Saturated-efferocytosis generates pro-resolving CD11b\textsuperscript{low} macrophages: Modulation by resolvins and glucocorticoids

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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 and pro-resolving lipid mediators promote the engulfment of apoptotic leukocytes by macrophages. Here, we present evidence for the emergence of pro-resolving, CD11b\textsuperscript{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\textsuperscript{high} to the CD11b\textsuperscript{low} phenotype upon interaction with apoptotic cells ex vivo. In addition, we found that the pro-resolving lipid mediators resolvin E1 and D1, and the glucocorticoid dexamethasone 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\textsuperscript{low} macrophage generation. These new phenotype and molecular pathway markers define the new satiated macrophage. Thus, we suggest that satisfying efferocytosis generates CD11b\textsuperscript{low} macrophages that are essential for complete nonphlogistic containment of inflammatory agents and the termination of acute inflammation.

Keywords: Apoptosis · Inflammation · Lipid mediators · Macrophages · Phagocytosis

Supporting Information available online

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 response are undergoing apoptosis [6, 7], and consequently, the apoptotic PMN are cleared by macrophages and other phagocytic cells in a nonphlogistic 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–\(\alpha_3\)\(\beta_3\)-integrin [9], and others (reviewed in [7, 11]).

Opsonization by iC3b leads to enhanced engulfment of apoptotic cells via the complement receptors 3 (CD18/CD11b) and 4 (CD18/CD11c) expressed on macrophages [12]. Moreover, lipoxin (LX) \(\Delta_4\) 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-\(\beta\) 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 resolvin E1 (RvE1) and RvD1 block PMN infiltration to inflated cavities (reviewed in [22]). RvE1 also promotes the 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, ALX/FPR2 [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 this study, we found that CD11b\textsuperscript{low} macrophages display a unique phenotype. CD11b\textsuperscript{low} macrophages differed from CD11b\textsuperscript{high} macrophages in the expression of functional proteins, such as iNOS, arginase-1, COX-2, 12/15-LO, and MMP-9. These cells engulfed significantly higher numbers of apoptotic PMN than CD11b\textsuperscript{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\textsuperscript{high} macrophages to their CD11b\textsuperscript{low} counterparts. Moreover, in vivo introduction of pro-resolving agents substantially enhanced CD11b\textsuperscript{low} macrophage emergence, despite diminished engulfment of apoptotic PMN that together define the new subpopulation as the satiated efferocytes.

Results

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

Whether previously noted pro-resolving, CD11b\textsuperscript{low} macrophages [26] express a different set of functional proteins than CD11b\textsuperscript{high} macrophages is not known. To define the cell populations of interest, we characterized and sorted F4/80\textsuperscript{+} macrophages according to their CD11b expression (Supporting Information Fig. 1A). We found that at 66 h after peritonitis initiation, 70% of the exudate cells were macrophages (Supporting Information Fig. 1B) of which 17% expressed low levels of CD11b (Supporting Information Fig. 1C and 2A). Expression of CD11b on CD11b\textsuperscript{high} macrophages was tenfold higher than its expression on CD11b\textsuperscript{low} macrophages (Supporting Information Fig. 1D) and the amount of CD11b in protein extracts of CD11b\textsuperscript{low} macrophages was significantly lower than its amount in the extracts from CD11b\textsuperscript{high} macrophages (Fig. 1A, 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\textsuperscript{high} and CD11b\textsuperscript{low} macrophages. Our results (Fig. 1) indicate that CD11b\textsuperscript{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\textsuperscript{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\textsuperscript{high} macrophages (Supporting Information Fig. 2), further indicating that these cells possess different properties in comparison to CD11b\textsuperscript{low} macrophages. Of interest, CD11b\textsuperscript{low} macrophages lysates contained lower levels of actin than CD11b\textsuperscript{high} cells (Fig. 1), suggesting a modulation of cytoskeletal dynamics in these cells. On the contrary, another housekeeping gene – tubulin – was equally expressed in both protein extracts (Fig. 1), indicating equal protein loading. Further analysis of surface receptor expression revealed reduced expression of CD206 and CD163 (both M2 markers) on CD11b\textsuperscript{low} macrophages, in comparison to their CD11b\textsuperscript{high} counterparts (19- and 17-fold difference, respectively; \(N = 3\), data not shown). Thus, CD11b\textsuperscript{high} and CD11b\textsuperscript{low} macrophages display different expression profiles of functional proteins and differentiation markers.

