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Decreased CD47 expression during spontaneous apoptosis targets neutrophils for phagocytosis by monocyte-derived macrophages

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ABSTRACT

Background: Neutrophils (PMN) are the primary leukocyte responders during acute inflammation. After migrating into the tissues, PMN undergo programmed cell death (apoptosis) and are subsequently removed via phagocytosis by resident macrophages during the resolution phase. Efficient phagocytosis of apoptotic neutrophils is necessary for successful resolution. CD47 plays a critical role in mediating the phagocytic response, although its role in the phagocytosis of apoptotic PMN is incompletely understood.
Aims: In the present study we tested the hypotheses that CD47 modulates the targeting of apoptotic PMN for phagocytosis, and that this process is altered in neonatal PMN.
Study design: Adult and neonatal PMN were examined for their expression of CD47. To investigate CD47-mediated functions, apoptotic adult and neonatal PMN were co-cultured with monocyte-derived macrophages (MDM) and the phagocytic index was determined using a flow cytometry-based assay.
Results: We observed lower basal surface CD47 levels on neonatal *vs.* adult PMN. In both groups, spontaneous apoptosis led to decreased surface and total cellular CD47 expression. Adult and neonatal MDM ingested apoptotic neonatal target PMN more avidly than apoptotic adult target PMN. Masking of surface CD47 on PMN with a monoclonal antibody enhanced MDM phagocytic activity.
Conclusions: Our results suggest that age-dependent expression of CD47 on PMN may account for differences in their ingestion by macrophages and in the resolution of inflammation.

1. Introduction

Following migration into the tissues during active inflammation, PMN must be removed by resident phagocytes during the resolution phase. Resolution of inflammation involves several steps including: cessation of PMN recruitment, initiation of PMN apoptosis and clearance of apoptotic PMN by resident macrophages [1]. Impairment of one or more of these processes could potentially prolong inflammation and exacerbate host tissue injury. The process of apoptosis, in addition to initiating systematic cell death, targets PMN for removal by macrophages. If apoptotic PMN within the tissues are not removed in an efficient and timely manner, they will become necrotic and release cytotoxic granule proteins that may perpetuate host tissue damage. Thus, PMN apoptosis is a critical limiting factor for the successful resolution of inflammation. In addition, we reported that apoptotic neonatal PMN retain their potent cytotoxic phenotype [2]. It is these inherent characteristics that promote the persistence of neonatal PMN at sites of inflammation.

Recent studies have uncovered three key concerted signaling mechanisms that facilitate phagocytosis of apoptotic cells. These signals, “come and get me” and “eat me”, in the absence of “don't eat me” signals, promote cellular ingestion of apoptotic cells by resident macrophages [3]. Mounting evidence suggests that viable cells prevent their own ingestion by displaying surface “don't eat me” signals. A common “don't eat me” signaling mechanism involves interactions between CD47 and signal regulatory protein alpha (SIRPα). CD47 is particularly abundant on leukocytes and red blood cells (RBC) [4]. *In vivo* studies have defined CD47 as a marker of self, as shown by the ingestion of CD47-null mouse RBC, but not wild-type RBC, by splenic red pulp macrophages after injection into wild-type mice [5]. Ligation of phagocyte SIRPα by surface CD47 on target cells, inhibits phagocytosis through the phosphorylation of tyrosine residues within the cytoplasmic ITIM (immunoreceptor tyrosine-based inhibitory motif) of SIRPα [6]. Phosphorylation of SIRPα provides binding sites for recruited tyrosine phosphatases, SHP-1 and SHP-2 [7]. As SHP-1 is predominately an inhibitory phosphatase, its involvement in phagocytic signaling serves to attenuate target cell engulfment. Indeed, alveolar macrophages treated with sodium stibogluconate (a protein tyrosine phosphatase inhibitor) or isolated from motheaten mice (SHP-1-defective) had an enhanced phagocytic capacity [8].

