

# Opsonization of Apoptotic Cells by Autologous iC3b Facilitates Clearance by Immature Dendritic Cells, Down-regulates DR and CD86, and Up-regulates CC Chemokine Receptor 7

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## Abstract

Immature dendritic cells (iDCs) do not mature after uptake of apoptotic cells and may play a role in the induction of peripheral tolerance to self antigens derived from apoptotic material. The integrins,  $\alpha\beta3$ ,  $\alpha\beta5$ , and the scavenger receptor, CD36, have been shown to mediate uptake of apoptotic cells by iDCs. However, it is not known whether the complement system, also takes part in this process. In this study we investigated the ability of iDCs to bind to apoptotic cells opsonized by iC3b. Monocyte-derived dendritic cells were offered apoptotic Jurkat cells opsonized by autologous iC3b and labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanineperchlorate. A significant increase ( $P < 0.001$ ) in the amount of cleared apoptotic cells was seen at low ratios. Despite increased efficiency of uptake, interaction between iC3b-opsonized apoptotic cells and iDCs down-regulated the expression of major histocompatibility complex class II, CD86, CC chemokine receptor (CCR)2, CCR5, and  $\beta2$ -integrins ( $P < 0.001$ ), and up-regulated expression of CCR7 ( $P < 0.001$ ). In addition, iDC maturation responses to CD40L and lipopolysaccharide were significantly inhibited. We conclude that opsonization of apoptotic cells by iC3b induces tolerant iDCs that are able to migrate to lymph nodes.

Key words: apoptosis • dendritic cells • complement • tolerance • autoimmunity

## Introduction

Immature dendritic cells (iDCs)\* engulf apoptotic cells and are able to acquire antigens found in the dying cells (1–5). Rubartelli et al., suggested that iDCs may use an integrin,  $\alpha\beta3$ , the vitronectin receptor, for uptake of apoptotic cells (6). Albert et al. showed that another integrin,  $\alpha\beta5$  with the scavenger receptor CD36, both mediate uptake of apoptotic cells by iDCs (7). Later, Nouri-shirazi et al. suggested that iDCs may have subpopulations that use different integrins (4).

The complement system has been shown to be activated by human apoptotic material (for a review, see reference 8). We were able to show activation of both the classical and alternative complement pathways by apoptotic cells with opsonization by iC3b (9). Taylor et al. suggested a hierarchical role for C1q in the ex vivo clearance of apoptotic thymocytes by thioglycolate-derived peritoneal murine macrophages (10), and Ogden et al. showed a role for CD91 and calreticulin in the uptake of apoptotic cells opsonized by mannose binding lectin and C1q (11). The possible importance of complement-mediated opsonization of apoptotic cells was further shown by the development of systemic lupus erythematosus in >90% of homozygous patients with C1q deficiency, in 75% of homozygous patients with C4 deficiency, and by the development of glomerulonephritis associated with increased apoptotic cells in the glomeruli of C1q deficient mice (12). Cr2, a complement degradation product receptor, found in mature B cells and

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\*Abbreviations used in this paper: DIL, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanineperchlorate; iDC, immature dendritic cell.

in follicular dendritic cells, was recently reported as a candidate gene in the murine Sle1c lupus susceptibility locus (13). Thus, C1q and complement degradation products may take part in normal clearance of apoptotic cells and, as such, may be involved in maintaining peripheral tolerance induction to auto antigens found in apoptotic material.

In this work we studied the ability of iDCs to bind efficiently to apoptotic cells opsonized by iC3b. Herein, we show that opsonization of apoptotic Jurkat cells by autologous iC3b increased the efficiency of their uptake by iDCs but inhibited maturation.

## Materials and Methods

**Media and Reagents.** Culture medium consisted of RPMI 1640, 1% L-glutamine, 1% penicillin/streptomycin (Biological Industries), 1% autologous human plasma, and recombinant human cytokines, GMCSF, and IL-4 (R&D Systems or Pepro-Tech). Phosphatidylserine was stained by annexin-V-FITC (R&D Systems). Mouse anti-human iC3b-PE was obtained from Quidel Corp. Mouse anti-human CD11b-FITC, CD11c-PE, CD14-APC, CD14-FITC, HLA-DR-FITC, CD1a-PE, CD1a-FITC, and isotype controls were obtained from IQ Products. Mouse anti-human CD83-PE was obtained from Immunotech, and mouse anti-human CD86-PE from Cymbus Biotechnology. CH11 was purchased at Upstate Biotechnology. PKH-67, complement depleted serum, latex beads, and LPS were obtained from Sigma-Aldrich, and 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanineperchlorate (DIL) was obtained from Molecular Probes.