CD11b\textsuperscript{low} macrophages engulfed more apoptotic PMN than CD11b\textsuperscript{high} macrophages

Clearance of apoptotic PMN from resolving inflammation sites is paramount for resolution and return to homeostasis [27]. Therefore, we examined whether CD11b\textsuperscript{low} macrophages differ in their efferocytosis capacity from CD11b\textsuperscript{high} cells. The results shown in Fig. 2A and B indicate that CD11b\textsuperscript{low} macrophages engulfed significantly more PMN on average than CD11b\textsuperscript{high} macrophages (12.2 ± 0.2 neutrophils/macrophone (N/M) and 2.2 ± 0.2 N/M, respectively). LysoTracker staining (Fig. 2A and Supporting Information Fig. 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\textsuperscript{high} and CD11b\textsuperscript{low} macrophages were distinguished by an engulfment threshold of seven PMN (Fig. 2C): 90.6 ± 4.2% of the CD11b\textsuperscript{low} macrophages engulfed seven or more PMN, whereas 97.5 ± 1.2% of the CD11b\textsuperscript{high} macrophages.
macrophages engulfed less than seven PMN. The results shown in Fig. 2D indicate that the differences between CD11b\textsuperscript{high} and CD11b\textsuperscript{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. Consequently, the threshold of engulfment is enforced in a very narrow range and hence, in this experimental setting only macrophages that engulfed six PMN have the same tendency to be either CD11b\textsuperscript{high} or CD11b\textsuperscript{low} macrophages.

**CD11b\textsuperscript{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\textsuperscript{low} macrophages are immune-silent, we stimulated CD11b\textsuperscript{low} and CD11b\textsuperscript{high} macrophages with the TLR3 and TLR9 ligands poly (I:C) and CpG-oligodeoxynucleotides (CpG-ODN), respectively (Fig. 4), and determined the secretion of TNF-\(\alpha\) and IL-1\(\beta\). The results indicate that both TNF-\(\alpha\) and IL-1\(\beta\) secretion were increased following exposure to poly (I:C) and CpG-ODN of CD11b\textsuperscript{high} macrophages, whereas the secretion of these cytokines by CD11b\textsuperscript{low} macrophages treated in the same manner was significantly lower. It is worth noting that no significant reduction in the expression of TLR3, 4, and 9 was found in CD11b\textsuperscript{low} macrophages, in comparison to their CD11b\textsuperscript{high} counterparts (N = 3, data not shown). Thus, CD11b\textsuperscript{low} macrophages are poorer responders to different TLR ligands in terms of cytokine and chemokine secretion, and therefore are immune-silent.

**CD11b\textsuperscript{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 that CD11b\textsuperscript{low} macrophages have lost their phagocytic function. To determine whether CD11b\textsuperscript{low} and CD11b\textsuperscript{high} macrophages differ in their ability to phagocyte external particles, the phagocyte-specific dye PKH2-PCL green was injected I.P. to mice undergoing peritonitis for 48 and 4 h later the peritoneal cells were recovered, immunostained, and analyzed for PKH2-PCL acquisition. The results shown in Fig. 5 indicate that CD11b\textsuperscript{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\textsuperscript{low} macrophages are indeed immune-silent, we stimulated CD11b\textsuperscript{low} and CD11b\textsuperscript{high} macrophages with LPS (Fig. 3B, E–G). Surprisingly, the secretion of IL-10, an anti-inflammatory cytokine [12], II-13, and CCL2, CCL3, and CCL5 was determined (Fig. 3B, E–G). Importantly, the secretion of these pro-inflammatory cytokines and chemokines IL-1\(\beta\), CCL2, CCL3, and CCL5 was determined (Fig. 3B, E–G). Surprisingly, the secretion of these cytokines increased following exposure to poly (I:C) and CpG-ODN of CD11b\textsuperscript{high} macrophages, whereas the secretion of these cytokines by CD11b\textsuperscript{low} macrophages treated in the same manner was significantly lower. It is worth noting that no significant reduction in the expression of TLR3, 4, and 9 was found in CD11b\textsuperscript{low} macrophages, in comparison to their CD11b\textsuperscript{high} counterparts (N = 3, data not shown). Thus, CD11b\textsuperscript{low} macrophages are poorer responders to different TLR ligands in terms of cytokine and chemokine secretion, and therefore are immune-silent.
indicate that most CD11b<sub>low</sub> macrophages did not acquire PKH2-PCL (Fig. 5A), whereas the majority of CD11b<sub>high</sub> macrophages acquired higher amounts of PKH2-PCL (Fig. 5B). As a result, the MFI of PKH2-PCL was 4-10-fold higher in CD11b<sub>high</sub> macrophages (Fig. 5C). Of interest, a small portion of the CD11b<sub>low</sub> macrophages was labeled with PKH2 to a low extent, suggesting that there is some residual phagocytic activity exerted by these cells, or that these macrophages were converted from the CD11b<sub>high</sub> to the CD11b<sub>low</sub> 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<sup>+</sup>F4/80<sup>+</sup> doublets expressed higher levels of CD11b and F4/80 than CD11b<sub>low</sub> macrophages (N = 8, data not shown), indicating that CD11b<sub>high</sub> macrophages are actively engulfing apoptotic PMN, whereas CD11b<sub>low</sub> macrophages are not. Thus, CD11b<sub>low</sub> macrophages are “satiated,” meaning that they lost their phagocytic potential upon meeting the apoptotic PMN engulfment threshold and reducing their CD11b expression.