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In the present study, we sought to define CD47 expression in neonatal PMN and to examine how CD47 might influence uptake of apoptotic PMN. We provide evidence that apoptotic neonatal and adult PMN exhibit age-related dissimilarities in their expression of cell surface signals involved in regulating phagocytosis by monocyte-derived macrophages (MDM). An alteration in these signals may promote the presence of neonatal PMN at inflammatory sites and exacerbate tissue injury.

2. Methods

2.1. Reagents

RPMI and CellTracker Green (5-chloromethylfluorescein; CMFDA) were purchased from Invitrogen. Fetal bovine serum and IMDM were from Hyclone. The antibodies anti-CD15-APC, -CD16-PE, IgG-FITC isotype control, and IgM-APC isotype control were purchased from Becton Dickinson. Anti-β actin was purchased from Sigma Aldrich. Monoclonal anti-CD47 (clone B6H12) is reactive with the IgV domain of CD47 and has been described previously [9,10].

2.2. Neutrophil and monocyte isolation and culture

Venous blood samples were obtained from healthy adult donors or placentas of uncomplicated deliveries and processed in parallel. Samples were obtained according to the guidelines of the Institutional Review Board for Human Studies at Saint Louis University. Neutrophils were isolated as described [11]. For the phagocytosis assay, PMN suspended in PBS (20×10^6/mL) were labeled with 10 μM CMFDA (20 min, 37 °C). To induce spontaneous apoptosis, CMFDA-labeled PMN in RPMI/2% FBS (4×10^6/mL) were cultured in round-bottom polypropylene tubes at 37 °C, 5% CO_2 for 24 h.

2.3. Monocyte-derived macrophages

Peripheral blood mononuclear cells (MNC) were collected following density centrifugation and isolated as previously described, with minor modifications [12]. After washing in PBS/2 mM EDTA, MNC were suspended (3×10^6/mL) in IMDM and plated in 24-well plates (1 h, 37 °C). Non-adherent cells were washed off and the monocytes were cultured for 7 days in IMDM/10% FBS containing 100 U/mL penicillin/streptomycin. Additional media was added at day 3.

2.4. Flow cytometry

Neutrophils were suspended (2×10^6/mL) in PBS/1% BSA (PBS/BSA). For each condition, 2×10^5 PMN were incubated with mAb anti-CD47 (20 min, 4 °C). After washing once in cold PBS/BSA the cells were incubated with donkey anti-mouse-FITC secondary or isotype control IgG (20 min, 4 °C). The labeled cells were washed in PBS/BSA, fixed in 1% formalin and analyzed by flow cytometry.

2.5. Flow cytometric phagocytosis assay

We measured the *in vitro* phagocytosis of apoptotic PMN using a previously described flow cytometric assay with minor modifications [12]. To obtain apoptotic target cells, 24 h PMN were labeled with anti-CD16-PE, and apoptotic PMN ($CD16^{low}$) were separated from non-apoptotic PMN ($CD16^{high}$) using the anti-PE EasySep kit from StemCell Technologies, as described [2]. Adherent MDM were co-cultured with CMFDA-labeled apoptotic PMN at a ratio of 5:1 (PMN to MDM) for 1 h at 37 °C. For each condition, cells were stained with anti-CD15-APC to exclude contaminating PMN from the analysis [12]. A minimum of 10,000 CD15-negative events were acquired. For the CD47 ligation studies, apoptotic PMN were treated with a F(ab')2 fragment of anti-CD47 clone B6H12 or F(ab')2 IgG control Ab (20 min, 4 °C), washed twice in PBS to remove excess antibody, then co-cultured with MDM as above. To determine the phagocytic index, the number of CMFDA-positive MDM within the CD15-negative gate was divided by the total number of MDM acquired. Flow cytometric analysis was performed with FlowJo software, version 7.2.2 (Tree Star).

