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Satiated-efferocytosis generates pro-resolving CD11b^{low} macrophages: modulation by resolvins and glucocorticoids

Sagie Schiff-Zuck¹, Nufar Gross¹, Simaan Assi¹, Ran Rostoker¹, Charles N. Serhan², and Amiram Ariel¹

^{*}Department of Biology, Faculty of Natural Sciences, University of Haifa, Haifa, 31905.

[†]Department of Anesthesiology, Center for Experimental Therapeutics and Reperfusion Injury, Perioperative and Pain Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA, USA.

Summary

During the resolution phase of inflammation apoptotic leukocytes are efferocytosed by macrophages in a nonphlogistic fashion that results in diminished responses to bacterial moieties and production of anti-inflammatory cytokines. Complement receptor 3 (CR3) and pro-resolving lipid mediators promote the engulfment of apoptotic leukocytes by macrophages. Here, we present evidence for the emergence of pro-resolving, CD11b^{low} macrophages *in vivo* during the resolution of murine peritonitis. These macrophages are distinct from the majority of peritoneal macrophages in terms of their functional protein expression profile, as well as pro-resolving properties, such as apoptotic leukocyte engulfment, indifference to TLR ligands, and emigration to lymphoid organs. Notably, we also found macrophages convert from the CD11b^{high} to the CD11b^{low} phenotype upon interaction with apoptotic cells *ex vivo*. In addition, we found that the pro-resolving lipid mediators resolvin (Rv) E1 and RvD1, and the glucocorticoid dexamethasone (Dex) regulated pro-resolving macrophage functions *in vivo*. This regulation culminated in a novel pro-resolving function, namely reducing the apoptotic leukocyte ingestion requirement for CD11b^{low} macrophage generation. These new phenotype and molecular pathway markers define the new satiated-macrophage. Thus, we suggest that satisfying-efferocytosis generates CD11b^{low} macrophages that are essential for complete non-phlogistic containment of inflammatory agents and the termination of acute inflammation.

Keywords

Inflammation; macrophages; phagocytosis; apoptosis; lipid mediators

Introduction

Macrophages are a highly diverse subtype of immune cells that while originating from a common precursor are also capable of metamorphosing to functionally distinct phenotypes that play key roles in acute and chronic inflammation, as well as the resolution of inflammation and fibrosis [1, 2]. During the active resolution of inflammation [3, 4] immune response elements are eliminated [5]. The leukocytes that elicited the acute inflammatory

Correspondence: Amiram Ariel, Department of Biology, Faculty of Natural Sciences, University of Haifa, Haifa, 31905 Israel. amiram@research.haifa.ac.il. Tel: 972-4-8288771, Fax: 972-4-8288763.

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response are undergoing apoptosis [6, 7], and consequently, the apoptotic PMNs are cleared by macrophages and other phagocytic cells in a non-phlogistic fashion [8, 9]. Apoptotic cell engulfment by phagocytes is mediated by signals that are expressed on the surface of apoptotic cells and their corresponding receptors, thrombospondin-CD36 [10], milk fat globule-EGF-factor 8 (MFG-E8)- $\alpha_v\beta_3$ -integrin [9], and others (reviewed in [7, 11]).

Opsonization by iC3b leads to enhances engulfment of apoptotic cells *via* the complement receptors CR3 (CD18/CD11b) and CR4 (CD18/CD11c) expressed on macrophages [12]. Moreover, lipoxin (LX) A₄ enhances uptake of apoptotic PMN by macrophages in a CD18-dependent manner [8]. Apoptotic cells also serve as resolution cues for macrophages, as their recognition evokes distinct signaling events [13] that block the release of pro-inflammatory mediators from macrophages. This release is activated by bacterial moieties, and its blockage, which is termed immune-silencing [14, 15], is accompanied by the production of TGF β and IL-10 [16–18], cytokines that can promote resolution and wound repair. The engulfment of apoptotic leukocytes by macrophages also leads to inhibition of inducible NO synthase (iNOS) expression and stimulates the expression of arginase-1 in the RAW 264 macrophage cell line [19], thereby preventing reactive NO production. In addition, the expression of 15-lipoxygenase (LO)-1, which is involved in the generation of pro-resolving lipid mediators [19, 20], as well as the production of angiogenic growth factors [21] by macrophages are consequent to the uptake of apoptotic cells. Pro-resolving lipid mediators, such as RvE1 and RvD1 block PMN infiltration to inflamed cavities (reviewed in [22]). RvE1 also promotes removal of apoptotic PMN by macrophages, and leukocyte emigration out of resolving inflammation sites [20]. Glucocorticoids are another set of naturally occurring pro-resolving mediators [23], that act, at least in part, through annexin-A1 release and activation of the LXA₄ receptor, FPR2/ALX [24, 25].

We identified earlier a new subset of macrophages that appeared during the resolution of murine peritonitis and that expressed lower levels of CD11b than the majority of the macrophage population [26]. In the present study we found that CD11b^{low} macrophages display a unique phenotype. CD11b^{low} macrophages differed from CD11b^{high} macrophages in the expression of functional proteins, such as iNOS, arginase-1, cyclooxygenase (COX) 2, 12/15-LO, and matrix metalloproteinase (MMP)-9. These cells engulfed significantly higher numbers of apoptotic PMN than CD11b^{high} macrophages, responded poorly to activation by different TLR ligands, in terms of cytokine and chemokine secretion, lost their phagocytic potential and were prone to migrate to lymphoid organs. Of interest, exposure to apoptotic cells *ex vivo* was sufficient to convert CD11b^{high} macrophages to their CD11b^{low} counterparts. Moreover, *in vivo* introduction of pro-resolving agents substantially enhanced CD11b^{low} macrophage emergence, despite diminished engulfment of apoptotic PMN that together define the new subpopulation as the satiated-efferocytes.

Results

CD11b^{low} macrophages express a distinct profile of functional proteins

Whether previously noted pro-resolving, CD11b^{low} macrophages [26] express a different set of functional proteins than CD11b^{high} macrophages is not known. To define the cell populations of interest we characterized and sorted F4/80⁺ macrophages according to their CD11b expression. We found that at 66 hrs after peritonitis initiation 70% of the exudate cells were macrophages (supplemental Figure 1A), of which 17% expressed low levels of CD11b (Supplemental figures 1B and 2A). Expression of CD11b on CD11b^{high} macrophages was 10-fold higher than its expression on CD11b^{low} macrophages (Supplemental figure 1C) and the amount of CD11b in protein extracts of CD11b^{low} macrophages was significantly lower than its amount in extracts from CD11b^{high} macrophages (Figure 1, top panel). Consequently, analysis of some proteins that are

functionally-relevant to inflammation and well characterized by their expression in classically (M1)- and alternatively (M2)- activated macrophages was performed in CD11b^{high} and CD11b^{low} macrophages. Our results (Figure 1) indicate that CD11b^{high} macrophages express low levels of iNOS, moderate levels of COX-2 and MMP-9 and high levels of arginase-1, but no 12/15-LO, whereas CD11b^{low} macrophages express low levels of COX-2 and MMP-9, moderate levels of 12/15-LO and no iNOS or arginase-1. In addition, the macrophage differentiation marker F4/80 was expressed to a higher extent on CD11b^{high} macrophages (Supplemental figure 2), further indicating that these cells possess different properties in comparison to CD11b^{low} macrophages. Of interest, CD11b^{low} macrophages lysates contained lower levels of actin than CD11b^{high} cells (Figure 1), suggesting a modulation of cytoskeletal dynamics in these cells. In contrast, another housekeeping gene – tubulin – was equally expressed in both protein extracts (Figure 1), indicating equal protein loading. Further analysis of surface receptor expression revealed increased expression of and reduced expression of CD206 and CD163 (both M2 markers) on CD11b^{low} macrophages, in comparison to their CD11b^{high} counterparts (19 and 17 fold difference, respectively; N=3, data not shown). Thus, CD11b^{high} and CD11b^{low} macrophages display different expression profiles of functional proteins and differentiation markers.

