

Received Date : 27-Oct-2015

Revised Date : 07-Jan-2016

Accepted Date : 27-Jan-2016

Article type : Original Article: Experimental Allergy and Immunology

Title

**sCD48 is anti-inflammatory in *S. aureus* Enterotoxin B induced
Eosinophilic inflammation.**

Short title

sCD48 in eosinophilic inflammation

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12851

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Abstract

Background

S. aureus, one of the most important pathogens, is heavily associated to allergy. *S. aureus* and its toxins interact with eosinophils through CD48 a GPI-anchored receptor important in allergy mainly as expressed by the eosinophils (mCD48). CD48 can exist in a soluble form (sCD48). Our aim was to investigate SEB induced regulation of eosinophil CD48 and the possible formation and role of sCD48 in SEB mediated eosinophil activation *in vitro* and *in vivo*.

Methods

Human peripheral blood eosinophils were activated by SEB with or without inhibitors for phospholipases (PL) (–C or –D), or cycloheximide or brefeldin A. We evaluated: eosinophil activation (CD11b expression or EPO/IL-8 release), mCD48 (flow cytometry), sCD48 (ELISA), SEB binding to sCD48 (ELISA) and chemotaxis towards SEB. C57BL/6 mice were pre-injected (ip.) with sCD48 then peritonitis was induced by SEB injection; peritoneal lavages were collected after 48 hr. and analyzed by flow cytometry and ELISA.

Results

SEB activated human eosinophils formed sCD48, directly correlating to CD11b expression, through cell-associated PL–C and –D. mCD48 remained stable due to up-regulation in CD48 transcription and cellular trafficking. sCD48 bound to SEB and down-regulated SEB stimulatory effects on eosinophils as assessed by EPO and IL-8 release and eosinophil

chemotaxis towards SEB. sCD48 showed anti-inflammatory activity in a SEB-induced mouse peritonitis model.

Conclusions

SEB regulates CD48 dynamics on eosinophils. Our data indicate sCD48 as a SEB induced “decoy” receptor derived from eosinophil and therefore as a potential anti-inflammatory tool in *S. aureus* induced eosinophil inflammation often associated with allergy.

Keywords

CD48; Eosinophils, Inflammation; Peritonitis; SEB

Introduction

Staphylococcus aureus is one of the most common human commensal bacteria found in skin, and in nasal and bronchial mucosa. Furthermore, it is the most important pathogen associated with atopic dermatitis (AD), and it is also found in allergic rhinitis and allergic asthma (1-3). Eosinophils, the main effector cells of the late and chronic phase of allergic inflammation are also important in host protection against parasitic, bacterial and viral infections. Eosinophils express several activating and inhibitory receptors and produce an array of the pro-inflammatory cytokines, cationic proteins, and mitochondrial and extracellular DNA-containing ‘traps’ (4, 5). Interestingly eosinophils have been demonstrated to have a role in inflammation resolution (6). The most studied allergic disease in which an interplay exists between the immune system and bacteria is AD that has been long known to be dominated by skin colonization of *S. aureus* (1). *S. aureus* produces exotoxins with superantigenic capability such as staphylococcal enterotoxin B (SEB), protein A (PtA), peptidoglycan (PGN) and the toxic shock syndrome toxins (7). These exotoxins amplify allergic reactions by direct activation of eosinophils, mast cells, T cells and other innate immune cells (8-12).

Specifically they inhibit eosinophil apoptosis, enhance oxidative burst and increase expression of receptors, and cause the release of basic proteins and cytokines (11, 12). Usually the cell surface receptors involved in cell/bacteria interactions are the toll like receptors TLRs (13). However, we showed that *S. aureus* and its exotoxins can bind to and activate human and murine eosinophils and also mast cells through CD48 (10, 11). CD48 is a GPI-anchored cell-surface protein belonging to the CD2 family expressed on several human and mouse hematopoietic cells (14). CD48 activates cells *via* its high affinity receptor 2B4 also expressed by the eosinophils (15, 16). CD48 functions as a co-stimulatory molecule in lymphocyte activation and facilitates cell adhesion (14). Its expression and function on both mast cells and eosinophils indicated CD48 as an important molecule in allergy mediating many pivotal cellular interactions (17). CD48 was found to be one of the asthma signature genes in murine asthma (18) and as expressed by mast cells' and interacting with eosinophils' 2B4 induces the formation and activation of the 'Allergic Effector Unit' (19). On eosinophils CD48 was found to be up-regulated by IL-3 *via* ORMDL3, an asthma candidate gene, by *S. aureus*/exotoxins and by allergen challenge in a murine model of experimental asthma model (11, 17, 20).