**CD11b<sub>low</sub> 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 LN and spleen [20, 30]. Our results indicate that CD11b<sub>low</sub> macrophages are not phagocytic (Fig. 5). Therefore, it is probable that 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<sub>low</sub> macrophages are prone to depart resolving inflammation sites and emigrate to lymphoid organs. To this end, we determined the relative distribution of CD11b<sub>high</sub> and

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**Figure 2.** CD11b<sub>low</sub> macrophages engulf higher numbers of apoptotic neutrophils than CD11b<sub>high</sub> macrophages. Sorted CD11b<sub>high</sub> and CD11b<sub>low</sub> macrophages were stained with Hoechst and LysoTracker, analyzed by confocal microscopy, and photographed as shown ((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 and C) or representative (A and D) from three experiments. Significant differences by Student’s t-test between CD11b<sub>high</sub> and CD11b<sub>low</sub> macrophages (*p<0.05, **p<0.005, and ***p<0.001) are shown.
CD11b\textsuperscript{low} macrophages 66 h post-peritonitis initiation at the peritoneum, inguinal LN, and spleen. Our results indicate that CD11b\textsuperscript{high} macrophages are the predominant macrophage subtype in the peritoneum during late resolution, whereas, at the same time CD11b\textsuperscript{low} macrophages are the predominant macrophage subtype at the LN and spleen (Fig. 6A). To examine whether CD11b\textsuperscript{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 h). The results shown in Fig. 6 indicate that the distribution of labeled CD11b\textsuperscript{high} and CD11b\textsuperscript{low} macrophages at the peritoneum, inguinal LN, and spleen 18 h after transfer (Fig. 6B) was similar to the distribution of unlabeled macrophages (Fig. 6A). Thus, during the late phase of resolution, peritoneal CD11b\textsuperscript{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\textsuperscript{high} to CD11b\textsuperscript{low} phenotype

The results shown in Fig. 2 and 6 suggest that CD11b\textsuperscript{high} macrophages convert to CD11b\textsuperscript{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\textsuperscript{high} and CD11b\textsuperscript{low} macrophages were incubated with or without apoptotic Jurkat cells and the changes in surface expression of CD11b were determined. The results shown in Fig. 7 indicate that CD11b expression on the surface of both CD11b\textsuperscript{high} and CD11b\textsuperscript{low} macrophages was significantly reduced.
following their incubation with apoptotic cells. It is worth noting that the level of expression of CD11b on both macrophage populations following interaction with apoptotic cells was lower than its level of expression on CD11b\textsuperscript{low} macrophages recovered from peritoneal exudates, and therefore we designated the ex vivo-generated population CD11b\textsuperscript{low}/C0 macrophages (Fig. 7C). The detection of CD11b\textsuperscript{low}/C0 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\textsuperscript{low}/C0 macrophages are not present in peritoneal cavities. On the other hand, the absence of exit routes in cell cultures allows CD11b downregulation to reach significantly lower levels of its expression. Interestingly, some reduction in CD11b expression on both CD11b\textsuperscript{high} and CD11b\textsuperscript{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 latex beads (LB) or IgG-opsonized LB (Supporting Information Fig. 4A). Of interest, the impact of apoptotic cells was partially mimicked by CD11b ligation with monoclonal antibodies (Supporting Information 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 downregulation. Notably, CD206 and CD163 surface expression was not modulated by apoptotic cells ex vivo, but was reduced by anti-CD11b antibodies (Supporting Information Fig. 4A). In addition, exposure to zymosan A, TGF-β, 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 mieties, 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\textsuperscript{high} and CD11b\textsuperscript{low} macrophages that are shown in Fig. 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 (Fig. 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<sup>high</sup> to the CD11b<sup>low</sup> 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 show reduced actin levels in lysates of CD11b<sup>low</sup> macrophages, in comparison to their CD11b<sup>high</sup> 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 LB or IgG-opsonized LB, resulted in a significant reduction in CD11b and arginase-1 expression (Supporting Information 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<sup>high</sup> to CD11b<sup>low</sup> macrophages.

