4, which uses both the MyD88 and TRIF pathways (23). We compared these results with those obtained with poly I:C, which stimulates TLR3 and uses the TRIF pathway only (24), and with CpG oligonucleotides, which stimulate TLR9 and use the MyD88 pathway only (25). CR3, but not CD36, ligation resulted in decreased cytokine production through all three TLRs (Fig. 4, A–D). These results indicate that CR3 ligation inhibits the production of cytokines induced by three different TLRs, which signal through two different pathways, and suggest that the target of CR3 inhibition does not lie on specific signaling pathways downstream of particular TLRs.

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All three TLRs require NF-κB activation to signal cytokine transcription (22). Blockade of either TLR pathway results in decreased phosphorylation and nuclear relocation of NF-κB p65 (26). To determine whether the decrease in cytokine production is due to blockade of TLR signaling at the common junction of the TRIF and MyD88 pathways, we measured NF-κB nuclear translocation and activation after LPS stimulation, with and without previous CR3 ligation. CR3 ligation did not change the NF-κB activation induced by TLR stimulation, nor did it induce activation before TLR stimulation, indicating that its effects lie...
outside of TLR signaling pathways leading to NF-κB activation (Fig. 4F).

Both CR3 and CD36 ligation increase DC production of TGF-β

TGF-β is an important immunomodulatory cytokine that is important in multiple DC functions including but not limited to: DC survival (27), suppression of IL-12 secretion (28), the generation of regulatory CD4<sup>+</sup> T cells (29), and the induction of Th17 cells (30).

Because the ligation of CR3 blunted the production of TLR-induced IL-10 and proinflammatory cytokines, we reasoned that CR3 ligation might also affect other immunomodulatory cytokines such as TGF-β. Isotype-ligated BMDCs did not produce TGF-β at baseline. However, CR3 ligation induced the production of TGF-β without further stimulus (Fig. 4G). Interestingly, CD36 ligation also resulted in TGF-β production to levels twice that seen with CR3 ligation. The TLR ligands LPS, poly I:C, or CpG also induced TGF-β. Ligation of BMDCs with CD36 before TLR stimulus caused a statistically significant increase in TGF-β compared with isotype-treated BMDCs after LPS stimulus. A similar trend was also seen in poly I:C-stimulated BMDCs, although this did not achieve statistical significance in our experiments. CR3 ligation did not produce a significant excess of TGF-β after LPS or poly I:C stimulation, and ligation of either receptor did not change TGF-β levels produced after CpG stimulus (Fig. 4G).

Thus, both CR3 ligation and CD36 ligation result in up-regulation of TGF-β in unstimulated BMDCs and CD36 ligation delivers a strong enough stimulus for TGF-β production to also have an additive effect on LPS and poly I:C stimulations. CR3 cannot further increase TLR-induced TGF-β. Importantly, it does not decrease TGF-β, as it does for IL-12, TNF-α, IL-6, and IL-10 (Fig. 4, A–D). These results suggest that CR3 does not inhibit cytokine secretion in general, but rather committees DCs to produce a specific pattern of cytokines with the functional outcome of a blunted Ag presentation (Fig. 3).

CR3 ligation increases CD86 and MHC II after stimulation with LPS, poly I:C, or CpGs

We have shown that CR3 ligation increases surface expression of costimulatory molecules on unstimulated BMDCs (Fig. 1). To determine whether CR3 has the same effect upon TLR stimulation,
we examined the effect of CR3 or CD36 ligation on the expression of costimulatory molecules following TLR stimuli. BMDCs ligated with CR3 and then stimulated with LPS showed a small trend toward increase in surface expression of CD86 and MHC II, compared with BMDCs ligated with isotype control Abs (Fig. 5, A–E). CD80, MHC I, and CD40 levels were unaffected. The lack of effect on CD40 expression in CR3-ligated/TLR stimulated BMDCs may be due to the already maximal expression induced by TLR stimulation. Data were also obtained from DCs stimulated with poly I:C or CpGs (Fig. 5, A–E) again demonstrating that CR3 has little effect after maximal up-regulation of these molecules by TLR stimulus. Thus, CR3 ligation has a similar effect with or without subsequent stimulation via LPS, i.e., the induction of a small but consistent up-regulation of costimulatory molecules.