CD48 like other GPI-anchored proteins (CD55, CD59, CD87, etc.) can be found both as mCD48 and sCD48. Indeed, to the best of our knowledge sCD48 was only reported in lymphoid leukemias and arthritis but its function and mechanism of formation remain unknown (21).

Due to CD48 and *S. aureus*' role in allergy we aimed to dissect the regulation of formation of sCD48 in SEB induced eosinophil activation *in vitro* and *in vivo*. We investigated whether SEB can regulate eosinophils' mCD48 and sCD48 expression and its pathophysiological role in SEB induced eosinophil activation *in vitro* and *in vivo*.

Methods

***In vitro* experiments with human peripheral blood eosinophils**

Eosinophils were purified (16) from the peripheral blood of atopic volunteers with written informed consent according to the guidelines of the Hadassah-Hebrew University Human Experimentation Helsinki Committee.

Eosinophils were cultured with medium supplemented with recombinant human GM-CSF (5 ng/ml; Peprotech, Rocky Hill, NJ) for their prolonged survival in all the *in vitro* experiments. To study the kinetics of sCD48 formation and mCD48 expression eosinophils were incubated with or without SEB (SEB was used at 10 µg/ml throughout the study unless otherwise stated) for up to 18 hr. Supernatant was analyzed for sCD48 formation (ELISA) and cells for membranal expression of CD48 and CD11b (flow cytometry). Eosinophil viability was determined and always found to be ≥95%. See supplemental methods online.

Mechanism of formation of sCD48 from SEB activated eosinophils

SEB was pre-incubated (30 min., 37 °C) with inhibitors of: phospholipase-C (1 µM, U73122) or phospholipase-D (10 µM, 1,10-phenanthroline) or vehicle control (DMSO) and then added to eosinophils for 18 hr. (37 °C, 5 % CO₂).

In parallel, eosinophils were pre-incubated under conditions similar to the above described with U73122 or 1,10-PNT; or brefeldin A (1 µg/ml) or cycloheximide (1 µg/ml) or vehicle controls (DMSO or ethanol), before SEB (10 µg/ml) addition (18 hr.).

After incubation supernatants and cells were separated by centrifugation and analyzed as above. CD48 transcription was studied by RT-PCR from SEB activated eosinophils and intracellular CD48 was examined by immunofluorescence and confocal microscopy. See complete procedures in supplemental methods online.

sCD48-SEB binding assays

sCD48 binding to SEB was analyzed by sandwich ELISA using anti-SEB mAb as capture Ab and biotin labelled anti-CD48 Ab as detection Ab. See detailed protocol and materials in supplemental methods online.

Anti-inflammatory effect of sCD48 on SEB eosinophil activation

SEB, pre-incubated with/without sCD48 (5 µg/ml) for 30 min (37 °C), was then added to eosinophils (2×10^5 cells/100 µl/well) for either 40 min or 18 hr. Cell activation was determined by quantifying supernatants for released EPO (40 min) (11) or IL-8 (18 hr.) (human IL-8 ELISA kit (900-K18, PeproTech Asia, Rehovot, Israel) as per the manufacturers' instructions). Mean values of each treatment were normalized with SEB treated cells in respective experiments.

Eosinophil transmigration assay

Eosinophils transmigration assay was carried out as previously described (22). See complete procedures in supplemental methods online.