**Dendritic Cells.** Immature monocyte-derived dendritic cells were generated from the CD14<sup>+</sup> selected fraction of PBMCs and from blood donors buffy coats. iDCs were isolated as described elsewhere (1) with modification in monocytes selection. Briefly, PBMCs were isolated using Ficoll as described previously (9) and anti-CD14 magnetic beads were used in order to isolate monocytes from PBMCs according to the manufacturer's instructions (Miltenyi Biotec). Monocytes were placed in wells at a concentration of  $2.5 \times 10^6/3$  ml culture media in the presence of 1% autologous plasma, GMCSF (1,000 U/ml), and IL-4 (1,000 U/ml). Every 2 d 0.3 ml were removed and 0.5 ml media containing plasma, IL-4, and GMCSF, 500 U/ml, were added. By day 6, >90% of the cells were CD14<sup>+</sup>, with low expression of DR, CD-83, and CD-86.

**Staining Cells, Induction, and Detection of Apoptosis.** Apoptotic cells were generated using a tumor cell line, Jurkat T cell lymphoma (9). Jurkat T cell lymphoma cells were washed with RPMI in the absence of serum and stained with DIL according to the manufacturer's instructions. In some experiments cells were stained with PKH-67. After staining, 2% FCS was added and the cells were incubated at 37°C for 2.5 h for the induction of serum-withdrawal apoptosis or 2.5 h with 0.5 µg/ml, CH11, for fas-induced apoptosis. Apoptosis was detected by double staining with annexin-V-FITC and propidium iodide, as well as by the percentage of hypodiploid region of propidium iodide stained cells (14).

**Opsonization by Complement.** Complement activation was shown by measuring the release of C3a by ELISA (The Binding Site). Complement opsonization with iC3b was verified using a FAC-Scan™ (Becton Dickinson) and costaining of annexin-V-FITC and iC3b-PE. In addition, membrane localization was evaluated using confocal microscopy.

**Interaction of Apoptotic Cells with iDCs.** Labeled apoptotic cells were added to iDCs on day 6 of culture, for 1 or 2 h/37°C in 200 µl volume of culture media in the presence or absence of 15% autologous human serum or plasma or complement depleted serum or heat inactivated serum or heat inactivated plasma. Heat inactivation of serum or plasma was performed at 56°C for 30 min. In most assays, iDCs were labeled with CD1a-PE, or CD14-APC. Uptake was read by a FACScan™ and validation of the results was performed using PKH-67 for staining apoptotic cells. Adhered, noninternalized apoptotic Jurkat cells were detected using anti-CD3-FITC. In additional experiments, unlabeled apoptotic cells were offered to iDCs and the expression of membrane molecules was examined 2–48 h later. In some interactions, αCD11b (αCR3), αCD11c (αCR4), or control mouse IgG, were added. Uptake of latex beads (Sigma), was used as a control for phagocytosis.

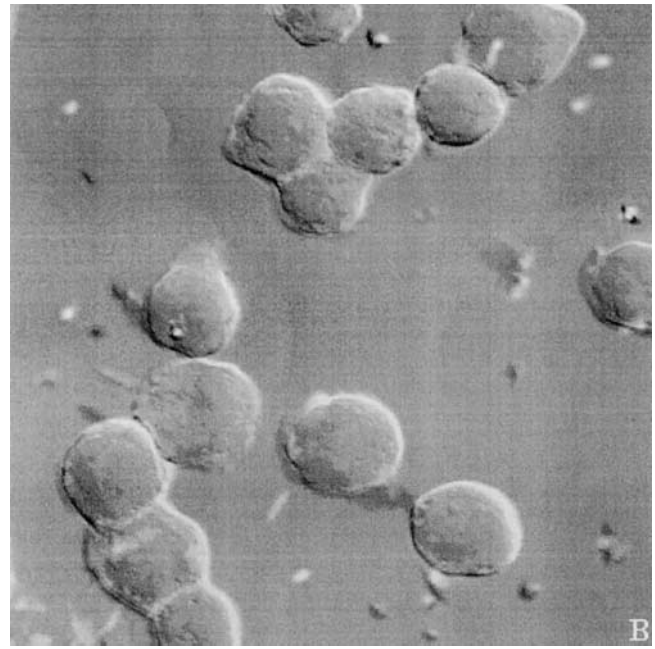
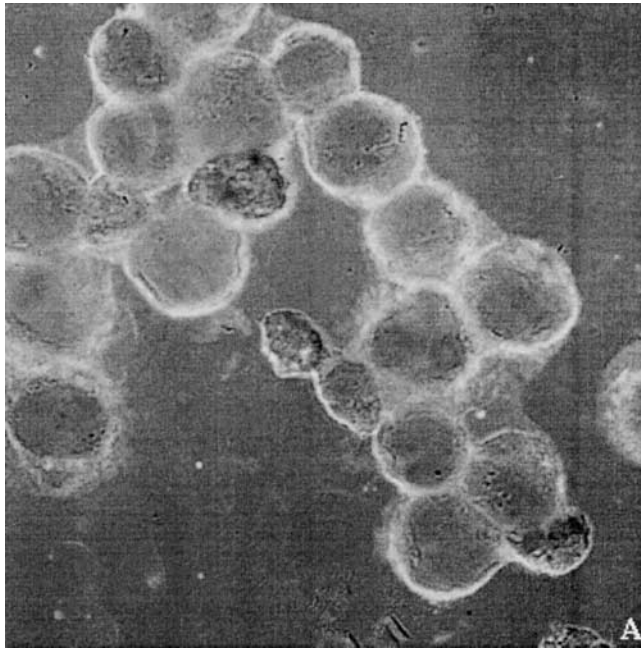
**Stimulation of iDCs via CD40 and LPS.** iDCs were incubated overnight with 10 µg/ml CD40L and 20 ng/ml IL-4 or 5 µg/ml anti-CD40, or 2 ng/ml LPS, and the expression of DR and CD86 was evaluated. In inhibition experiments, iDCs were first exposed for 2 h to apoptotic cells and then exposed to CD40L or anti-CD40, or LPS.