Furthermore, these results show that CR3 ligation is not a general inhibitor of TLR signaling in BMDCs, but has a selective inhibitory effect on the production of some TLR-induced cytokines.

**CR3 ligation increases MHC II expression after TLR-independent stimulation**

IFN-α up-regulates costimulatory molecules independent of TLR signaling (13, 31). We asked whether CR3 ligation further increased signals 1 and 2 induced by an activator of DCs that does not use TLRs. CR3 ligation of BMDCs ligated with isotype control Abs (Fig. 5, A–E). CD80, MHC I, and CD40 levels were unaffected. The lack of effect on CD40 expression in CR3-ligated/TLR stimulated BMDCs may be due to the already maximal expression induced by TLR stimulation. Thus, CR3 ligation has a similar effect with or without subsequent stimulation via LPS, i.e., the induction of a small but consistent up-regulation of costimulatory molecules. Furthermore, these results show that CR3 ligation is not a general inhibitor of TLR signaling in BMDCs, but has a selective inhibitory effect on the production of some TLR-induced cytokines.

**CR3 ligation increases IFN-β production upon triggering of TRIF-dependent TLRs**

Increased expression of costimulatory molecules could be the result of increased autocrine type I IFN production (31–33). To determine whether the CR3-induced increase of costimulatory molecules is accompanied by, and/or due to, induction of type I IFNs, we measured type I IFN production following CR3 ligation and TLR stimulation. We assayed IFN-β since it is the member of the family of type I IFNs primarily produced by BMDCs (34). We found that CR3 ligation increased LPS-induced IFN-β transcription (Fig. 6, A and B). CR3 ligation also had a similar effect in conjunction with a poly I:C stimulus, while it did not increase the IFN-β induced by CpGs (Fig. 6B). CD36 ligation also increased IFN-β transcription in response to the TRIF-dependent TLR ligands, LPS and poly I:C, albeit to a lesser degree (Fig. 6B). Because of the widely varying absolute amounts of RNA measured from experiment to experiment, we were unable to achieve statistical significance from these data. Nonetheless, there is a consistent, reproducible trend of increased IFN-β message production after CR3 ligation followed by TRIF-dependent TLR stimulation.

**CR3 ligation up-regulates costimulatory molecules independently of type I IFNs**

The increased IFN-β induced by CR3 ligation, upon stimulation with LPS and poly I:C, could suggest that the autocrine production of this cytokine is involved in CR3 up-regulation of costimulatory molecules. An increase in IFN-β would not, however, explain the
effect of CR3 on expression of costimulatory molecules in CpG-treated BMDCs, which did not exhibit increased IFN-β production. To evaluate the requirement of type I IFNs for CR3-mediated up-regulation of signals 1 and 2, we prepared BMDCs from type I IFN-RI-deficient mice (IFN-αβR−/−). IFN-αβR−/−BMDCs are unable to respond to type I IFNs, as demonstrated by their inability to up-regulate costimulatory molecules upon IFN-α stimulation, compared with BMDCs from littermate control mice (Fig. 6C). CR3 ligation was still able to up-regulate MHC and costimulatory molecules on IFN-αβR−/−BMDCs, to a level comparable to wild-type BMDCs (compare Fig. 6D with Fig. 1). Indeed, we saw an effect not only on CD80, MHC II, and CD40, as in normal mice, but even an effect on CD80 and MHC I. We suggest that this is due to the lower levels of CD80 and MHC I present on IFN-αβR−/−BMDCs, levels that are normally regulated by constitutive production of autocrine type I IFNs (35). Just as the addition of LPS or IFN-α masks the effect of CR3 on CD40 and CD86, respectively, basal autocrine type I IFN signaling may partially mask the effect of CR3 on CD80 and MHC I. In conclusion, our results suggest that the induction of costimulatory molecules by CR3 is type I IFN independent.