CD11b^{low} macrophages engulfed more apoptotic PMN than CD11b^{high} macrophages

Clearance of apoptotic PMN from resolving inflammation sites is paramount for resolution and return to homeostasis [27]. Therefore, we examined whether CD11b^{low} macrophages differ in their efferocytosis capacity from CD11b^{high} cells. The results in Figure 2A–B indicate that CD11b^{low} macrophages engulfed significantly more PMN on average than CD11b^{high} macrophages (12.2 ± 0.2 neutrophils/macrophage (N/M) and 2.2 ± 0.2 N/M, respectively). LysoTracker staining (Fig. 2A and Supplemental figure 3) indicates that the apoptotic PMN visualized in macrophages are indeed phagocytosed and not merely attached to the macrophage surface. In fact, in this experimental setting CD11b^{high} and CD11b^{low} macrophages were distinguished by an engulfment threshold of seven PMN (Figure 2C): $90.6 \pm 4.2\%$ of the CD11b^{low} macrophages engulfed seven or more PMN, whereas $97.5 \pm 1.2\%$ of the CD11b^{high} macrophages engulfed less than seven PMN. The results in Figure 2D indicate that the differences between CD11b^{high} and CD11b^{low} macrophages in terms of engulfment of apoptotic PMN are distinct not only when engulfment thresholds are depicted but rather that there is very little overlap between the populations, meaning that the threshold of engulfment is enforced in a very narrow range and that in this experimental setting only macrophages that engulfed 6 PMN have the same tendency to be either CD11b^{high} or CD11b^{low} macrophages. Thus, CD11b^{low} macrophages exhibited distinguishable pro-resolving properties, such as greater engulfment of apoptotic PMN.

CD11b^{low} macrophages display reduced responsiveness to TLR ligands

Apoptotic cell engulfment by macrophages leads to their immune-silencing [14, 15, 28], which prevents unwanted excessive inflammatory responses during the resolution phase and catabasis. To determine whether CD11b^{low} macrophages are immune-silent, we activated sorted macrophages with LPS, and determined the secretion levels of TNF α , IL-1 β , IL-10, and TGF β by CD11b^{high} and CD11b^{low} macrophages. The results (Figure 3A) indicate that CD11b^{high} macrophages secreted twice as much TNF α as CD11b^{low} macrophages, in the absence of LPS. Moreover, stimulation with LPS at 500 ng/ml resulted in a significantly elevated and maximal secretion of TNF α by CD11b^{high} macrophages, whereas CD11b^{low} macrophages did not increase their TNF α secretion. Similar results were obtained when the secretion of the pro-inflammatory cytokines and chemokines IL-1 β , CCL2, CCL3, and CCL5 was determined (Figures 3B, 3E–G). Surprisingly, the secretion of IL-10, an anti-inflammatory cytokine, was also increased in CD11b^{high} macrophages stimulated with LPS

(Figure 3C). Of interest, the secretion of the resolution-promoting cytokine TGF β was higher in CD11b^{low} macrophages, but did not increase following activation with LPS (Fig. 3D).

Immune-silent macrophages respond to a lower extent to different TLR ligands in terms of pro-inflammatory cytokine production [14, 29]. To determine whether CD11b^{low} macrophages are indeed immune-silent, we stimulated CD11b^{low} and CD11b^{high} macrophages with the TLR3 and TLR9 ligands poly (I:C) and CpG-ODN, respectively (Figure 4), and determined the secretion of TNF α and IL-1 β . The results indicate that both TNF α and IL-1 β secretion were increased following exposure to poly (I:C) and CpG-ODN of CD11b^{high} macrophages, whereas the secretion of these cytokines by CD11b^{low} macrophages treated in the same manner was significantly lower. Of note, no significant reduction in the expression of TLR3, 4, and 9 was found in CD11b^{low} macrophages, in comparison to their CD11b^{high} counterparts (N=3, data not shown). Thus, CD11b^{low} macrophages are poorer responders to different TLR ligands in terms of cytokine and chemokine secretion, and therefore are immune-silent.

CD11b^{low} macrophages are "satiated"

Since CD11b was found to be essential for the engulfment of iC3b-opsonized apoptotic cells by macrophages [12], its reduced expression in macrophages that engulfed high number of apoptotic cells suggests CD11b^{low} macrophages have lost their phagocytic function. To determine whether CD11b^{low} and CD11b^{high} macrophages differ in their ability to phagocytose external particles, the phagocyte-specific dye PKH2-PCL green was injected I.P. to mice undergoing peritonitis for 48 hrs and 4 hrs later the peritoneal cells were recovered, immune-stained, and analyzed for PKH2-PCL acquisition. The results in Figure 5 indicate that most CD11b^{low} macrophages did not acquire PKH2-PCL (Fig. 5A), whereas the majority of CD11b^{high} macrophages acquired higher amounts of PKH2-PCL (Fig. 5B). As a result the mean fluorescence intensity of PKH2-PCL was >10 fold higher in CD11b^{high} macrophages (Fig. 5C). Of interest, a small portion of the CD11b^{low} macrophages were labeled with PKH2 to a low extent, suggesting there is some residual phagocytic activity exerted by these cells, or that these macrophages converted from the CD11b^{high} to the CD11b^{low} phenotype during the assay period, and therefore lost the phagocytic capacity after acquiring a low amount of PKH2-PCL. Of interest, macrophage-PMN conjugates identified by FACS analysis as Ly-6G⁺F4/80⁺ doublets expressed higher levels of CD11b and F4/80 than CD11b^{low} macrophages (N=8, data not shown) indicating that CD11b^{high} macrophages are actively engulfing apoptotic PMN, while CD11b^{low} macrophages are not. Thus, CD11b^{low} macrophages are "satiated", meaning they lost their phagocytic potential upon meeting the apoptotic PMN engulfment threshold and reducing their CD11b expression.

CD11b^{low} macrophages are prone to emigrate to lymphoid organs

Most of the macrophages that are differentiating from monocytes in tissues during inflammation are emigrating to the draining lymph nodes and spleen [20, 30]. Our results indicate that CD11b^{low} macrophages are not phagocytic (Fig. 5). Therefore, it is probable they are no longer required for resolution at the inflammation site, and might play a role in communicating the resolving state in a systemic manner. Hence, we sought to determine whether CD11b^{low} macrophages are prone to depart resolving inflammation sites and emigrate to lymphoid organs. To this end, we determined the relative distribution of CD11b^{high} and CD11b^{low} macrophages 66 hrs post peritonitis initiation at the peritoneum, inguinal lymph node, and spleen. Our results indicate that CD11b^{high} macrophages are the predominant macrophage subtype in the peritoneum during late resolution, whereas, at the same time CD11b^{low} macrophages are the predominant macrophage subtype at the LN and

spleen (Figure 6A). To examine whether CD11b^{low} macrophages at the LN and spleen originated in the peritoneum, adoptive transfer experiments were performed in which peritoneal macrophages were isolated and labeled fluorescently, and then transferred to the peritoneum of mice undergoing peritonitis at the same period (48 hrs). The results in Figure 5B indicate that the distribution of labeled CD11b^{high} and CD11b^{low} macrophages at the peritoneum, inguinal LN, and spleen 18 hrs after transfer (Figure 6B) was similar to the distribution of unlabeled macrophages (Figure 6A). Thus, during the late phase of resolution peritoneal CD11b^{low} macrophages are prone to emigrate to lymphoid organs and possibly transfer resolution-phase signals to the acquired immune system.

Interaction with apoptotic cells converts macrophages from the CD11b^{high} to CD11b^{low} phenotype

The results in Figures 2 and 6 suggest that CD11b^{high} macrophages convert to CD11b^{low} ones as they interact with apoptotic PMN and engulf them. To determine whether interaction with apoptotic cells is sufficient to reduce CD11b expression on macrophages, sorted CD11b^{high} and CD11b^{low} macrophages were incubated with or without apoptotic Jurkat cells and the changes in surface expression of CD11b were determined. The results in Figure 7 indicate that CD11b expression on the surface of both CD11b^{high} and CD11b^{low} macrophages was significantly reduced following their incubation with apoptotic cells. Of note, the level of expression of CD11b on both macrophage populations following interaction with apoptotic cells (AC) was lower than its level of expression on CD11b^{low} macrophages recovered from peritoneal exudates, and therefore we designated the *ex vivo*-generated population CD11b⁻ macrophages (Figure 7C). The detection of CD11b⁻ macrophages *ex vivo*, but not *in vivo*, suggests that the initial reduction in CD11b expression detected *in vivo* might be sufficient to trigger macrophage departure of resolving tissues, and therefore CD11b⁻ macrophages are not present in peritoneal cavities. On the other hand, the absence of exit routes in cell cultures allows CD11b down-regulation to reach significantly lower levels of CD11b expression. Interestingly, some reduction in CD11b expression on both CD11b^{high} and CD11b^{low} macrophages occurred following *ex vivo* culture in the absence of apoptotic cells, possibly due to the delayed effects of intracellular signals downstream of apoptotic cell recognition *in vivo*. Importantly, no significant reduction in surface expression of F4/80 or CD11b was observed following macrophage incubation with LB or IgG-opsonized LB (Supplemental Fig. 4A). Of interest, the impact of apoptotic cells was partially mimicked by CD11b ligation with monoclonal antibodies (Supplemental Fig. 4A), which resulted in a decrease in CD11b, but not F4/80, surface expression, thus suggesting CD11b is involved in the signaling cascade that leads to its down-regulation. Notably, CD206 and CD163 surface expression was not modulated by AC *ex vivo*, but was reduced by anti-CD11b antibodies (Supplemental Fig. 4A). In addition, exposure to zymosan A, TGFβ, or live cells did not result in a significant reduction in the surface expression of CD11b (N=2, data not shown). Thus, the reduction in macrophage CD11b expression *ex vivo* is specific for interaction with apoptotic cells, and could not be achieved by treatment with other phagocytic targets, prototypic activating bacterial moieties, or pro-resolving cytokines, and is not due to the cell type of the apoptotic cells.