**Statistics.** Student's *t* test was used to compare uptake and Kolmogorov-Smirnov analysis was used to compare the expression of surface receptors on iDCs.

## Results

The conditions for serum-withdrawal apoptosis were selected because 60–70% of cells were annexin-V positive and <7% were positive for propidium iodide staining (unpublished data). Activation of complement by apoptotic Jurkat cells was shown previously by us and by others (9, 15, 16). However, in order to verify that apoptotic Jurkat cells can activate complement under the specific conditions that we used for induction of apoptosis, we measured release of C3a in the supernatants. C3a release measured in the supernatants of interacting iDCs and apoptotic Jurkat cells was found to be  $1,990 \pm 198$  ng/ml in the presence of 15% fresh serum as compared with  $220 \pm 38$  when the interaction occurred in the presence of heat-inactivated serum ( $P < 0.001$ ). To further verify that apoptotic cells were opsonized by iC3b we stained apoptotic Jurkat cells for iC3b deposition and found membrane staining using confocal microscopy (Fig. 1). These results were verified using flow cytometry, where deposition of iC3b was shown in >90% of annexin-V-positive cells but in <3% of annexin-V-negative cells (not shown).

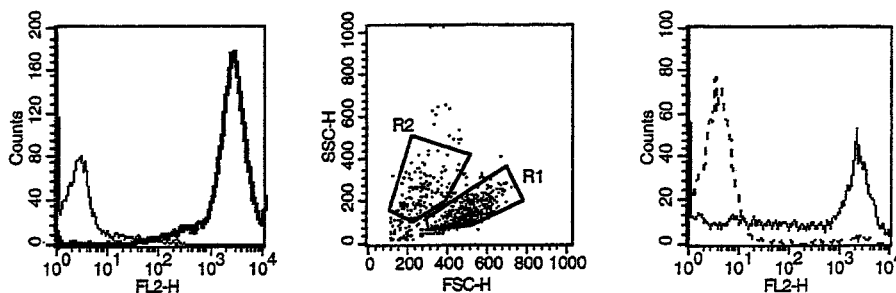
We generated iDCs that were >90% CD14<sup>+</sup>CD1a<sup>+</sup>, as well as low DR, CD83, and CD86 (see below). To determine whether complement activation increased uptake of apoptotic cells, we measured uptake by iDCs by FAC-Scan™ using similar methodology to that described (4, 7, 17), and as explained in Fig. 2. As shown in Fig. 3 A, at a ratio of 1:1, iDC:apoptotic cell,  $44.25 \pm 2.21\%$  of iDCs bound/engulfed apoptotic Jurkat cells in the absence of serum compared with  $83.25 \pm 4.3\%$ , in the presence of serum ( $P < 0.001$ , triplicates, representative of six experiments). At a ratio of 1:2 the gap decreased but there was still a significant difference in all experiments,  $59.2 \pm 4.3\%$  versus  $93 \pm$



**Figure 1.** Apoptotic Jurkat cells are opsonized by iC3b. Jurkat cells were induced to undergo serum withdrawal apoptosis, followed by incubation and exposure to 15% fresh serum from donor dendritic cells for 1 h/37°C/5% CO<sub>2</sub>. Cells were stained with annexin V-FITC (A), or iC3b-PE (B). As seen by confocal microscopy (Nomarsky, 40, zoom ×2), cells show membranous staining of both Phosphatidylserine and iC3b. Viable cells or apoptotic cells exposed to heat-inactivated serum did not stain for iC3b.