Excess cross-linking via SA is not necessary for M1/70 to trigger CR3-mediated phenotypic changes

Because CR3 triggering increased costimulatory molecules in our system, where others have reported the opposite effect (36), we examined whether this difference might be due to excessive cross-linking produced by our biotin-SA system. BMDCs were treated with isotype Ab, biotinylated or directly FITC-conjugated anti-CR3 Ab alone, or biotinylated anti-CR3 Ab followed by SA-FITC as in previous experiments. Directly FITC-conjugated M1/70 Abs produced a rim surface staining pattern similar to the biotin-SA-FITC-stained cells (Fig. 7A). After 18 h from the initial exposure to the Abs, both treatments showed similar internalization of the FITC signal, with the directly conjugated Ab producing a homogenous pattern and the biotinylated Ab-SA-FITC combination producing the same granular pattern from previous experiments (Figs. 7A and 2E). Biotinylated M1/70 alone was able to induce up-regulation of CD86, MHC II, and CD40 similar to that seen with BMDCs treated with the biotinylated M1/70 and SA-FITC combination (Fig. 7B). Furthermore, there was no statistically significant difference in the inhibition of TNF-α or IL-12 after LPS stimulation between biotinylated M1/70 alone or with SA-FITC (Fig. 7C). Therefore, M1/70 has the capacity to trigger CR3-induced changes without the need for further cross-linking with SA-FITC, suggesting that the changes in costimulatory molecules and cytokines induced by Ab ligation of CR3 are not due to excessive cross-linking by SA-FITC treatment.

Discussion

CR3 has been described as both a pro- and anti-inflammatory molecule (6, 37). In part, these conflicting results are due to the fact that CR3 binds many ligands, among them iC3b (38) and LPS (39). Apoptotic cells opsonized with iC3b bind to CR3 (10), raising the possibility that CR3 mediates their widely reported immunosuppressive effects (40). In this study, the direct cross-linking of CR3 with a specific Ab induced in DCs the phenotype of high signal 1 (surface expression of MHC) and signal 2 (costimulatory molecules), along with an altered signal 3 (suppressed production of inflammatory cytokines such as IL-12 and increased production of immunomodulatory TGF-β), that lead to impaired stimulation of a Th1/Th2 T cell response. These results suggest that the specific activation of CR3 plays an anti-inflammatory role, leading to suppression of DC stimulatory capacity.

CR3 ligation resulted in the up-regulation of multiple costimulatory molecules, including CD86, CD40, and MHC II, on the DC surface. These effects were independent of TLR activation since they occurred in unstimulated BMDCs, and the further increase in costimulatory molecules seen after TLR or IFN-α stimulation of CR3-ligated BMDCs may represent the additive nature of these two signals.

We found that CR3 ligation increased MHC and costimulatory markers differently depending on the second stimulus used. We explain these differences, namely the lack of an incremental increase of CD40 in the case of LPS and both CD40 and CD86 in the case of IFN-α, with the idea that maximal expression of these molecules is obtained with the stimulus alone, masking further effects from CR3. Similarly, we suspect that the greater up-regulation of CD80 and MHC I in the IFN-αβR−/−BMDCs may be due to the initial lower levels of CD80 and MHC I present in these cells, levels that are normally regulated by autocrine type I IFNs produced constitutively by DCs (35).