To determine whether macrophage interaction with apoptotic cells also triggers the major differences in protein expression distinguishing CD11b^{high} and CD11b^{low} macrophages that were indicated in Figure 1, peritoneal macrophages were incubated with apoptotic cells and changes in the cytoplasmic content of CD11b, arginase-1, 12/15-LO, and actin were determined. The results (Figure 7D) show that macrophages incubated with apoptotic cells expressed reduced levels of CD11b and arginase-1, and increased levels of 12/15-LO, as expected of conversion from the CD11b^{high} to the CD11b^{low} phenotype. Interestingly, the levels of detergent-soluble actin in macrophages were also reduced following incubation

with apoptotic cells. These findings are consistent with the findings in Fig. 1 that showed reduced actin levels in lysates of CD11b^{low} macrophages, in comparison to their CD11b^{high} counterparts. These differences are probably due to the increased numbers of actin-associated phagosomes formed in these cells. Importantly, *ex vivo* exposure of macrophages to senescent neutrophils, but not to latex beads (LB) or IgG-opsonized LB, resulted in a significant reduction in CD11b and arginase-1 expression (Supplemental Fig. 4B), whereas 12/15-LO expression was increased in this setting by all phagocytic targets. Together, these results indicate that interaction with apoptotic cells is sufficient to drive the conversion of CD11b^{high} to CD11b^{low} macrophages.

RvD1, RvE1, and Dex reduce the PMN engulfment threshold required for macrophage immune-silencing

Resolvins are resolution-phase-generated mediators derived from ω -3 polyunsaturated fatty acids; they were found to promote resolution by acting on PMN and macrophages [20, 31, 32]. Similar properties were attributed to glucocorticoids, such as Dex, that inhibit leukocyte infiltration to inflammation sites and promote the clearance of apoptotic PMN by macrophages (reviewed in [33]). To determine whether pro-resolving mediators regulate emergence of CD11b^{low} macrophages and thereby promote resolution, RvD1, RvE1, or Dex were introduced into peritonitis-affected mice, and the recovered leukocytes were collected and analyzed for macrophage and neutrophil numbers, macrophage CD11b expression, engulfment of PMN, and responsiveness to LPS. Our results indicate (Figure 8A) that RvD1, RvE1 and Dex induced a reduction in neutrophil numbers, whereas RvD1 and Dex, but not RvE1 also reduced the number of macrophages in peritoneal cavities. In addition, RvD1, RvE1 and Dex enhanced (Fig. 8B) the appearance of CD11b^{low} macrophages ($32.8 \pm 8.8\%$, $46.2 \pm 1.8\%$, and $39.9 \pm 4.6\%$ increases over vehicle treatment, for RvD1, RvE1, and Dex, respectively) in peritoneal exudates. Of interest, RvD1 and RvE1, but not Dex, also reduced CD11b expression on CD11b^{high} macrophages (Figure 8C). To determine whether RvD1, RvE1, and Dex regulate apoptotic PMN engulfment by macrophages during resolution, exudate cells were enumerated for PMN engulfment and analyzed as in Fig. 2. Surprisingly, the results in Figure 8D indicate that RvD1 and, to a greater extent, RvE1 and Dex, reduced the numbers of apoptotic PMN engulfed by macrophages present in the peritoneum. A detailed analysis of engulfment according to thresholds (Figure 8E) indicated that RvE1 and Dex, but not RvD1, induced the appearance in the peritoneum of low-engulfing macrophages, designate “inexperienced” since they phagocytosed less than 2 N/M.

To validate the improvement in immune-silencing of macrophages that followed treatment with RvD1, RvE1 and Dex, macrophages were activated with LPS, and cytokine secretion was determined. The results in Figure 8F indicate that RvD1 and RvE1, and, to a lesser extent, Dex, inhibited the secretion of TNF α from unstimulated and LPS-stimulated macrophages. A similar response was observed (Figure 8G) with RvD1, RvE1 and Dex when IL-1 β secretion from LPS-stimulated macrophages was determined. Of interest, the secretion of IL-10, a pro-resolving cytokine generated following the ingestion of apoptotic cells [17], was upregulated by each of the pro-resolving mediators, in unstimulated and LPS-stimulated macrophages (Figure 8H). Thus, treatment with RvD1, RvE1, and Dex, promoted macrophage immune-silencing, as well as the secretion of pro-resolving cytokines from these cells.

Discussion

Macrophages are currently believed to display two functionally distinct phenotypes: classically- and alternatively- activated macrophages, termed M1 and M2, respectively. M2 macrophages are also subdivided into M2a, M2b, and M2c, based on different profiles of

responses following exposure to different macrophage-polarizing mediators, such as immune complexes, T_H2 cytokines, or glucocorticoids [1, 2]. The encounter of macrophages with apoptotic cells was proposed as an additional polarizing trigger that evokes T_H2-like responses, as well as the expression of arginase-1, and 12/15-LO [19]. A seminal study from Bystrom and colleagues [29] has identified hybrid resolving-phase macrophages (rMs) that exert properties of M1 macrophages, such as the expression of iNOS and COX 2, and at the same time express the mannose receptor and arginase-1 and secrete lower levels of inflammatory cytokines, but high levels of IL-10, which are properties of M2 [2]. In addition, rMs were found to possess a greater propensity to stay in the peritoneum and promote homeostatic responses at the resolving tissue. Here, we describe a distinct subtype of macrophages that appear to populate the peritoneal cavity simultaneously to rMs. However, these CD11b^{low} macrophages express neither iNOS nor arginase-1, while expressing lower levels of COX 2 and MMP9 (M1 enzymes) than their CD11b^{high} counterparts (Figure 1). Of note, CD11b^{low}, but not CD11b^{high} macrophages express the lipid converting enzyme 12/15-LO, which is involved in the production of pro-resolving lipid mediators, such as 15-hydroxydocosahexaenoate (HETE), lipoxins, protectins, and resolvins [3, 19, 34, 35].

Although the clearance of apoptotic cells by macrophages and its consequent immune-silencing of macrophages has been extensively described [14, 17, 36, 37], no identification of a nonphlogistic macrophage population *in vivo* has been provided. Moreover, evidence for a quantitative requirement for macrophages engulfment of apoptotic PMN to result in their immune-silencing *in vivo* is still lacking. CD11b was found to be involved in complement-mediated engulfment of apoptotic cells by macrophages [12], and recent studies indicate its binding to different ligands results in a significant immune-suppression in macrophages [38, 39]. In addition, CD11b expression on immature dendritic cells was downregulated following the iC3b-mediated ingestion of apoptotic cells [40]. Our findings determine that CD11b^{low} macrophages that appear in resolving inflammatory sites during the late resolution phase [26] engulf more apoptotic PMN than CD11b^{high} macrophages (Figure 2) and secrete lower amount of pro-inflammatory cytokines and chemokines, but not TGFβ in response to TLR ligands (Figures 3 and 4), thereby fulfilling the designation of pro-resolving macrophages. Of interest, CD11b^{low} macrophages did not produce IL-10, in accord with earlier studies that examined the responses of macrophages to apoptotic cell instillation *in vivo* [14].

Notably, CD11b^{low} macrophages were found to be the prominent F4/80⁺ macrophage subtype in the LN and spleen, in particular in the population that originated in the peritoneum (Figure 6), where the CD11b^{high} macrophages were the prevalent macrophage subtype, indicating a conversion from the CD11b^{high} to CD11b^{low} phenotype is required for the emigration of macrophages to lymphoid organs during the resolution of inflammation. Indeed, we also found that incubation of CD11b^{high} macrophages with apoptotic cells *ex vivo* resulted in a significant enhancement of CD11b down-regulation (Figure 7). Moreover, this reduction in CD11b expression is associated with changes in the protein expression signature characteristic of the CD11b^{high} to CD11b^{low} conversion, thereby indicating a complete phenotype change driven by satisfying macrophage interaction with apoptotic cells. Overall, our results show that CD11b^{high} macrophages seem to display mixed properties of M1 and M2, while CD11b^{low} macrophages, although originating in the CD11b^{high} population, express a unique phenotype. Putting our current study in perspective with previous works that characterized macrophages during resolution [20, 29] and following their interaction with apoptotic cells [19], it is tempting to suggest that the phenotype displayed by the macrophage depends on the number of apoptotic PMN that it engulfed, as well as the environment it is operating in. Hence, the functional scheme presented in figure 9A–C takes place.