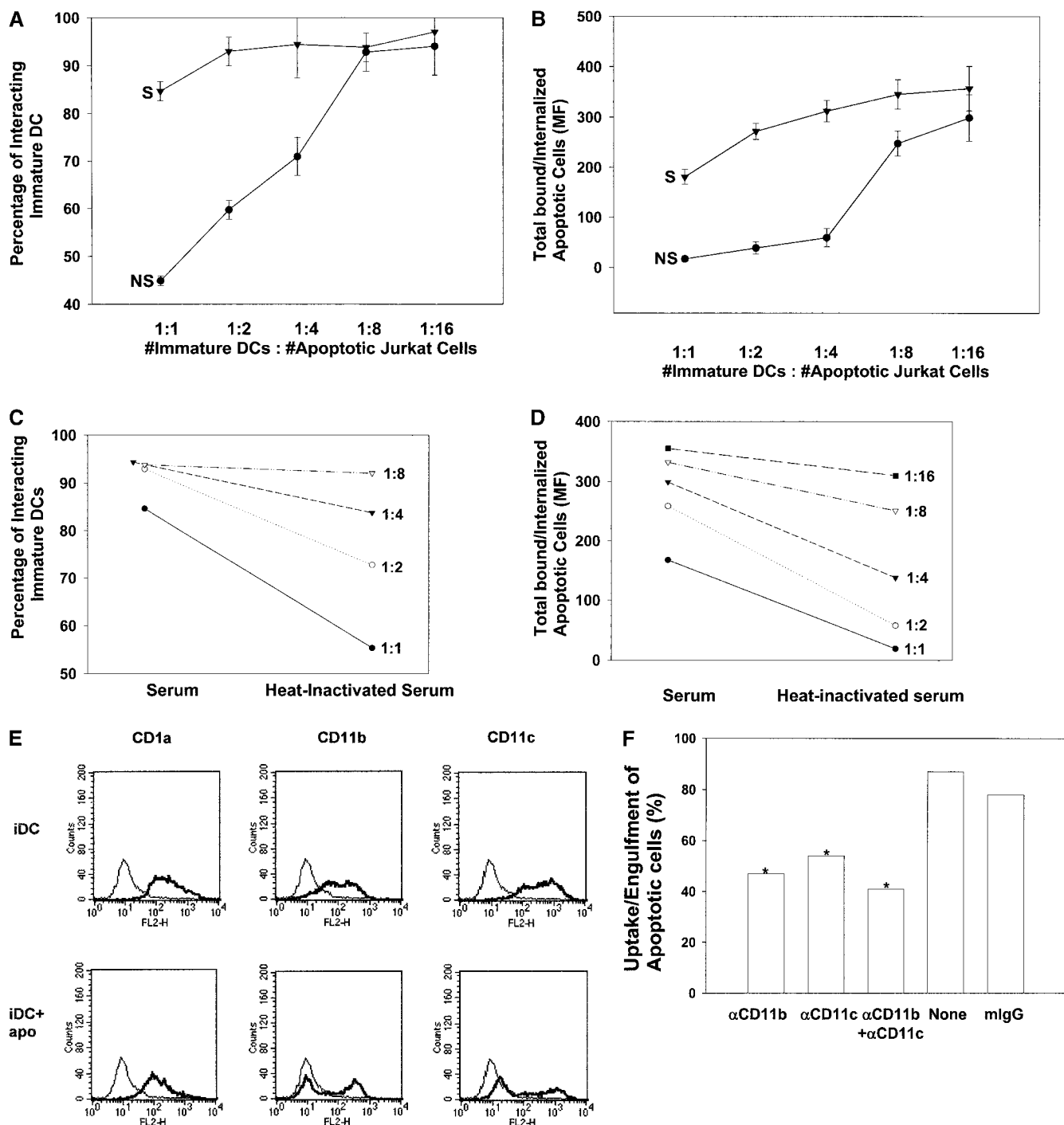
5.3%, respectively ( $P < 0.001$ ). However, at 1:4 there was a further decrease and the difference was nonsignificant in one out of six experiments. At ratios of 1:8 and 1:16 there was no difference in the number of iDCs that bound/engulfed apoptotic Jurkat cells in the absence or presence of autologous fresh serum. To further evaluate the relative number of apoptotic cells bound or engulfed by iDCs, we used the median fluorescence of DIL detected in the area of the gated iDCs. As shown in Fig. 3 B, median fluorescence, at a ratio of 1:1, was increased up to eightfold in the presence of au-

tologous serum. This difference decreased gradually upon increasing the ratio but was still elevated (1.3–1.8-fold) at a ratio of 1:16. To further verify that this effect was due to complement activation we simultaneously examined interactions with autologous heat-inactivated serum. Heat-inactivation abolished the effect of serum, the result being similar to that seen in the absence of serum (Fig. 3, C and D). The experiments were repeated using autologous plasma instead of serum, with similar results (not shown). To verify that all interacting cells were phagocytosed, staining with



**Figure 2.** Evaluation of interaction between iDCs and apoptotic Jurkat cells using flow cytometry. (Left) Jurkat cells were stained with DIL and then were induced to undergo apoptosis. DIL-stained apoptotic cells (bold line) and unstained apoptotic cells (solid line) are shown. (Middle) When iDCs and Jurkat cells were incubated together at 4°C, immediately before flow cytometry acquisition, two subpopulations were recognized. R1 consisted of >93% CD1a<sup>+</sup>CD3<sup>+</sup>DIL<sup>+</sup> cells, whereas R2 shows >94% CD1a<sup>+</sup>CD3<sup>+</sup>DIL<sup>+</sup> cells. Thus, R1