Mice and SEB-induced peritonitis

Female C57BL/6J, 6-8 weeks old (WT) mice were purchased from Harlan Laboratories Inc., Israel and housed in the specified pathogen free animal facilities of The Hebrew University of Jerusalem. All murine experiments were approved by the Animal Experimentation Ethics Committee of the Hebrew University of Jerusalem and performed in accordance with the guidelines of the committee. Peritonitis in WT mice was induced either by SEB injection (1

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 $\mu\text{g}/200\text{ }\mu\text{l}/\text{mice}$) as described previously (11). After 48 hr. mice were sacrificed and peritoneal lavages were collected and analyzed. See supplemental methods online.

Results

SEB activation of human eosinophils induces sCD48 formation and mCD48 cleavage by a phospholipase mechanism

To examine the regulation of CD48 on eosinophils by SEB, cells were incubated with or without SEB up to 18 hr. and both the mCD48 expression and sCD48 formation were evaluated. As shown in Figure 1, sCD48 formed in SEB activated eosinophils from 1 hr. onwards and its levels increased with time. sCD48 was significantly higher than the one in the medium incubated cells from 3 hr. up to 18 hr. (Fig. 1A). mCD48 expression on SEB incubated eosinophils was found to be nearly stable with a slight increase with time (Fig. 1B). Interestingly the expression of CD11b, an eosinophil activation marker, was significantly high on SEB incubated eosinophils by 3 hr. post SEB incubation (Fig. 1C). Moreover, a significant direct correlation was observed between the sCD48 and CD11b (Fig. 1D). Importantly eosinophils activated by platelet activating factor (PAF) also showed a significant increase in sCD48 in supernatants over the control (Fig. 1E).

Some strains of *S. aureus* and some bacterial toxins were reported to have phospholipase (PL) activity (23-26). In consequence we examined whether SEB by itself possesses PL-C or -D activity. Therefore, SEB was pre-incubated with inhibitors of PL-C or -D (U73122 or 1,10-PNT respectively) and then added to the eosinophils. This procedure had no effect on sCD48 concentrations in the eosinophil supernatants thus ruling out SEB intrinsic PL-C or PL-D activity (Fig. 2A). Since eosinophils are a known source of PLs (27, 28), eosinophils were pre-incubated (30 min) with U73122 or 1,10-PNT and then incubated with SEB (18

hr.). In eosinophils that were pre-incubated with these PL inhibitors, a significant decrease in sCD48 levels (Fig. 2B), but no difference in mCD48 (Fig. 2C), was observed indicating that sCD48 is formed because of eosinophils' intrinsic PL-C/ PL-D activity.

Next we assessed the formation of sCD48 by incubating eosinophils with phosphatidylinositol-specific PL-C (PI-PLC). This resulted in up to 80 % cleavage of mCD48 within 1 hr. and up to >90 % by 18 hr. (Fig. S1A) and increased sCD48 in supernatants of these cultures (>300 pg/ml of sCD48 was detected after 1 hr. and >700-pg/ml by 18 hr.) (Fig. S1B).

SEB activation of human eosinophils induces cellular trafficking and up-regulation of CD48 transcription

As SEB activation of human eosinophils caused mild up-regulation of mCD48 expression with time (Fig. 1B), we checked if preformed CD48 is found inside eosinophils and whether transport of preformed CD48 and/or *de novo* protein synthesis could be implied in the maintenance of mCD48 levels. Eosinophils that adhered to poly L-Lysine were stained with anti-human CD48 Ab and examined by confocal immunofluorescence microscopy. As shown in Figure 3A, CD48 staining was observed as previously reported on the plasma membrane (19) and in addition in the cytoplasm. As observed in Figure 1E, higher sCD48 levels were found in supernatants of PAF activated eosinophils. Therefore, to assess whether CD48 is contained in eosinophil granules, eosinophils were double stained with anti-EDN Ab (Fig. 3B) and with anti-CD48 Ab. As shown in Figure 3C some of the CD48 staining co-localized with these granular proteins. The colocalized CD48 staining was confirmed by another eosinophil granule protein ECP and similar results were obtained as shown in (supplementary Fig. S2). Further, to evaluate whether blockade of protein transport or of *de novo* protein synthesis could influence mCD48 and sCD48 formation, eosinophils were pre-incubated with