As the engulfment of apoptotic PMN is not a synchronized event and macrophages continue to infiltrate into the resolving inflammation site during resolution, the macrophage population in the peritoneum at any given time is probably heterogeneous. Integrating the results from Bystrom *et al.* with our study we conclude that the majority of the macrophages present at the peritoneum during late resolution (rMs or CD11b^{high} macrophages) are either M1-like or M2-like, although a hybrid or M1-to-M2 transition phenotype cannot be excluded. These macrophages are distinct from CD11b^{low} macrophages that are apparently more adept at migration to lymphoid tissues and delivery of resolution cues to the acquired immune system.

Importantly, CD11b^{low} macrophages were also found to lose their phagocytic activity (Figure 5) upon conversion from CD11b^{high} ones, and therefore were termed "satiated macrophages". This is, to the best of our knowledge, the first indication that the engulfment of apoptotic PMN by macrophages is self-limiting, and therefore serves as a feedback mechanism that can modulate macrophage pro-resolving functions and site of action (the resolving tissue or the lymphatics). Therefore, once a macrophage reaches the engulfment threshold for apoptotic PMN uptake it is undergoing immune-silencing and satiation. Consequently, the macrophage departs the resolving tissue through the lymphatics, as it can make no further contribution to resolution on site.

RvD1 and RvE1 exhibit a range of anti-inflammatory and pro-resolving actions (reviewed in [22]). Dex is also a potent regulator of acute immune responses, and mediates pro-resolving functions, primarily through the generation of annexin A1 peptides, which act through the LXA₄ receptor [24]. Notably, both Dex and LXA₄, as well as RvE1 promote the engulfment of apoptotic PMN by macrophages during the resolution of inflammation [8, 20, 25]. The present report reveals a novel pro-resolving function for RvE1, RvD1 and Dex, namely, reducing the number of engulfment-related events required for immune-silencing of macrophages, illustrated in Fig. 9. In fact, RvE1 and Dex reduced the number of macrophages that engulfed seven or more apoptotic PMN by 84.6% and 100%, respectively, while increasing the number of CD11b^{low} macrophages by 46.2% and 39.9%, respectively (Fig. 8). This function enables enhanced immune-silencing of macrophages at resolving sites, and might promote their departure from resolving sites, as was previously reported for RvE1 [20]. Moreover, the emergence of "inexperienced" macrophages that engulfed less than 2 PMNs following the introduction of RvE1 and Dex at resolving cavities (Fig. 8E), suggests these compounds increase turnover of macrophages at the resolving site to "compensate" for the reduced engulfment by each macrophage and thereby to enhance apoptotic cell clearance. Therefore, the reduction in apoptotic PMN found in peritoneal macrophages during resolution after treatment with RvE1 and Dex presumably stems from the cumulative actions of each of these compounds that include induction of infiltration of additional macrophages to the peritoneum, as well as promotion of the departure of satiated macrophages following their immune-silencing. Of note, RvE1 and RvD1 inhibited LPS-stimulated TNF α production from peritoneal macrophages to a higher extent than Dex, despite being used at a 250 times lower dose, which suggests that resolvins will exhibit improved actions over glucocorticoids in treatment of pathologies associated with hampered resolution.

In sum, our study revealed a new population of macrophages that display pro-resolving properties important for completing the resolution sequel and for communicating the return to a homeostatic state at lymphoid organs during resolution of the acute inflammatory response. In addition, we uncovered novel actions for resolvins and glucocorticoids on macrophages during the resolution of inflammation, which could indicate new therapeutic benefits in acute inflammatory disorders.

Materials and Methods

Reagents

ELISA kits for mouse TNF α , IL-1 β , IL-10, TGF β , CCL2, CCL3, and CCL5 were obtained from R&D Systems. Carboxyfluorescein succinimidyl ester (CFSE), staurosporine (STS), LPS (from *e.coli*, clone 055:B5), PKH2-PCL green fluorescence linker kit, and Dex from Sigma. Poly (I:C) and CpG-oligodeoxynucleotides (CpG-ODN) from InvivoGen. RvE1 (5S, 12R, 18R-trihydroxy-4Z, 8E, 10E, 14Z, 16E-eicosapentaenoic acid) and RvD1 (7S, 8R, 17S-trihydroxy-4Z, 9E, 11E, 13Z, 15E, 19Z-docosahexaenoic acid) were obtained from Cayman Chemicals and the synthetic resolvins were matched according to previously published biological and physical material [32, 41].

Murine peritonitis

Briefly, male C57BL/6 mice (6–8 wk; protocol approved by the Committee of Ethics, University of Haifa, authorization no. 088/07) were injected I.P. with zymosan A (1 mg). After 66 hrs, peritoneal exudates were collected and exudate cells were stained with FITC-conjugated rat anti-Ly-6G, PE-conjugated rat anti-F4/80, and PerCP-conjugated rat anti-mouse CD11b (Biolegend) and analyzed by FACSCalibur (Beckton-Dickinson). In some experiments the macrophages were sorted to CD11b^{high} and CD11b^{low} populations of >95% purity, using FACSaria (Beckton-Dickinson), and the separate populations were used for microscopic analysis and for *ex vivo* stimulation. In some experiments, PKH2-PCL green (0.25 μ M, 0.5 ml) was injected I.P. at 48 hrs and peritoneal cells were recovered 4 hrs later, immuno-stained for F4/80 and CD11b as above and analyzed for fluorescence intensity of different macrophage populations. In relevant experiments, vehicle, RvD1, RvE1 (100 ng/mouse each), or Dex (25 μ g/mouse) were introduced to the peritoneum 48 hrs after peritonitis initiation, and the exudates were recovered after 66 hrs and analyzed as before. For detailed methodologies please see "supporting information".

Western blot analysis

Protein extracts of sorted populations (>95% purity) of CD11b^{high} and CD11b^{low} macrophages were run using 10% SDS-PAGE (5 μ g/lane), transferred to nitrocellulose membranes and immuno-blotted with either goat anti-mouse CD11b (SantaCruz biotechnology), rabbit anti-mouse iNOS (Abcam), goat anti-mouse araginase-1 (Abcam) Rabbit anti-mouse COX-2 (Cayman chemicals), rabbit anti-mouse 15-lipoxygenase-1 (Cayman chemical), goat anti-mouse MMP-9 (R&D systems), goat anti-mouse β -actin (SantaCruz biotechnology) or goat anti mouse tubulin (SantaCruz biotechnology). Then, the membranes were washed, and incubated with the appropriate HRP-conjugated secondary antibody. The blots were washed and developed using EZ-ECL (Biological Industries).

Macrophage transfer

Macrophages were isolated from peritoneal exudates 48 hrs post peritonitis initiation, stained with CFSE (1 μ M) and injected into the peritoneum of mice that underwent peritonitis for 48 hrs. After an additional 18 hrs, cells from peritoneal exudates, inguinal lymph nodes and spleen were recovered from the recipient mice, immuno-stained as above, and the percentage of CD11b^{high} and CD11b^{low} macrophages in each site was determined.

Confocal microscopy

Sorted CD11b^{high} and CD11b^{low} macrophages were isolated and loaded with 50 nM LysoTracker Red DND 99 dye (Molecular Probes) for 2h at 37 °C in RPMI. Cells were then fixed with 2% paraformaldehyde at room temperature and stained with Hoechst (Molecular Probes) and FITC-conjugated anti-mouse Ly-6G. Mounted slides were kept in the dark at

4°C until analyzed by confocal microscopy. Confocal images were acquired using Z-sections of 1 µm thickness. The images were processed with Zeiss LSM Image Browser software.

Apoptotic PMN engulfment enumeration

Exudate cells or sorted CD11b^{high} and CD11b^{low} macrophages were stained with Hoechst, and enumerated under a fluorescent microscope (Zeiss). Two areas of two cover slips, each containing at least 50 (overall 200) macrophages were analyzed, and the average number of PMN engulfed per macrophage, as well as the number of macrophages with cutoff numbers of engulfed PMN were calculated. In sorted cells, F4/80⁺/Ly-6G⁺ entities (identified as macrophages that are attached to but did not fully engulf a PMN by forward vs. side scatter analysis) were excluded from the samples to avoid counting of attached PMNs.