determined population of noninteracting iDCs and R2 determined population of unbound Jurkat cells. During a 2-h interaction between iDCs and DIL-stained apoptotic Jurkat cells at 37°C, iDCs, acquired DIL from bound or phagocytosed apoptotic cells. Thus, after interaction at 37°C, R1 contains both noninteracting iDCs and interacting iDCs. (Right) R1 of the former figure after a 2-h interaction between iDCs and DIL-stained apoptotic Jurkat cells, at 37°C. Most of the interacting iDCs (bold line) acquired DIL in this experiment as compared with unstained iDCs (dotted line). iDCs that did not bound or phagocytosed apoptotic cells are found in the area of unstained iDCs. During 2 h of interaction no passive staining of iDCs occurred as demonstrated by 2 chambers incubation (not shown). The percentage of interacting iDCs that acquired DIL, as well as, mean and median fluorescence, were measured. The interaction was performed in different iDC:Jurkat ratios and in the presence or absence of fresh serum or heat inactivated serum. In inhibition studies, iDCs were preincubated with monoclonal antibodies for 30 min before interaction. To distinguish between bound apoptotic Jurkat cells and internalized cells, we stained with anti-CD3-FITC and determine what percentage of iDCs that acquired DIL, stained for CD3. CD3, expressed on apoptotic Jurkat cells (and not on iDCs), stained only noninternalized, adhered, apoptotic Jurkat cells. Similar results were obtained upon validation of this system, using Jurkat cells stained with PKH, or double staining of CD1a-FITC, DIL-apoptotic Jurkat cells.



**Figure 3.** Interaction of apoptotic cells with iDCs is facilitated by autologous complement and complement receptors. After 2 h of incubation with apoptotic cells, iDCs were analyzed for interaction using flow cytometry as explained in Fig. 2. (A) The percentage of iDCs that interacted with DIL-stained, iC3b-opsonized, apoptotic cells, is given for different ratios of iDCs:apoptotic cells. (B) Median fluorescence of DIL as expressed in iDCs is given for different ratios. (C and D) Heat-inactivation abolished the effect of serum seen in A and B, and brought it to the level of interaction seen in the absence of serum. Average of triplicates measures is given (error bars). Representative of six experiments. (E) iDCs express CD11b/CD18 and CD11c/CD18 (iDC, bold line). Marked down-regulation of both CD11b/CD18 and CD11c/CD18 but not in CD1a, was observed after interaction with iC3b-opsonized apoptotic cells (iDC+apo, bold line). (F) 91% of iC3b-opsonized apoptotic cells were cleared by iDCs (None). No significant increase in the remaining noncleared apoptotic cells was seen when control mouse IgG was added (mIgG), compared with significant, 53% inhibition, in clearance seen when αCD11b ( $P < 0.05$ ), or αCD11c (46% inhibition,  $P < 0.05$ ), or both (59% inhibition,  $P < 0.01$ ), were added. Representative of six experiments. DC, dendritic cells; MF, median fluorescence; NS, no serum, i.e., apoptotic cells were not exposed to fresh serum from iDC donor; S, serum, i.e., apoptotic cells were exposed to 15% fresh serum from iDC donor.



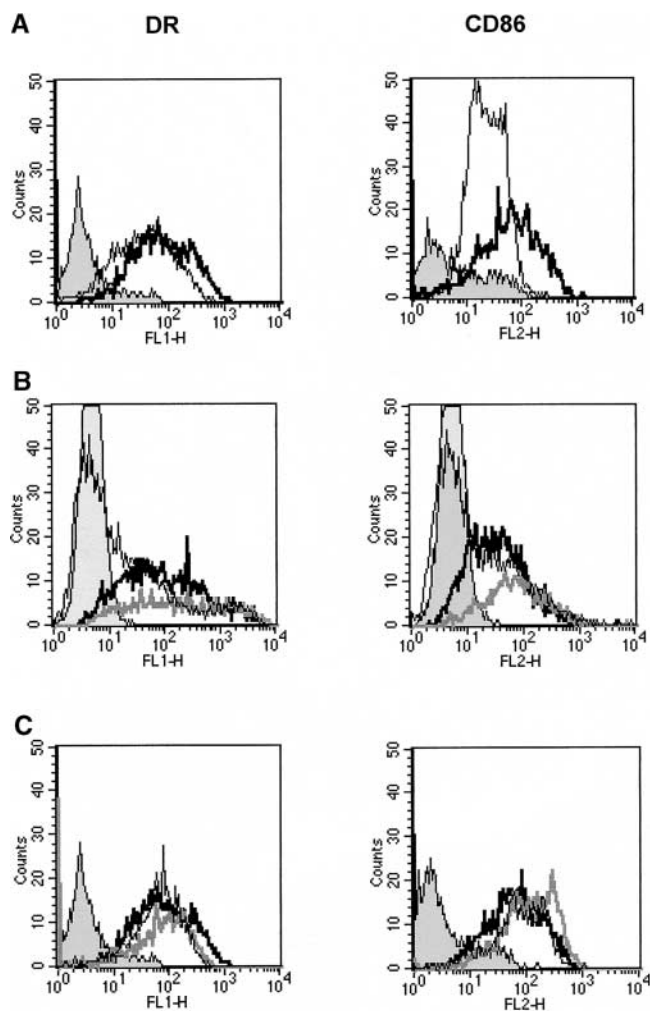
anti-CD3 was performed. 2 h after interaction, between 18–35% of the interacting cells still had bound Jurkat cells (at ratio 1:4). However, by 18 h after the interaction, <10% of iDCs that acquired DIL, stained for CD3.