The role of type I IFNs in the outcome of CR3 ligation is of interest: IFN-β production was increased by CR3 triggering followed by TLR stimulus. We cannot draw any conclusions as to the effect of CR3 on constitutive levels of IFN-β, because these were below our limits of detection (data not shown). Despite the increased IFN-β production after CR3 ligation and TLR stimulus, type I IFNs were not required to mediate the effects of CR3 ligation. IFN-β was up-regulated by CR3 ligation upon stimulation of TLR3 and TLR4, both of which use TRIF. However, IFN-β was not increased when CR3 ligation was followed by TLR9 stimulation, which uses only the MyD88 pathway. These results suggest that CR3 ligation may increase IFN-β production in a TRIF-dependent fashion. These signaling differences may have important consequences for regulation of the immune response. Because type I IFN may play a role in providing signal 3 as an alternative to IL-12 (41), we envision that the excess IFN-β produced by CR3 activation followed by TLR3 and TLR4 stimulation allows effective immune responses to bacteria and other pathogens opsonized by iC3b. In contrast, the lack of excess of IFN-β in response to TLR9 stimulation could suppress signal 3 in the presence of DNA from apoptotic cells, which can trigger TLR9, leading to peripheral tolerance to these self-Ags. Alternatively, because IFN-α-producing plasmacytoid DCs are able to induce CD4+ T cells to become CD25+ regulatory T cells (42, 43), it is possible that CR3 induces type I IFNs in myeloid DCs for a similar purpose. The role of type I IFNs involved in the provision of signal 3 in vivo is still unclear and needs further exploration in this system.

A complex interplay between multiple signaling molecules is likely required to achieve the complete effect of CR3 both at rest and after activation. Suppression of TLR-induced production of cytokines by anti-CR3 Ab is not explained by blockade of TLR ligand binding to CR3, because only LPS, and not poly I:C and CpG, have been shown to interact with CR3 (44). This suppression does not involve inhibition of TLR-signaling pathways, because it does not show preference for either the TRIF or MyD88 pathway, and allows the up-regulation of costimulatory molecules and NF-κB p65 activation, both of which require intact TLR signaling. We found that CR3 ligation inhibited LPS-induced ERK1/2 phosphorylation (our unpublished data), and because ERK inhibition could only partially recapitulate CR3 effects (our unpublished data), we must hypothesize that other molecules are involved in CR3 signaling. Given that apoptotic cells affect other MAPK pathway molecules (45, 46), future efforts will be directed toward studying these enzymes.

The functional outcome of the changes induced by CR3 ligation is to effectively blunt the capacity of BMDCs to stimulate both
CD4 and CD8 primary T cell responses, as measured by decreased IFN-γ production. Because we were unable to measure IL-4 production in our CD4 cocultures, we propose that the decrease in IFN-γ is not due to a skewing toward a Th2 phenotype, but rather to the functional inhibition of DC stimulatory capacity by CR3 ligation. The recent report by Skoberne et al. (36) showed a similar CR3-induced inhibition of T cell stimulatory activities in human DCs, although, in their case, CR3 ligation also prevented the up-regulation of costimulatory molecules in response to LPS or a mixture of proinflammatory cytokines. The differences in our report may be due to the use of an Ab to directly ligate CR3, as opposed to an RBC system (i.e., RBCs coated with anti-CR3 Abs). We demonstrate that M1/70, the Ab we used to cross-link CR3, is an activating Ab, because it can trigger CR3 internalization and anti-inflammatory effects without requiring cross-linking with SA. Because the system used by Skoberne et al. (36) did not test the ability of their mAb in solution alone to effect DC changes, it is possible that the difference in our results are due to differences in receptor activation by a soluble molecule vs that seen when the ligand is fixed to a platform such as an RBC. Further investigation into the mechanisms operating in the two systems will answer important questions regarding the exact mechanisms used by CR3 to suppress DC immunogenicity.