TLR-mediated responsiveness *ex vivo*

Exudate macrophages were sorted or separated using PE selection magnetic beads (StemCell Technologies) and incubated (1×10^6 cells in 0.5 ml of culture media) with LPS (0–1000 ng/ml), poly (I:C) (4 µg/ml), or CpG-ODN (1 µM). After 16 hrs the supernatants were collected and their TNFα, IL-1β, IL-10, TGFβ, CCL2, CCL3, and CCL5 contents were determined by standard ELISA.

Regulation of macrophage phenotype by apoptotic cells *ex-vivo*

Jurkat cells were treated with 1 µM staurosporine (4 hrs, Sigma) to induce apoptosis, and washed. Then, peritoneal macrophages or sorted subpopulations thereof were incubated in the presence or absence of apoptotic Jurkat cells (1:5 macrophage to apoptotic cell ratio). At the beginning of the incubation and after 20 hrs, macrophages were immuno-stained for CD11b and analyzed by FACS analysis. Alternatively, protein extracts were prepared from the macrophages after the incubation period and run by SDS-PAGE followed by Western blot analysis for CD11b, arginase-1, 12/15-LO, actin, and tubulin, as above.

Statistical analysis

Ex vivo and *in vivo* experiments were performed at least 3 times with at least 4 replicates. Results were analyzed by one-way analysis of variance (for multiple groups) or Student's *t* test (for comparison between 2 groups) with *p* values < 0.05 considered as statistically significant. Results are expressed as means ± SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CR3	complement receptor 3
Rv	resolvin
Dex	dexamethasone
MFG-E8	milk fat globule-EGF-factor 8
LO	lipoyxygenase

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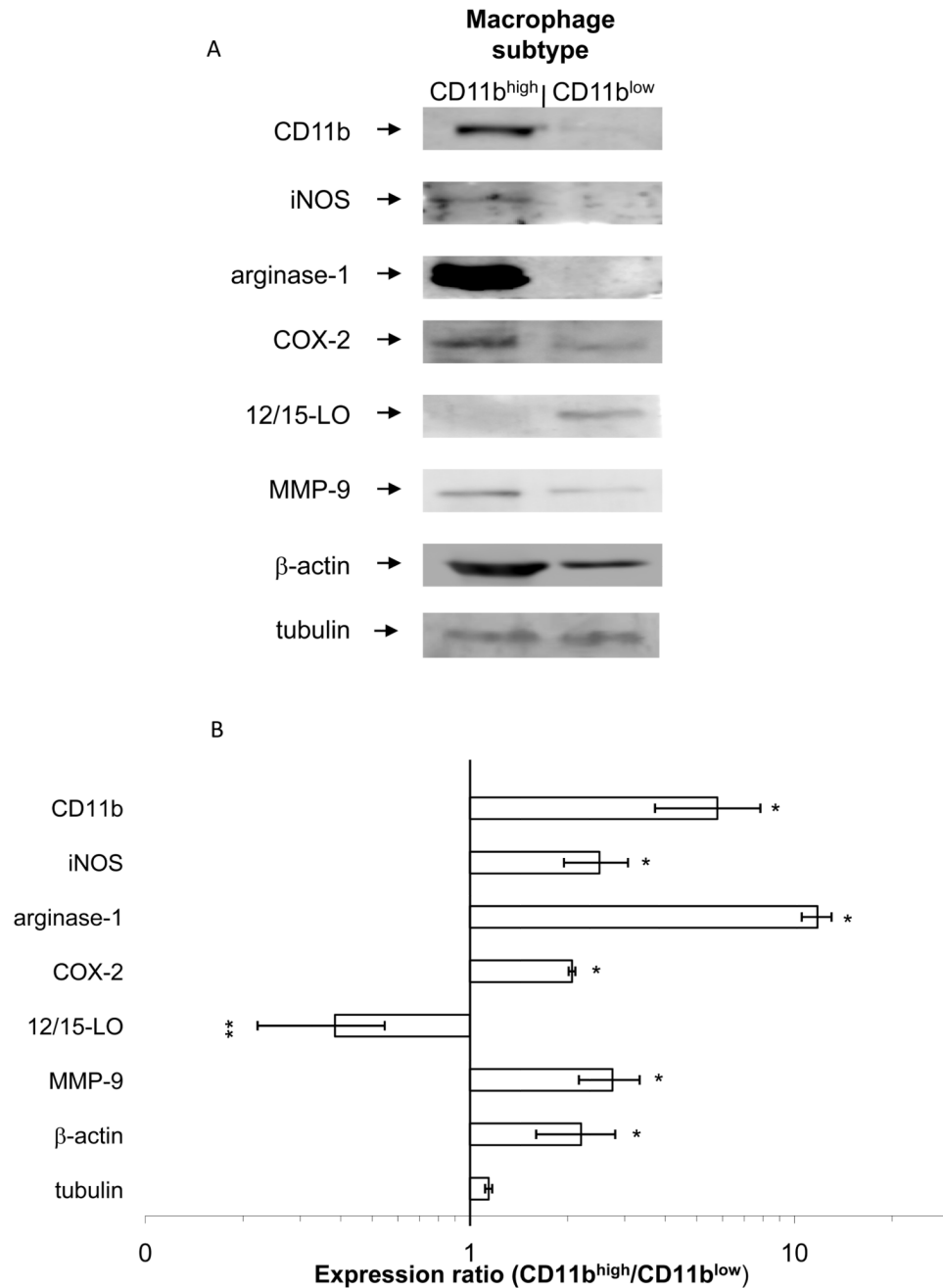


Figure 1. CD11b^{low} macrophages differ in their protein expression signature from CD11b^{high} macrophages

Resolving peritoneal exudates were recovered 66 hrs after zymosan A (1 mg) injection into mice, the cells were immuno-stained for Ly-6G, F4/80, and CD11b, and the Ly-6G⁺/F4/80⁺ macrophages were sorted to CD11b^{high} and CD11b^{low} populations. The recovered macrophages were lysed and their protein content was analyzed by SDS-PAGE and Western blotting for the indicated proteins. Results are representative blots (A) and mean \pm SE of three independent experiments (B). Fold difference in expression was calculated according to the following formula: Densitometric units (DU) of CD11b^{high} extract/DU of CD11b^{low} extracts from three experiments. Significant differences by *Student's t* test between

CD11b^{high} and CD11b^{low} extracts (* *P* value < 0.05, ** *P* value < 0.005, *** *P* value < 0.001) are indicated.