CD11b/CD18 and CD11c/CD18 are known receptors for complement degradation products and as shown in Fig. 3 E, all iDCs expressed the complement receptors with marked down-regulation of these receptors after exposure to iC3b-opsonized apoptotic cells. To test the role of  $\beta$ 2 integrins in mediating interaction with apoptotic cells we further exposed iDCs to monoclonal antibodies to these receptors before interaction with iC3b-opsonized apoptotic cells. As shown in Fig. 3 F, uptake of apoptotic cells by iDCs was significantly reduced when exposed to  $\alpha$ CD11b/CD18 and  $\alpha$ CD11c/CD18 but not to control antibody.

Next we attempted to verify that the increased uptake of iC3b opsonized apoptotic cells did not induce maturation of iDCs. iDCs were stained for CD1a, DR, CD83, and CD86, before and after exposure to apoptotic cells opsonized by autologous iC3b. In Fig. 4 A, we show that iDC did not up-regulate surface DR or CD86 (as well as CD83, not shown) after uptake of apoptotic cells opsonized by iC3b. In fact, as shown in Fig. 4 A, in most experiments we observed down-regulation of DR and CD86. In addition, we measured IL-12 secretion in supernatants of iDCs exposed to opsonized apoptotic cells. No increase in baseline level was observed in up to 48 h (data not shown). To further test whether iDCs exposed to iC3b-opsonized apoptotic cells were in a state of anergy we exposed iDCs to CD40L or to anti-CD40. We observed marked inhibition of the maturation response to CD40L after exposure to iC3b-opsonized apoptotic cells (see Fig. 4 B). Remarkably, in most experiments, the expression of DR and CD86 was down-regulated to levels below levels that were seen in iDCs that were not exposed to CD40L. This immune-paralysis induced by apoptotic cells was shown also during LPS administration (Fig. 4 C). However, LPS-induced inhibition was to the basic levels of iDCs. Uptake of latex particles did not inhibit maturation after LPS and did not down-regulate the expression of CD86 and MHC class II with median fluorescence of 88 and 124, after phagocytosis, and 91 and 118 without phagocytosis, respectively.

We further strove to determine whether the down-regulation of maturation molecules such as CD86, CD83, and MHC class II was linked to the absence CCR7-mediated migration to lymph nodes. We were surprised to see, as shown in Fig. 5, that after interaction with autologous iC3b-opsonized apoptotic Jurkat cells, iDCs demonstrated mild but significant up-regulation in the expression of CCR7 but down-regulation of CCR2 or CCR5. Phagocytosis of latex beads did not up-regulate the expression of CCR7 as median fluorescence was  $3.66 \pm 0.8$  in iDCs and  $3.88 \pm 0.92$  after phagocytosis of latex beads (three experiments).

Taken together, activation by complement and opsonization of apoptotic cells by autologous iC3b, were associated with a significant increase in uptake by iDCs at low ratios of iDCs:apoptotic cells. This effect not only was not associated with “maturation,” but rather, induced

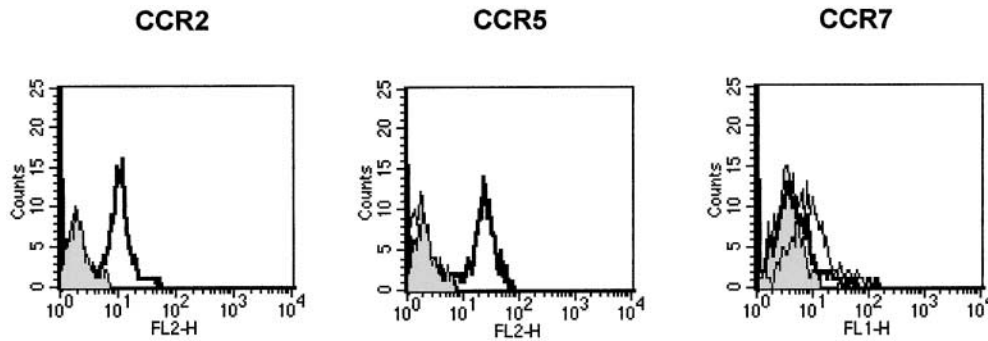


**Figure 4.** Apoptotic cells opsonized by iC3b, down-regulate MHC class II and CD86, and immune-paralyze the effect of CD40L or LPS, on iDCs. (A) Expression of DR-FITC and CD86-PE (bold lines) on iDCs were down-regulated upon exposure to apoptotic cells (solid lines, median fluorescence 67 to 40, and 54 to 17, respectively,  $P < 0.001$ ). (B) DR-FITC and CD86-PE, expressed on iDCs (bold lines, median fluorescence 62, and 22, respectively), were up-regulated (gray lines, median fluorescence 133, and 58, respectively,  $P < 0.001$ ) after exposure to CD40L. However, if iDCs were exposed to apoptotic cells, before CD40L (solid lines), up-regulation was inhibited (median fluorescence 7.4, and 7.84, respectively,  $P < 0.001$ ). (C) Similarly to CD40L, DR-FITC, and CD86-PE, expressed on iDC (bold lines, median fluorescence, 57 and 55, respectively), were up-regulated (gray lines, median fluorescence 80, and 117, respectively,  $P < 0.001$ ), after exposure to LPS. Again, if iDCs were exposed to apoptotic cells, before LPS (solid lines), up-regulation was inhibited (median fluorescence 60, and 71, respectively;  $P < 0.001$ ).

down-regulation of MHC class II and CD86, as well as CCR2, CCR5, CD11b/CD18, and CD11c/CD18. Despite lack of classical maturation, iDCs expressed CCR7.