We found that CR3 ligation suppresses DC capacity to stimulate naive T cell responses and that T cells exposed to CR3-ligated DCs have a blunted ability to produce a secondary response compared with T cells that were initially exposed to isotype-ligated BMDCs. These outcomes did not require a difference in T cell proliferation and absolute numbers, suggesting that lack of Ag presentation and/or deletion do not play a role. Rather, CR3-ligated DCs may either prime memory T cells to only make a partial response, or only a fraction of T cells are fully primed by CR3-ligated BMDCs, again leading to a partial response. Thus, while CR3-ligated BMDCs cannot tolerate T cells in culture, the secondary response of the T cell population is not as high as would be expected from T cells initially stimulated by normal BMDCs. It is likely that the simultaneous activation of multiple apoptotic cell receptors such as CD36, or mer (47), is required to commit DCs to tolerance. This may be in part due to the fact that CR3 acts in an inflammatory manner when activated in nonapoptotic cell contexts such as when ligated by certain bacterial products (37). Thus, it would not be beneficial for the DC to commit to tolerization on the basis of CR3 ligation alone. Future work will be directed at examining the effects of CR3-ligated DCs in an in vivo system to confirm our results and better define the physiologic significance of the blunted secondary response.

Both CR3 and CD36 ligation produce a TGF-β response by BMDCs. TGF-β is a potent immunomodulatory cytokine that has been associated with regulatory T cell induction (29) as well as multiple other effects (27, 28, 30). We could speculate a role for this TGF-β in the blunted T cell responses seen in CR3-ligated BMDCs, though it is difficult to invoke a role for regulatory T cells in the blunted CD8 response as there were no CD4 cells present in these cultures. Future investigation into the specific requirement for TGF-β will be needed to address these questions.

CD36, while previously described as mediating some of the anti-inflammatory effects of apoptotic cells via IL-10 production, had little effect in this study. This may be explained in several ways. In the original studies by Voll et al. (9), the effect of CD36 was demonstrated via blocking CD36 ligation by apoptotic cells. In our study, we attempted to activate CD36 directly. Furthermore, we have shown that ligation of CD36 in our system ultimately leads to decreased levels of the molecule itself, as measured by fluorescence intensity, perhaps limiting its effect. CR3 levels, despite internalization of the molecule, did not decrease, maybe by localizing in different intracellular compartments and possibly providing a stronger continuous signal. It is becoming clear that different apoptotic cell receptors have divergent effects, because ligation of αvβ3, another DC apoptotic cell receptor important for phagocytosis, also did not result in changes in cytokine production (36).

Moreover, CD36 ligation induced a strong TGF-β response in unstimulated BMDCs and upon TLR stimulation in our study, altering the cytokine profile of these cells despite its lack of effect on IL-12 and the other cytokines investigated.

We have identified CR3 as an apoptotic cell receptor that is able to deliver a nondanger signal to DCs by actively inducing them to up-regulate MHC, CD40, CD80, and CD86 expression, while suppressing proinflammatory cytokines and stimulating immunomodulatory TGF-β production. The net outcome of these phenotypic changes is the decreased capacity of DCs to stimulate naive T cells. Defects in this process could lead to autoimmunity, in which a deficient clearance of apoptotic cells has been suggested to be pathogenic (48). We envision a scenario where qualitatively or quantitatively abnormal apoptotic cells might not bind CR3 properly, therefore failing to deliver the nondanger signals that otherwise would suppress APC activation and inhibit their presentation of self-Ags from apoptotic cells, ultimately disrupting the maintenance of peripheral tolerance. Thus, greater understanding of these pathways may help to elucidate the origins of autoimmune disease and provide potential targets for intervention.

Furthermore, the increase in allograft transplantation procedures pushes the search for novel immune modulators that can prevent rejection of the allograft. The suppression of proinflammatory cytokines induced by CR3 on DCs may be beneficial in these cases. CR3 triggering is often obtained using particulated systems such as RBC opsonized with iC3b (6, 8). The ability to induce CR3 inhibitory effects using direct ligation via mAbs, as we achieved, is encouraging for its ready feasibility in testing this approach therapeutically.

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Disclosures

The authors have no financial conflict of interest.

References