**RvD1, RvE1, and dexamethasone reduce the efferocytic requirement for macrophage immune-silencing**

Rv 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 dexamethasone (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<sup>low</sup> 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 (Fig. 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<sup>low</sup> macrophages (32.8 ± 8.8, 46.2 ± 1.8, and 39.9 ± 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 the numbers of apoptotic PMN engulfed by macrophages in peritoneal cavities. In addition, RvD1, RvE1, and Dex enhanced the engulfment of PMN in the peritoneum. A detailed analysis of engulfment according to thresholds (Fig. 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 shown in Fig. 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 (Fig. 8G) with RvD1, RvE1, and Dex when IL-1β 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 (Fig. 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, Th2 cytokines, or glucocorticoids [1, 2]. The encounter of macrophages with apoptotic cells was proposed as an additional polarizing trigger that evokes Th2-like responses, as well as the expression of arginase-1, and 12/15-LO [19]. A seminal study from Bystrom et al. [29] has identified hybrid resolution-phase macrophages (rM) that exert properties of M1 macrophages, such as the expression of iNOS and COX-2, and secret lower levels of inflammatory cytokines, but high levels of IL-10, which are properties of M2 [2]. In addition, rM 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 rM. However, these CD11b<sup>low</sup> macrophages express neither iNOS nor arginase-1, whereas expressing lower levels of COX-2 and MMP-9 (M1 enzymes) than their CD11b<sup>high</sup> counterparts (Fig. 1). It is worth noting that CD11b<sup>low</sup>, but not CD11b<sup>high</sup> macrophages express the lipid converting enzyme 12/15-LO, which is involved in the production of pro-resolving lipid mediators, such as 15-hydroxydocosahexaenoate, LX, protectins, and Rv [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 that its binding to different ligands results in a significant immune suppression in macrophages [38, 39]. In addition, CD11b expression on immature DC was downregulated, following the iC3b-mediated ingestion of apoptotic cells [40]. Our findings determine that CD11b<sub>low</sub> macrophages that appear in resolving inflammatory sites during the late resolution phase [26] engulf more apoptotic PMN than CD11b<sub>high</sub> macrophages (Fig. 2) and secrete lower amounts of pro-inflammatory cytokines and chemokines, but not TGF-β in response to TLR ligands (Fig. 3 and 4), thereby fulfilling the designation of pro-resolving macrophages. Of interest, CD11b<sub>low</sub> macrophages did not produce IL-10, in accordance with the earlier studies that examined the responses of macrophages to apoptotic cell instillation in vivo [14].

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 h after peritonitis initiation. At 66 h, the peritoneal exudate cells from all treatments were recovered, enumerated, and immunostained 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<sub>low</sub> macrophages (B), and the level of CD11b expression on CD11b<sub>high</sub> macrophages (C). Some of the exudate cells were also stained with Hoechst and apoptotic PMN engulfment by macrophages was determined (D and E). In addition, macrophages were isolated from all treatments and stimulated ex vivo with LPS (500 ng/mL). After 24 h, the secretion of TNF-α (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 < 0.05, **p < 0.005, and ***p < 0.001) are shown.
Notably, CD11b<sub>low</sub> macrophages were found to be the prominent F4/80<sup>+</sup> macrophage subtype in the LN and spleen, in particular in the population that originated in the peritoneum (Fig. 6), where the CD11b<sup>high</sup> macrophages were the prevalent macrophage subtype, indicating a conversion from the CD11b<sup>high</sup> to the CD11b<sup>low</sup> phenotype is required for the emigration of macrophages to lymphoid organs during the resolution of inflammation. Indeed, we also found that incubation of CD11b<sup>high</sup> macrophages with apoptotic cells ex vivo resulted in a significant enhancement of CD11b downregulation (Fig. 7). Moreover, this reduction in CD11b expression is associated with changes in the protein expression signature characteristic of the CD11b<sup>high</sup> to CD11b<sup>low</sup> conversion, thereby indicating a complete phenotype change driven by satisfying macrophage interaction with apoptotic cells. Overall, our results show that CD11b<sup>high</sup> macrophages seem to display mixed properties of M1 and M2, whereas CD11b<sup>low</sup> macrophages, although originating in the CD11b<sup>high</sup> population, express a unique phenotype. Putting our current study in perspective with the previous studies 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 shown in Fig. 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 (rM or CD11b<sup>high</sup> 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<sup>low</sup> macrophages that are apparently more adept at migration to lymphoid tissues and delivery of resolution cues to the acquired immune system.