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either brefeldin A or with cycloheximide prior to SEB addition. These treatments resulted in a significant decrease in sCD48 (Fig. 3E). Also mCD48 in these settings was significantly decreased (40–50 %) (Fig. 3F). This would imply that transport of intracellular CD48 and new protein synthesis are both involved. To strengthen our findings, eosinophils pre-incubated (30 min) with brefeldin A and then activated by SEB (18 hr.) were stained for intracellular CD48 and examined by confocal microscopy (Fig. 3H–K). As expected eosinophils that were pre-incubated with brefeldin A before SEB activation showed higher content of intracellular CD48 (Fig. 3K) than those that were not pre-treated with brefeldin A (Fig. 3J). In addition, we evaluated transcription levels of CD48 in SEB activated eosinophils at different time points. A significant up-regulation in CD48 mRNA was observed at 3 hr. and 18 hr. (Fig. 3L & M). These results show that mCD48 levels are maintained by a combination of *de novo* protein synthesis and cellular trafficking.

Anti-inflammatory effects of sCD48 *in vitro*

We recently reported that SEB interacts with human and mouse eosinophil through their mCD48 (11). Since sCD48 is the mCD48 without the GPI-anchor, we hypothesized that sCD48 would also bind to SEB. sCD48–SEB binding was evaluated by two sandwich ELISAs, and it was found that sCD48 binds both to free SEB in solution (Fig. 4A) as well as plate bound SEB captured on anti-SEB Ab (Fig. 4B).

Next we investigated the role of sCD48 hypothesizing that by binding to SEB it might decrease SEB potential to activate the eosinophils. Thus, SEB was pre-incubated with sCD48 before addition to the cells. It is noteworthy that this resulted in a significant decrease of EPO and IL-8 release (Fig. 4C & D). These results indicate that sCD48 binding to SEB prevents its further interaction with mCD48 expressed on the eosinophils. We then assessed in an eosinophil transmigration assay towards SEB, the effect of sCD48 pre-incubation with SEB.

This treatment significantly reduced eosinophils migration to nearly basal level, as compared to SEB alone (Fig. 4E).

SEB induces sCD48 formation in a mouse peritonitis model

We assessed in a SEB-induced mouse peritonitis with rich eosinophils infiltrate (11) whether sCD48 could be detected in the peritoneal lavage and in the serum. Forty-eight hours after SEB injection when eosinophil peritoneal infiltration peaks, sCD48 was measured and found to be significantly increased as compared to PBS injected controls (Fig. 5A). This significantly correlated ($p < 0.005$, $R^2 \geq 0.73$) with the eosinophils and lymphocytes counts (Fig. 5B & C). sCD48 also correlated to granulocytes, mononuclear cells and total cells but to a lesser extent (Fig. S2). These results indicate that eosinophils and lymphocytes should be the main source of sCD48 in this model with other cells contributing. sCD48 was also detected in the serum of these mice though there was no significant difference observed between PBS or SEB groups (not shown).

Anti-inflammatory effect of sCD48 on SEB-induced mouse peritonitis

To assess the role of sCD48 *in vivo*, the peritonitis model was carried out by injecting ip. sCD48 or PBS 30 min. prior to SEB injection. Mice that were pre-treated with sCD48 before SEB challenge (sCD48-SEB group) at 48 hr. showed a significant reduction in overall inflammation as indicated by the highly significant decrease in total infiltrating cells including lymphocytes, mononuclear cells and eosinophils (Fig. 6A and B) in comparison to the control group (PBS-SEB). Interestingly the CD107a (Lamp-1) positive cells (mostly lymphocytes and granulocytes) that were increased because of SEB injection, were significantly reduced in sCD48-SEB mice (Fig. 6C). The peritoneal lavage levels of the pro-

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inflammatory cytokine TNF- α that showed an increase in the PBS-SEB treated mice were also attenuated significantly by sCD48 treatment (Fig. 6D). Moreover, PBS-SEB injection caused a significant increase in spleen weight, and remarkably, pre-treatment of mice with sCD48 completely prevented this (Fig. 6E).