2.6. Immunoblot analysis

PMN cell pellets were suspended in a hypotonic lysis buffer (10 mM Tris; pH 7.5, 10 mM NaCl, 0.5% TX-100, 2 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin) for 10 min at 4 °C. Lysates were normalized for total protein concentration, and transferred to PVDF membranes. Total CD47 was detected using monoclonal anti-CD47 (B6H12, 1:2000), followed by detection with the appropriated horseradish peroxidase-conjugated secondary Ab. Parallel blots were run for β-actin and the densitometric ratio of CD47 to β-actin was calculated.

2.7. Statistical analysis

All statistical calculations were performed using Microsoft Excel (Redmond, WA). Data were analyzed by Student's *t*-test. A p value <0.05 was considered statistically significant.

3. Results

3.1. CD47 expression in neonatal and adult PMN

We assessed CD47 surface expression on freshly isolated adult and neonatal PMN using flow cytometry. As shown (Fig. 1A, B), we observed a nearly 2-fold difference in baseline CD47 surface expression between adult and neonatal PMN (MFI: adults, 6.8 ± 3.3 *vs.* neonates, 3.5 ± 1.9; X ± SD, $p<0.05$). To determine if CD47 expression is altered during spontaneous apoptosis, we examined CD47 surface levels on adult and neonatal PMN under basal (0 h) or cultured (24 h) conditions. As shown in Fig. 1A, adult PMN expressed less surface CD47 at 24 h than at baseline (MFI: 24 h, 2.2 ± 1.8 *vs.* 0 h, 8.2 ± 2.0, $p<0.05$). CD47 expression was also reduced in neonatal PMN undergoing apoptosis relative to baseline values (24 h, 1.2 ± 0.45 *vs.* 0 h, 4.2 ± 0.75, $p<0.05$) (Fig. 1B).

The association of CD47 with secondary granules suggests the possibility that surface expression does not reflect total cellular CD47 expression [13]. To address this, we performed Western blot analysis on lysates from 0 h and 24 h adult and neonatal PMN. Our immunoblot results (Fig. 2) showed that total cellular levels of CD47 were similar in neonatal and adult PMN under resting conditions. In addition, CD47 expression decreased under conditions that induce apoptosis (24 h culture) in adult and neonatal PMN, thus confirming our flow cytometry data.

3.2. Phagocytosis of apoptotic PMN by MDM

To address the involvement of CD47 in regulating phagocytosis of apoptotic PMN, we treated cultured adult and neonatal PMN with a F(ab')2 fragment of monoclonal anti-CD47 (clone B6H12) prior to co-culturing with MDM. As shown in Fig. 3, blockage of surface CD47 on apoptotic adult or neonatal target PMN increased the phagocytic index of adult MDM (adult PMN targets: IgG control, 24.8 ± 8.0 *vs.* B6H12 34 ± 10.7, $p<0.05$; neonatal PMN targets: IgG control, 29.2 ± 7.2 *vs.* B6H12 40.4 ± 10.0, $p<0.05$).

The population of cultured (24 h) apoptotic PMN used for the previous phagocytosis assay contained a mixture of apoptotic and surviving PMN. As neonatal PMN are inherently resistant to apoptosis [11], the overall percentage of eligible target PMN within each population will differ in comparison to apoptotic adult PMN. To better