Importantly, CD11b<sup>low</sup> macrophages were also found to lose their phagocytic activity (Fig. 5) upon conversion from CD11b<sup>high</sup> 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 LXA<sub>4</sub> receptor [24]. Notably, both Dex and LXA<sub>4</sub>, as well as RvE1 promote the engulfment of apoptotic PMN by macrophages during the resolution of inflammation [8, 20, 25]. This 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 shown 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, whereas increasing the number of CD11b<sup>low</sup> 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 two PMN following the introduction of RvE1 and Dex at resolving cavities (Fig. 8E) suggests that 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 immunosilencing. It is worth noting that 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 Rv will exhibit improved actions over glucocorticoids in treatment of pathologies associated with hampered resolution.

In summary, 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 Rv 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-1β, IL-10, TGF-β, CCL2, CCL3, and CCL5 were obtained from R&D Systems. CFSE, staurosporine, LPS (from *Escherichia coli*, clone O55:B5), PKH2-PCL green fluorescence linker kit, and Dex from Sigma. Poly (I:C) and 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 Rv were matched according to the 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 h, peritoneal exudates were collected and exudate cells were stained with FITC-conjugated rat anti-mouse Ly-6G, PE-conjugated rat anti-mouse F4/80, and PerCP-conjugated rat anti-mouse CD11b (Biolegend) and analyzed by FACSCalibur (Beckton-Dickinson). In some experiments, the macrophages were sorted to CD11bhigh and CD11blow populations of 495% purity, using FACSaria (Beckton-Dickinson), and the separate populations were used for microscopic analysis and for ex vivo stimulation. In some experiments, PHK2-PCL green (0.25 μM, 0.5 mL) was injected I.P. at 48 h and peritoneal cells were recovered 4 h later, immunostained 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 μg/mouse) were introduced to the peritoneum 48 h after peritonitis initiation, and the exudates were recovered after 66 h and analyzed as before. For detailed methodologies, see “Supporting Information”.

Western blot analysis

Protein extracts of sorted populations (>95% purity) of CD11bhigh and CD11blow macrophages were run using 10% SDS-PAGE (5 μg/lane), transferred to nitrocellulose membranes, and immunoblotted with either goat anti-mouse CD11b (Santa-Cruz Biotechnology), rabbit anti-mouse iNOS (Abcam), goat anti-mouse arginase-1 (Abcam), rabbit anti-mouse COX-2 (Cayman Chemicals), sheep anti-mouse 12/15-LO (Cayman Chemicals), 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 h post-peritonitis initiation, stained with CFSE (1 µM), and injected into the peritoneum of mice that underwent peritonitis for 48 h. After an additional 18 h, cells from peritoneal exudates, inguinal LN, and spleen were recovered from the recipient mice, immunostained as above, and the percentage of CD11b<sup>high</sup> and CD11b<sup>low</sup> macrophages in each site was determined.

**Confocal microscopy**

Sorted CD11b<sup>high</sup> and CD11b<sup>low</sup> macrophages were isolated and loaded with 50 nm LysoTracker Red DND 99 dye (Molecular Probes) for 2 h 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<sup>high</sup> and CD11b<sup>low</sup> 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<sup>+</sup> Ly-6G<sup>+</sup> entities (identified as macrophages that are attached to but did not fully engulf a PMN by forward versus side scatter analysis) were excluded from the samples to avoid counting of attached PMN.

**TLR-mediated responsiveness ex vivo**

Exudate macrophages were sorted or separated using PE selection magnetic beads (StemCell Technologies) and incubated (1 x 10<sup>6</sup> 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 h, the supernatants were collected and their TNF-α, IL-1β, IL-10, TGF-β, 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 h, 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 h, macrophages were immunostained 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 three times with at least four replicates. Results were analyzed by one-way analysis of variance (for multiple groups) or Student’s t-test (for comparison between two groups) with p-values<0.05 considered as statistically significant. Results are expressed as means± SEM.

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**Conflict of interest:** C. N. S. is inventor on patents covering resolvins as biotemplates for novel therapeutic development assigned to Brigham and Women’s Hospital, Partners Healthcare and are licensed for clinical development. The other authors declare no other competing financial interests.

**References**


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Abbreviations: CpG-ODN: CpG-oligodeoxynucleotides · Dex: dexamethasone · iNOS: inducible NO synthase · LB: latex beads · LO: lipoxygenase · LX: lipoxin · rM: resolving-phase macrophages · Rv: resolvins

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