## Discussion

As a part of innate immunity, the main purpose of opsonization by complement was traditionally ascribed to



**Figure 5.** CCR7 is expressed on iDCs after interaction with iC3b-opsonized apoptotic cells. iDCs were stained for the expression of chemokine receptors (bold lines), CCR2, CCR5, and CCR7 and compared with iDCs that were exposed to apoptotic cells (solid lines). On day 6, iDCs stained for CCR2 and CCR5 but not for CCR7. Upon exposure to opsonized apoptotic cells marked down-regulation of CCR2 and CCR5 was observed (median fluorescence

were down-regulated (from 9.14 to 1.88, and from 21 to 1.55, respectively,  $P < 0.001$ ). In contrast, median fluorescent of CCR7 was up-regulated from 3.31 to 6.92 ( $P = 0.001$ ). Representative of four experiments. Range of up-regulation of CCR7, 60–300% increase.

coating of microorganisms that penetrated the mammalian sterile milieu with subsequent clearance of opsonized particles by specific receptors on the surface of leukocytes. Thus, in this context, the requirement for complement activation to ensure efficient uptake of apoptotic cells by iDCs, appears contrary to the notion that phagocytosis of apoptotic cells is a noninflammatory process (18, 19). However, as shown here, opsonization of apoptotic cells by iC3b down-regulated the expression of MHC class II or CD86 on iDCs, and therefore would not be expected to increase T cells priming and autoimmunity in “normal” circumstances, and may actually have a role in maintaining tolerance in a steady-state (20, 21).

A role for complement and complement receptors in clearance of apoptotic cells by macrophages, have been shown previously (9–11, 16, 22). As shown here, the use of ratios equal to or higher than 1:8 could miss this effect. Furthermore, using the number of phagocytes participating in uptake rather than the actual relative number of apoptotic cells being bound or engulfed, masks the complement contribution, even at ratios of 1:4. When total bound/engulfed apoptotic cells were measured, as expressed in median fluorescence, the complement effect was still evident at 1:8. These observations may provide an explanation for the findings in some previous studies that used high ratios or percentage of phagocytic cells for evaluation of uptake, and in which serum or complement did not seem to have an effect. In addition, when certain batches of sera were used, we noted inhibitory effects on phagocytosis that were eliminated only when we used autologous sera. Thus, the use of nonautologous sera may have both a facilitating effect related to complement and an inhibitory effect of uncertain cause.

CD11b/CD18 and CD11c/CD18 that were shown to be receptors for iC3b (23) may function in several ways. First, in adhesion as down-regulation of CD11b/CD18 and CD11c/CD18 may facilitate de-adhesion and migration. Second, as shown in this study, opsonization of apoptotic cells by iC3b may facilitate peripheral or tissue specific self-antigen capture either by tethering, phagocytosis, or both. It may also help to compete with less potent APCs like B cells that do not express CD11b/CD18 and CD11c/CD18

at the level of lymph node. Third, integrins also elicit signal transduction events (for a review, see reference 24). Ligation of iC3b-opsonized particles to complement receptors does not have a proinflammatory effect and was shown to signal for an antiinflammatory response, as demonstrated by down-regulating IL-12 and  $\gamma$ -interferon production by human monocytes (25–27). In addition, binding and phagocytosis via macrophage CD11b/CD18, did not trigger a respiratory burst (28, 29), or leukotriene release (30). Transdominant suppression was described for  $\alpha\beta 5$  by ligand to integrin receptor (31) and it is not known if, similarly, ligation of  $\beta 2$  integrins may mediate down-regulation of phagocytic function or surface expression of molecules such as CD86 or MHC class II. Fourth, iDCs that capture apoptotic material were suggested to be short-lived dendritic cells (32) but ligation of integrins may serve as an antiapoptotic signal (33). Indeed, CD11b/CD18-deficient granulocytes are relatively protected from apoptosis (34). Fifth,  $\beta 2$  integrin are known for their bacteria and LPS binding properties (35), and down-regulation may avoid interactions that lead to proinflammatory consequences and recruitment to inflammatory sites.