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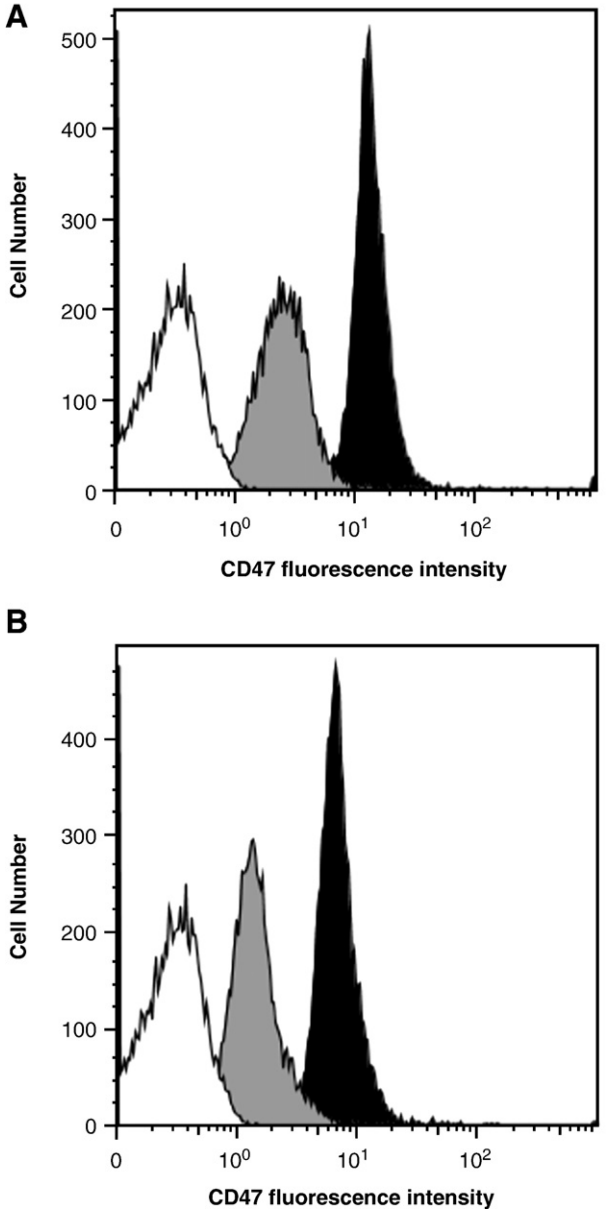

Fig. 1. CD47 expression levels on baseline and cultured PMN. CD47 expression on 0 h (black peak) and 24 h (gray peak) adult (A) and neonatal (B) PMN are shown. Cells stained with control IgG are represented by white peaks. Histograms are representative of 4 independent studies.

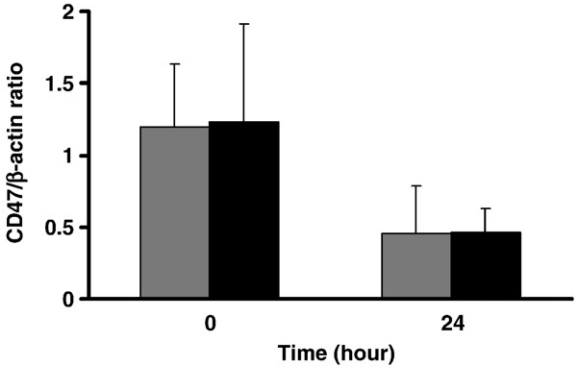

Fig. 2. Whole cell PMN CD47 expression in baseline and culture PMN. Densitometric ratios of CD47 immunoblots normalized to β-actin for 0 h and 24 h adult (gray column) and neonatal (black column) PMN. Data are representative of 3 independent, paired experiments.

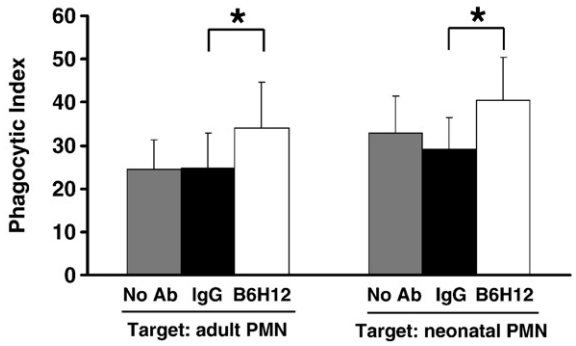

Fig. 3. Enhancement of PMN phagocytosis by target cell CD47 blockade. Adult or neonatal target PMN were untreated (gray column), treated with control IgG F(ab')2 (black column), or anti-CD47 (clone B6H12) F(ab')2 (white column), then co-cultured with adult MDM. Data (phagocytic index) represent the percentage of MDM containing ingested PMN. Data are mean ± SD. *$p < 0.05$; $n = 5$ paired studies.