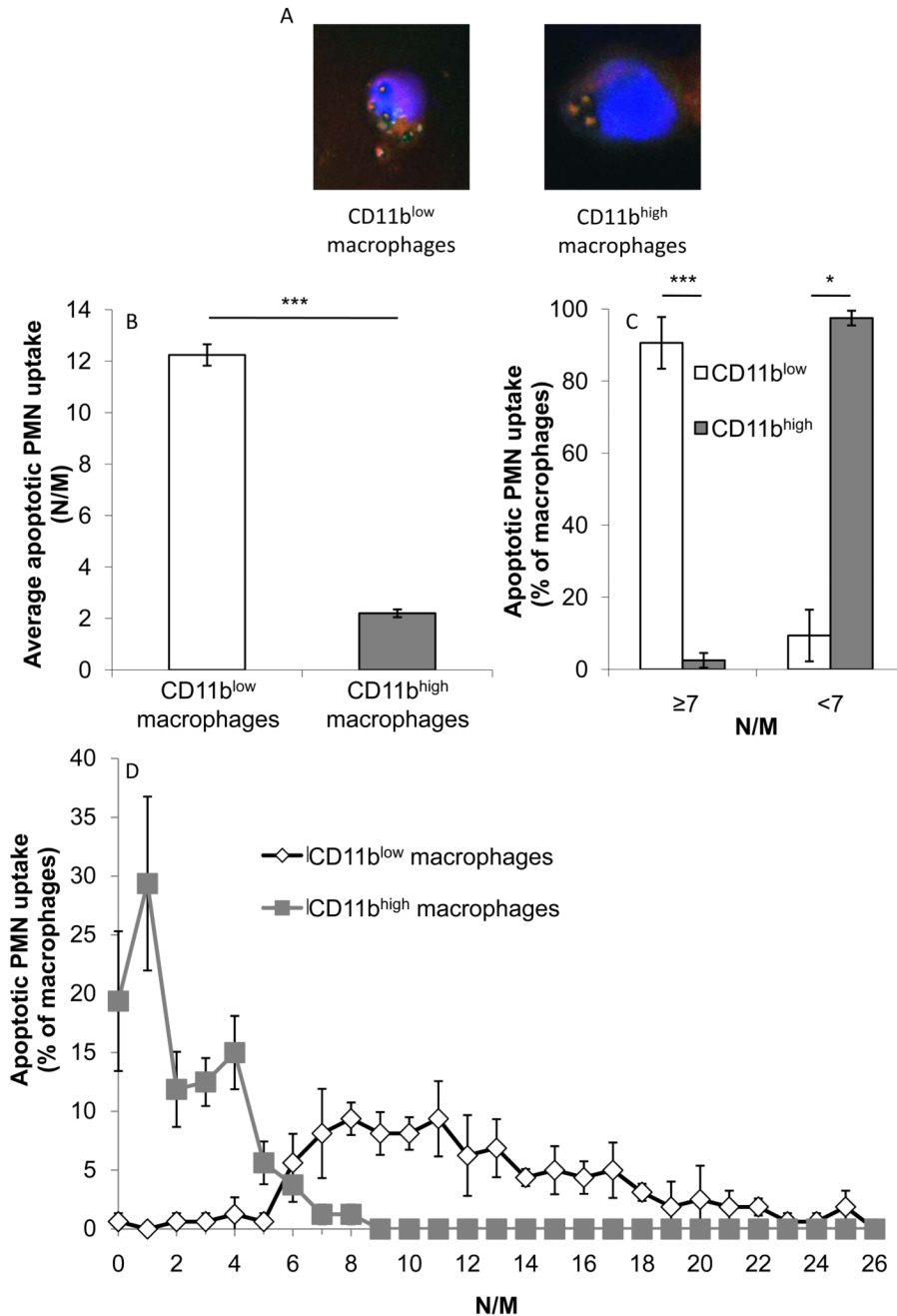


Figure 2. CD11b^{low} macrophages engulf higher numbers of apoptotic neutrophils than CD11b^{high} macrophages

Sorted CD11b^{high} and CD11b^{low} macrophages were stained with Hoechst and LysoTracker, analyzed by confocal microscopy and photographed as indicated (A; merged pictures). Similar preparations were enumerated for apoptotic PMN uptake and analyzed according to average (B) neutrophils engulfed per macrophage (N/M), and percentage of cells reaching engulfment threshold (C) or engulfing the indicated number of apoptotic PMN (D). Results are mean ± SE (B, C) or representative (A, D) from three experiments. Significant differences by *Student's t* test between CD11b^{high} and CD11b^{low} macrophages (* *P* value < 0.05, ** *P* value < 0.005, *** *P* value < 0.001) are indicated.

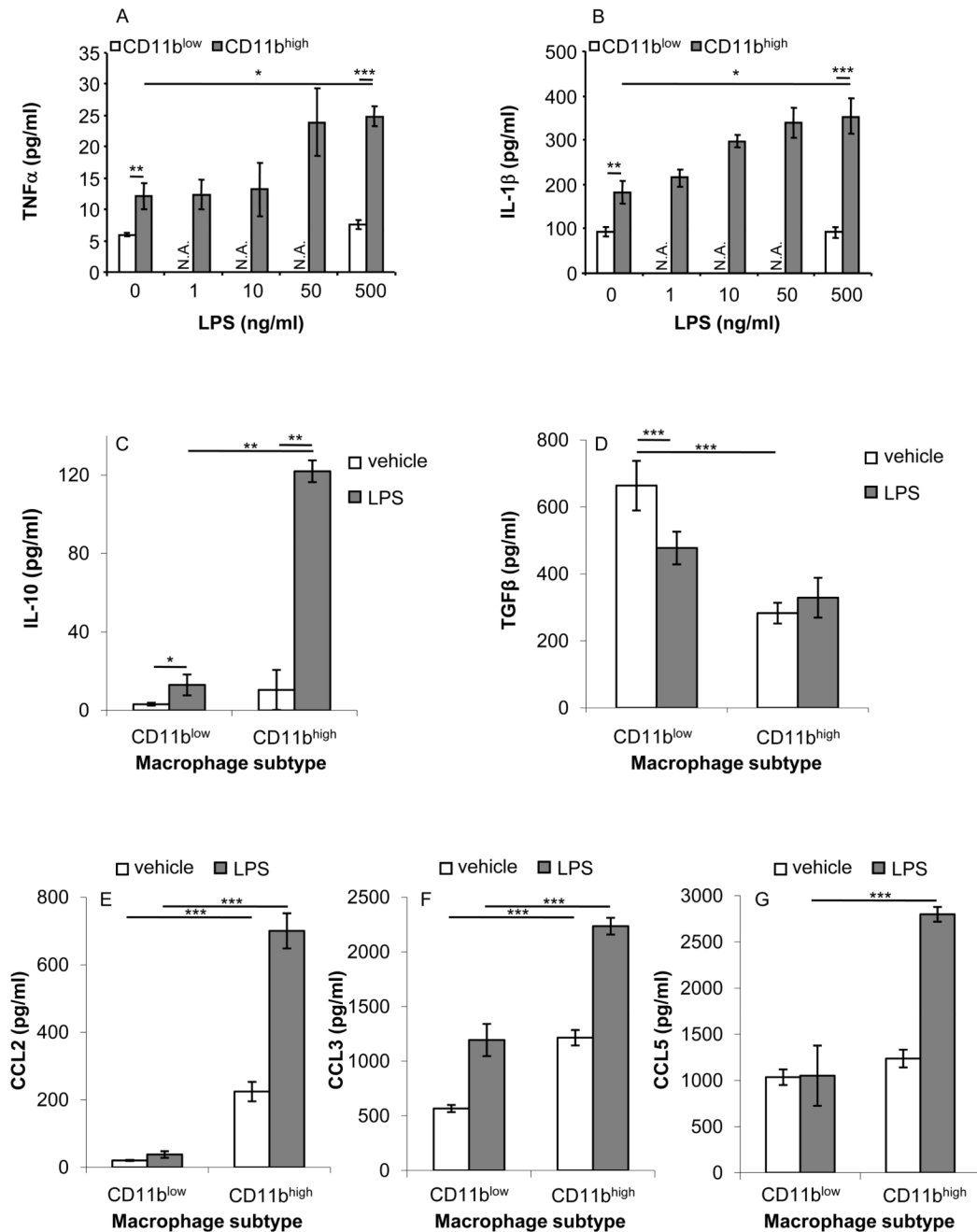


Figure 3. CD11b^{low} macrophages secrete lower levels of pro-inflammatory cytokines and chemokines, but higher levels of TGFβ, in comparison to CD11b^{high} ones
Sorted CD11b^{high} and CD11b^{low} macrophages were activated with the indicated concentrations (A–B) or 1 μg/ml (C–G) of LPS, and evaluated for secreted TNFα (A), IL-1β (B) IL-10 (C), TGFβ (D), CCL2 (E), CCL3 (F), and CCL5 (G) using standard ELISA. N.A.-not available. Results are mean ± SE of four replicates from a representative of three experiments. Significant differences by *Student's t* test between CD11b^{high} and CD11b^{low} macrophages or between vehicle and LPS-stimulated macrophages (* *P* value < 0.05, ** *P* value < 0.005, *** *P* value < 0.001) are indicated.