Some limitation of our study are noteworthy. First, it is possible that the function of complement receptors and CD36 represent adherence or tethering whereas other receptors described, such as  $\alpha\beta 5$ ,  $\alpha\beta 3$ , Mer, or the phosphatidylserine receptor (36), mediate phagocytosis. We did not directly address the question as to whether complement facilitation is mediated by increased adherence or increased phagocytosis but we were able to show that complement enhanced interaction of apoptotic cells with iDC and that by 16–18 h >90% of cells were internalized, at low ratios. In addition, cytokines, chemokines, autacoids, and other factors may function in tethering and/or engulfment. In the same line, phagocytosis of apoptotic cells involves a complex of receptors and we cannot attribute the down-regulation of costimulatory receptors only to the presence of complement degradation products or ligation of complement receptors. Second, it is possible that some cells and/or some modes of apoptosis do not activate complement and therefore some apoptotic cells are neither opsonized nor bound nor engulfed through a complement-dependent mechanism.

iDCs ingest antigens by phagocytosis, macropinocytosis or receptor-mediated endocytosis. After ingestion, iDCs are triggered to undergo a developmental program called maturation (37). In this study we were able to show that after interaction with apoptotic cells, iDCs not only did not up-regulate, but rather down-regulated MHC class II and CD86, emphasizing the possible role of dendritic cells in inducing anergy rather than priming autoimmune T cells. This is further illustrated by the state of anergy of iDCs exposed to iC3b-opsonized apoptotic cells that did not up-regulate MHC II and CD86 when stimulated by CD40L or LPS and by the lack of IL-12 secretion. The mechanism of down-regulation of CD86 and MHC class II is not known but could be related, in the case of MHC class II, to the high capacity of endocytosis (38), or by regulation of proteolysis by cytokines (39).

An additional functional modification, usually attributed to mature dendritic cells, is the ability to migrate from peripheral tissues to secondary lymphoid organs, achieved through the modification of chemokine receptors, most notably, the up-regulation of CCR7 (EBI1; references 40–43). CCL19 (MIP-3 $\beta$ , ELC), a ligand for CCR7, have been shown to be expressed in various lymphoid tissues (44–46) and in T cell-rich area of lymph nodes (42). Furthermore, CCL19 expression was restricted to paracortical areas, where either generation of primary T cell response or tolerance is induced. As CCR7 was reported to be expressed mainly in mature dendritic cells, a question that arises is, how might anergized “immature” dendritic cells migrate to lymph nodes in order to tolerize T cells (20, 21)? In this study, CCR7 was weakly expressed on iDCs on day 6 and was up-regulated after exposure to apoptotic cells despite the absence of maturation properties. Indeed, CD14-derived iDCs showed a weak but clear migratory response to CCL19 (47), and bone marrow-derived murine dendritic cells expressed CCR7 even in the absence of  $\alpha$ CD40 treatment (48). On the other hand, Yanagihara et al. showed that CD14<sup>+</sup>-derived iDCs did not migrate in response to CCL19 (40), when CD86<sup>−</sup> iDCs were used. The present study and the former observations lead us to suggest that “young” iDCs, characterized by CD86<sup>absent</sup> (40), do not express CCR7 and do not migrate toward CCL19. However, iDCs, defined by CD86<sup>low</sup>DR<sup>low</sup> that were perhaps exposed to apoptotic dendritic cells during the 6 d of incubation (unpublished data), weakly up-regulate CCR7. It is tempting to speculate that CCR7-expressing iDCs that ingested apoptotic material may migrate to peripheral lymph nodes, constitutively, and at a lower rate (where opsonization by complement is most efficient) compared with inflammatory stimuli, in order to maintain tolerance (20, 21, 32). Further studies are needed in order to determine whether responsiveness to CCR7 chemokines is functional.

The pro-, non-, or antiinflammatory consequences of complement activation via complement receptors likely depend upon the specific ligands encountered, the coligation of other receptors such as Toll receptors and FcR, the presence of molecules such as CRP and factor H (16), and

the extent of generation of active fragments such as C3a, C5a, and membrane attack complex. Ligation of CD11b/CD18 and CD11c/CD18, receptors for iC3b-opsonized apoptotic cells, probably represent an antiinflammatory or noninflammatory consequence of limited complement activation (9). On the other hand, due to the great potential of iDCs in priming an immune response, a great danger of autoimmunity exists (21) in the presence of a proinflammatory milieu. Proinflammatory milieu may include ligands for Toll receptors, proinflammatory cytokines as found in conditioned media, absence of facilitating factors such as complement proteins, or the presence of autoantibodies (17). Opsonization by IgG autoantibodies rather than natural IgM autoantibodies may lead to uptake via Fc $\gamma$ R that was shown to promote iDC maturation, presentation on both MHC class I and II molecules, and priming of both CD4<sup>+</sup> helper and CD8<sup>+</sup> cytotoxic T lymphocytes (49). Any of these conditions and other unknown ones may shift the limited complement activation to full activation with generation of C5 convertase, C5a, and membrane attack complex. These may dictate higher expression of MHC class II and costimulatory molecules in interacting iDCs and may cause priming of autoimmune T cell clones in lymph nodes. This may be a possible mechanism for persisting inflammation or accelerating autoimmunity in diseases such as systemic lupus erythematosus (22) or may even explain the increased autoimmunity seen in diseases such as AIDS, in which a high turnover of apoptotic cells exists.

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