address target cell-specific modulation of the phagocytic response, we enriched the target PMN populations for apoptotic ($CD16^{low}$) PMN using immunomagnetic sorting. Neutrophils that express low levels of CD16 are significantly more apoptotic than $CD16^{high}$ PMN [14]; therefore the PMN targets for this assay were predominantly apoptotic PMN. As shown in Fig. 4, we found that adult MDM had a higher phagocytic index when ingesting neonatal target PMN (48 ± 11%) than adult target PMN (24 ± 12%, $p < 0.05$). Neonatal MDM also had a higher phagocytic index when co-cultured with neonatal target PMN (neonatal target PMN; 52.8 ± 15% *vs.* adult target PMN 39.5 ± 16%, $p < 0.05$).

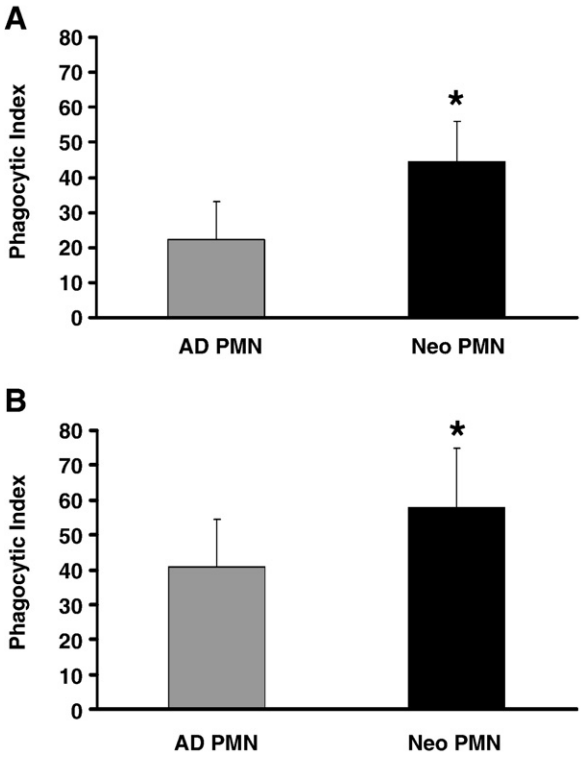

Fig. 4. Phagocytosis of $CD16^{low}$ adult and neonatal PMN by adult and neonatal MDM. Apoptotic adult and neonatal PMN populations were enriched for $CD16^{low}$ expressing PMN then co-cultured with adult (A)and neonatal (B) MDM. Data (phagocytic index) represent the percentage of MDM containing ingested PMN. Data are mean ± SD. *$p < 0.05$; $n = 3$ separate studies.

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4. Discussion

During the onset of infection, activated PMN migrate to sites of inflammation in order to mount a protective response against invading pathogens. Neutrophils that migrate into tissues during inflammation are subsequently removed by resident macrophages during the resolution phase. The initiation of resolution is a multifaceted process, involving interactions between soluble and cellular components, and the efficient phagocytosis of PMN by macrophages during resolution depends on the timely progression of PMN apoptosis. Since CD47 plays a prominent role in the uptake of apoptotic PMN (via interactions with SIRPα) [15], we hypothesized that changes in CD47 surface expression on apoptotic neonatal PMN might affect their phagocytosis by MDM. To test this hypothesis, we first compared CD47 expression on adult and neonatal PMN under basal conditions. We observed less surface but not total cellular expression of CD47 in neonatal PMN compared to adult PMN (Fig. 2). Our observation suggests that neonatal neutrophils differentially express CD47 on their surface. The precise function of this is unknown; however, reduced CD47 expression on PMN may serve to dampen PMN-mediated immune responses in infants during a time when they are constantly challenged with a vast array of foreign pathogens not present during gestation. Reduced CD47 surface levels may partly explain the observed diminished neonatal PMN migratory response [16], as CD47 is critically important for PMN transepithelial [17] and transendothelial [18] migration.