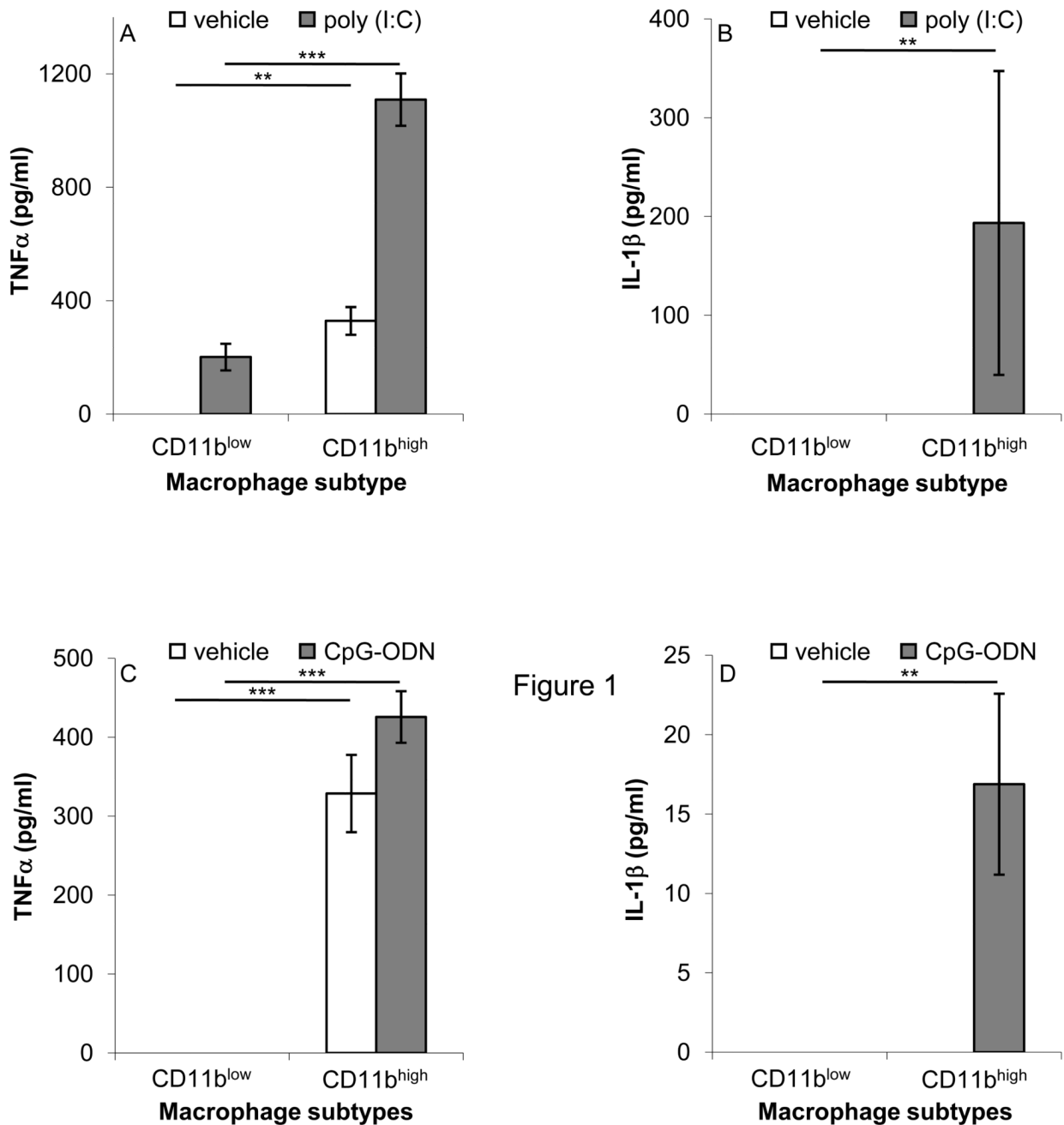


Figure 4. CD11b^{low} macrophages secrete lower levels of pro-inflammatory cytokines in response to various TLR ligands

Sorted CD11b^{high} and CD11b^{low} macrophages were activated with poly (I:C) (4 μ g/ml; A–B), or CpG-ODN (1 μ M; C–D) and evaluated for secreted TNF α (A,C), IL-1 β (B,D) using standard ELISA. Results are mean \pm SE of four replicates from a representative of three experiments. Significant differences by *Student's t* test between CD11b^{high} and CD11b^{low} macrophages or between macrophages stimulated with vehicle, poly (I:C), or CpG-ODN (* P value < 0.05, ** P value < 0.005, *** P value < 0.001) are indicated.

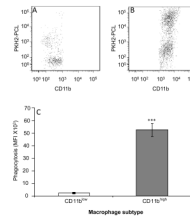


Figure 5. CD11b^{low} macrophages are satiated

PKH2-PCL-green was injected I.P. to mice 48 hrs post peritonitis initiation. After 4 hrs, the peritoneal cells were recovered, immune-stained for F4/80 and CD11b and analyzed using FACSCalibur. Results are representative dot plot for CD11b^{low} (A) and CD11b^{high} macrophages (B), or mean MFI values \pm SE of 5 experiments (C). Significant differences by *Student's t* test between CD11b^{high} and CD11b^{low} macrophages (***) *P* value < 0.001) are indicated.

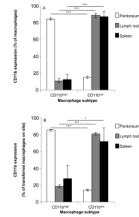


Figure 6. CD11b^{low} macrophages are prone to migrate to lymphoid organs

(A) Cells were recovered from the peritoneum, inguinal LN and spleen of mice undergoing peritonitis for 66 hrs. The cells were stained as above and CD11b expression on macrophages was determined by flow cytometry.

(B) Macrophages were recovered from peritoneal exudates 48 hrs post peritonitis initiation, labeled with CFSE, and transferred to recipient mice undergoing peritonitis for the same period. After 18 hrs, the cells from the peritoneum, inguinal LN, and spleen were recovered and the expression of CD11b on transferred macrophages was determined as above. Results presented in (A) and (B) are mean \pm SE from three experiments, 5 mice per experiment. Significant differences by *Student's t* test between CD11b^{high} and CD11b^{low} macrophages (* P value < 0.05, ** P value < 0.005, *** P value < 0.001) are indicated.

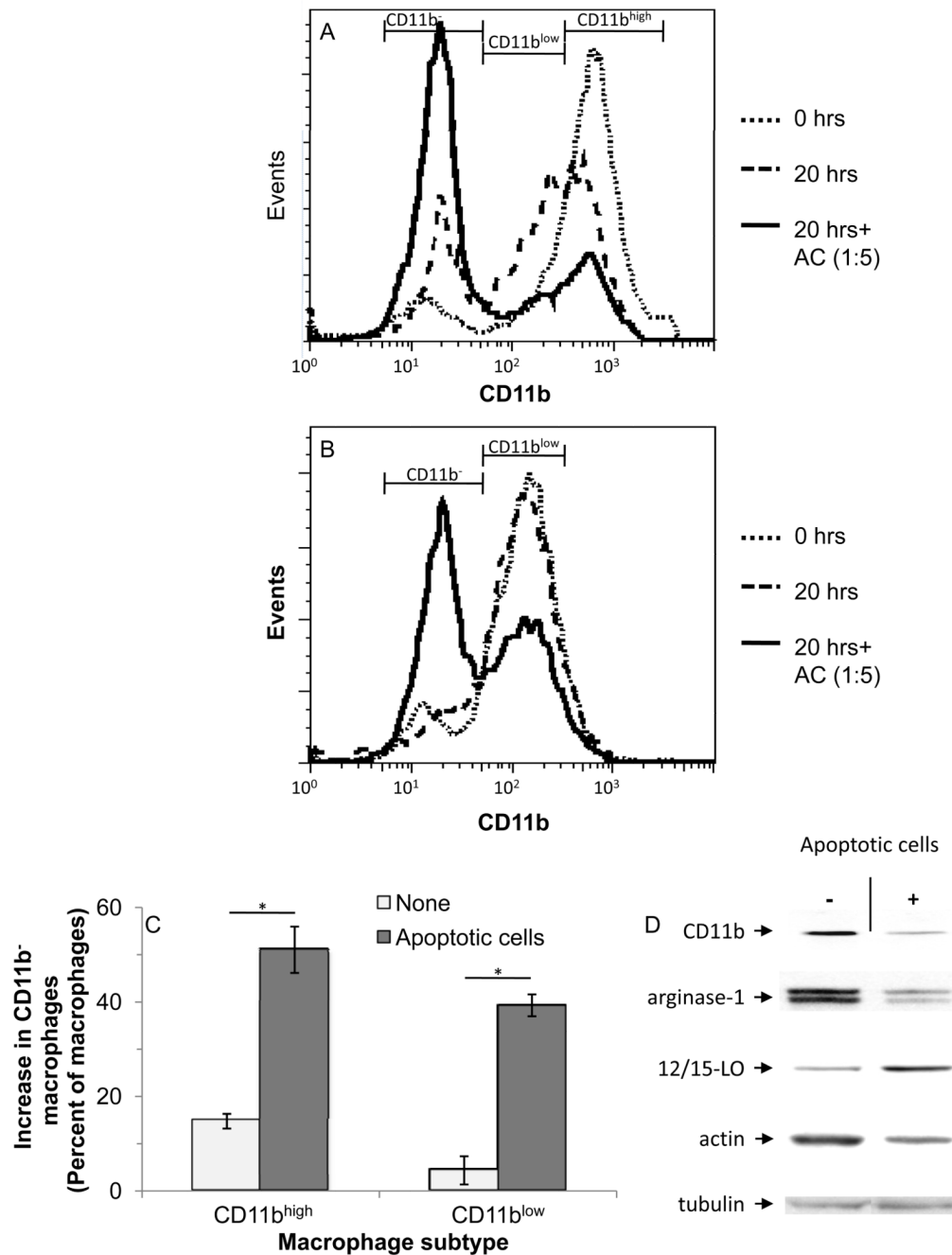


Figure 7. Interaction with apoptotic leukocytes converts CD11b^{high} macrophages to CD11b^{low} ones

Sorted CD11b^{high} (A) or CD11b^{low} (B) macrophages were immune stained for CD11b immediately (A–B, dotted line), or incubated for 20 hrs without cells (A–B, hashed line) or with apoptotic Jurkat cells (1:5 ratio; A–B, continuous line). Then the unbound apoptotic cells were washed and the macrophages were recovered and immuno-stained for CD11b expression on their surface. Results are representative histograms (A, B; depicted macrophage populations indicated) or average MFI values \pm SE (C) of 3 experiments. Significant differences by *Student's t* test between CD11b^{high} and CD11b^{low} macrophages (* P value < 0.05, ** P value < 0.005, *** P value < 0.001) are indicated.