A number of other cell surface adhesion molecules have lower expression levels in neonatal PMN in comparison to adult PMN. Neonatal PMN have lower surface levels of L-selectin and are deficient in the shedding of L-selectin after stimulation [19,20]. Likewise, FcRII (CD32) and FcRIII (CD16) expressions are also significantly reduced in the immature PMN [21]. The expression of CD11b in neonatal PMN has been found to be somewhat variable. Some investigators showed that CD11b was increased on neonatal PMN [2,19], while McEvoy et al. found that neonatal PMN had decreased CD11b expression [22]. These discrepancies are likely due to the fact that cord blood contains a mixture of both mature and immature PMN [23]. Reduced expression of proteins key to innate immunity may help explain PMN dysfunction in neonates and their predisposition for infection.

Gardai et al. showed decreased CD47 surface expression in PMN undergoing UV light-induced apoptosis [15]. In the present report we also showed a decrease of CD47 expression (surface and total cellular amounts) in adult and neonatal PMN during spontaneous apoptosis. Although the relationship between PMN apoptosis and CD47 is incompletely defined, these observations suggest that the progression of apoptosis is linked to the reduction of cellular CD47 levels independent of the apoptotic stimulus. The mechanism underlying this reduction of PMN CD47 is unknown, although protease cleavage is a possibility. Allen et al. showed that matrix metalloprotease 2 (MMP-2) cleaved CD47 [24], and proteolysis removed the SIRPα binding site [25]. Human PMN contain MMP-2 in their secondary granules [26]; however, proteolysis of CD47 by neutrophil MMP-2 has not been reported. Thus, CD47 proteolysis by MMP-2 as a pro-resolution mechanism is an attractive theory; as such cleavage would remove the SIRPα binding site from CD47 and target the PMN for phagocytosis.

The systematic removal of apoptotic inflammatory cells is critical for inflammation resolution. Impairment of this process may prolong the presence of surviving functional PMN and subject the host to cytotoxic and inflammatory PMN mediators, as we previously reported [2]. CD47 has been previously described as a marker of self [5], and as such, removal or masking of CD47 targets a cell for phagocytosis [15]. As shown in Fig. 3, treating adult or neonatal PMN with a F(ab')2 fragment of anti-CD47 increased their uptake by MDM, which is consistent with the proposed role of CD47 as a targeting molecule for macrophage-mediated phagocytosis.

Finally, we utilized a population of PMN that was predominantly apoptotic ($CD16^{low}$) to better examine any target cell-specific characteristics that might modulate phagocytosis. When we examined adult and neonatal MDM individually and compared their respective ingestion of adult or neonatal apoptotic PMN targets, we observed a higher phagocytic index in the presence of apoptotic neonatal PMN targets (Fig. 4A,B). We attributed this increase in target PMN ingestion to the lower surface levels of CD47 on neonatal *vs.* adult PMN (Fig. 1). Our observation is in agreement with that of Gardai et al. who demonstrated that increased ingestion of apoptotic PMN correlated with diminished PMN surface CD47 levels [15]. Although co-culturing adult MDM with neonatal PMN and vice versa, is not a physiological scenario, our studies provide new insight into the uptake of phagocytic cells based on CD47 surface expression. The results presented here provide further evidence of the inhibitory role of CD47 in phagocytosis.

In summary, we determined that neonatal PMN have an age-related reduction of surface CD47 levels. In addition, conditions that promote spontaneous apoptosis decrease CD47 expression in both adult and neonatal PMN. Our data suggest that the reduction of CD47 levels during apoptosis targets PMN for removal by resident macrophages. In support of this concept, we observed that apoptotic neonatal PMN were ingested more avidly than apoptotic adult PMN, presumably due to the lowered CD47 expression on the neonatal PMN. Collectively, our observations provide insight into the age-dependent role of CD47 in PMN apoptosis and set the stage for investigations of mechanisms regulating neonatal macrophage-mediated phagocytosis.

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