(D). Peritoneal macrophages were recovered 66 hrs post peritonitis initiation and incubated with apoptotic Jurkat cells or without cells as indicated. After 20 hrs the macrophages were recovered and lysed. The protein extracts were run by SDS-PAGE and analyzed by Western blot for the indicated proteins. Results are a representative set of blots from 3 experiments.

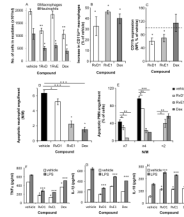


Figure 8. RvD1, RvE1, and Dex modulate CD11b expression, apoptotic PMN engulfment, and cytokine secretion by macrophages

Mice undergoing peritonitis were injected I.P. with RvD1, RvE1 (100 ng/mouse), Dex (25 µg/mouse) or vehicle 48 hrs after peritonitis initiation. At 66 hrs, the peritoneal exudate cells from all treatments were recovered, enumerated and immuno-stained for Ly-6G, F4/80, and CD11b, as above, and analyzed by flow cytometry to determine the number of macrophages and neutrophils (A), percentage of CD11b^{low} macrophages (B) and the level of CD11b expression on CD11b^{high} macrophages (C). Some of the exudate cells were also stained with Hoechst and apoptotic PMN engulfment by macrophages was determined (D–E). In addition, macrophages were isolated from all treatments and stimulated *ex vivo* with LPS (500 ng/ml). After 24 hrs, the secretion of TNFα (F), IL-1β (G), and IL-10 (H) from the macrophages was determined as above. Results are mean ± SE of four replicates from a representative of three experiments. Significant differences by ANOVA between leukocytes and macrophages from mice treated with vehicle and macrophages treated with RvD1, RvE1, or Dex (* *P* value < 0.05, ** *P* value < 0.005, *** *P* value < 0.001) are indicated.

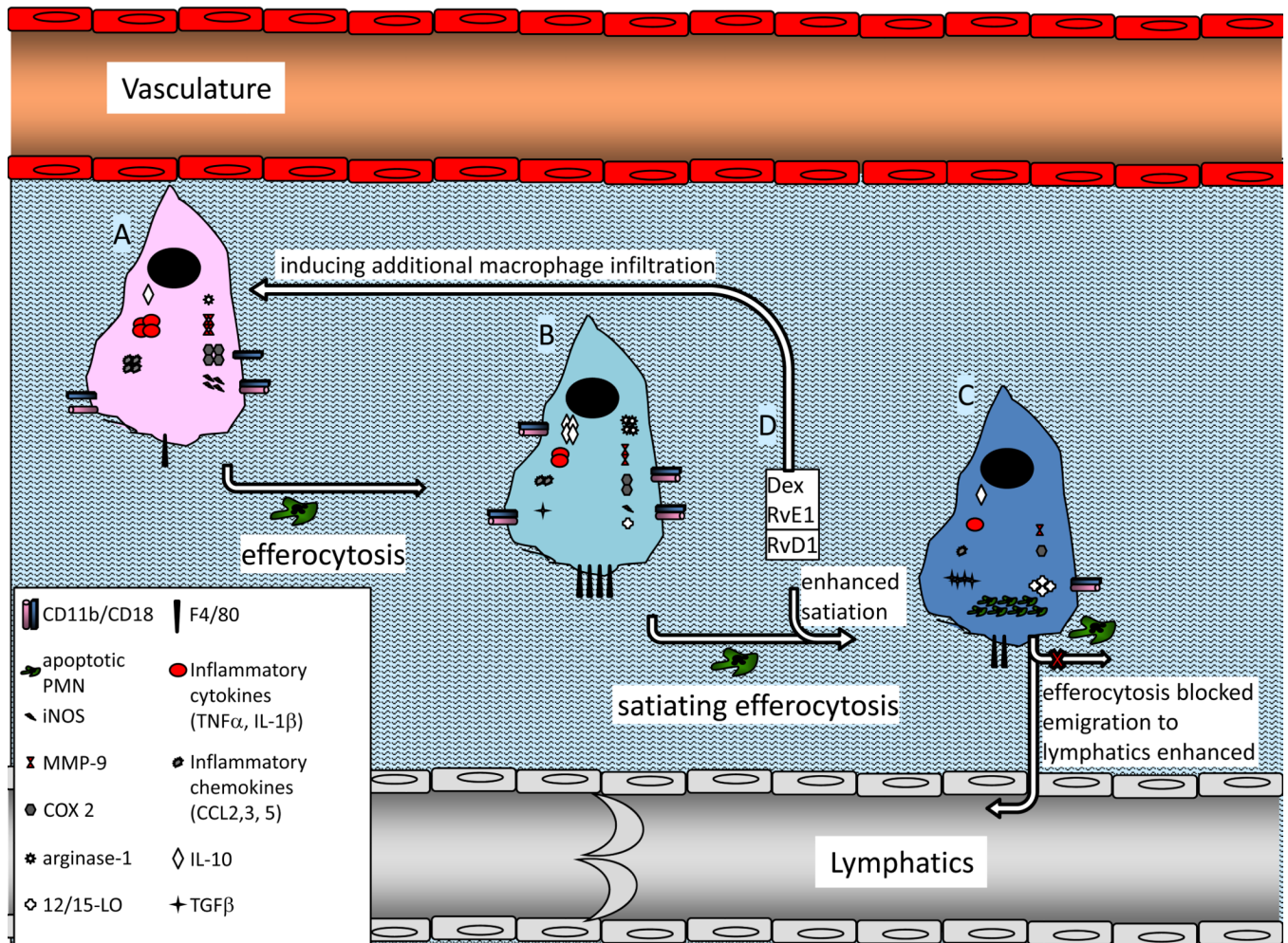


Figure 9. A functional scheme for macrophage phenotype conversions during resolution-phase efferocytosis

A macrophage that infiltrated an inflamed tissue adopts an M1-like phenotype (including expression of inflammatory cytokines and chemokines, iNOS, and COX 2 [1]) previous to encounter with apoptotic PMNs (A). Once it encounters apoptotic PMN and starts to engulf them, the macrophage switches to an M2-like phenotype that is involved in tissue repair and return to homeostasis. This switch is accompanied by an increase in the expression of arginase-1 and IL-10, whereas a decrease in the expression of pro-inflammatory cytokines, as well as COX-2, and iNOS also takes place (B). Reports documenting a role for M2 cytokines in the resolution of inflammation and efferocytosis [42, 43] seem to support this part of our scheme. As the engulfment of apoptotic PMN by the macrophage continues and reaches a threshold level (sating efferocytosis) the macrophage undergoes another switch to the CD11b^{low} phenotype (C) that secretes neither pro-inflammatory cytokines and chemokines nor IL-10, but does secrete higher levels of TGF β . CD11b^{low} macrophages also do not express iNOS or arginase-1, and express only low levels of COX-2. Interestingly, this switch leads to the expression of 12/15-LO, which is exclusively expressed by CD11b^{low} macrophages and was previously reported to be expressed in a TGF β -dependent manner following the interaction of macrophages with apoptotic cells [19]. The second phenotype switch also seems to stop CD18/CD11b-mediated apoptotic PMN engulfment [8, 12] thereby allowing rapid macrophage departure of the resolving tissue and emigration to lymphoid organs. At these target organs CD11b^{low} macrophages presumably produce 12/15-

LO-derived pro-resolving lipid mediators, and deliver homeostatic signals to antigen presenting cells and lymphocytes [34, 44]. Satiating efferocytosis can be modulated by pro-resolving mediators, such as RvD1, RvE1, and Dex (D). This modulation enhances the immune-silencing and departure of pro-resolving CD11b^{low} macrophages to the lymphatics, where they can contribute to the termination of the acquired immune response. RvE1 and Dex also promote the infiltration of new macrophages to the resolving tissue to continue the necessary clearance of apoptotic PMN and return to homeostasis.