Discussion

Particularly as expressed by eosinophils, CD48 is a central molecule in allergic responses that are commonly associated with *S. aureus* infections. Moreover eosinophils have recently gained much attention for their potential role in fighting microbes (29, 30). Our previous studies indicated that heat killed *S. aureus* as well as SEB, PtA and PGN interact with both human and murine eosinophils' CD48 (11). In the present work we have shown for the first time that SEB regulates CD48 dynamics and formation of sCD48 from eosinophils *in vitro* and *in vivo*. Furthermore, sCD48 exerts the anti-inflammatory activity on SEB activation of eosinophils *in vitro* and *in vivo* in a SEB peritonitis model.

In this study we first questioned whether SEB activated eosinophils could be a source of sCD48. We found that upon eosinophils incubation with SEB, sCD48 was formed and increased significantly at 3-18 hr. while mCD48 expression displayed a non-significant trend to up-regulate. sCD48 levels directly correlated with the activation state of eosinophils. Eosinophils incubated with PAF also showed increased levels of the sCD48. Interestingly we have found increased plasma sCD48 levels in human asthma patients (31).

We next investigated the mechanism of sCD48 formation. Being a GPI-anchored protein CD48 structure includes a phosphoethanolamine linker, glycan core and phospholipid tail that can be enzymatically cleaved by GPI-specific PLs. We therefore, evaluated whether SEB could be directly responsible for mCD48 cleavage. Pre-incubation of SEB with inhibitors of PL-C and PL-D ruled out this possibility. Other GPI-anchored proteins (32, 33) have been

reported to be cleaved by cell associated GPI-PLD. *S. aureus* and its toxins have been reported to stimulate cells' phospholipase (A/C and D) (34-36) and SEB was shown to induce the activation of PL-C in human T cells (37). Therefore, we hypothesized that eosinophil associated PLs could be involved in this cleavage process and SEB might be activating eosinophils' PLs. Indeed eosinophils' pre-incubation with U73122 or 1,10-PNT followed by SEB activation resulted in significantly decreased sCD48 production indicating that cell associated PL-C and -D are involved in mCD48 cleavage. Moreover as expected PI-PLC cleaved all the eosinophils' mCD48, which was evident from increased sCD48 levels in the supernatants, and nearly no mCD48 expression after 18 hr. Noteworthy, eosinophils incubated with PI-PLC formed more sCD48 than those incubated with SEB. Therefore, in an allergic inflammation complicated by *S. aureus* infection enhanced sCD48 formation may be found since eosinophil and *S. aureus* associated PLs induce sCD48 formation. Notably the finding of sCD48 formation because of eosinophil PLs shows the potential of this cell to cleave other GPI-anchored molecules as well.

mCD48 expression was not decreased but instead up-regulated in SEB incubated eosinophils. Indeed we found that an intracellular pool of CD48 is present in eosinophils partially colocalized with the granule proteins EDN and ECP. This would indicate that eosinophil activation might cause some direct release of CD48 from this pool and also replenish mCD48. Eosinophils, pre-incubated with either brefeldin A or cycloheximide and then activated by SEB, displayed a significant decrease in both the mCD48 and sCD48. This was also observed by immunofluorescence microscopy, as brefeldin A pre-incubated SEB-activated eosinophils show increased intracellular CD48 staining. Moreover CD48 mRNA was found to be significantly high in SEB incubated eosinophils at 3 hr. and 18 hr. Notably it has been claimed that alternate splicing of the mRNA of CD48 is not responsible for the sCD48 (21). In summary, all these results bolster the hypothesis that *de novo* synthesis and

protein transport maintains the mCD48 despite the cleavage by cell-associated phospholipases.

Next we investigated the role of sCD48 on eosinophil activation in inflammation. Soluble receptors have been shown to be decoy receptors or antagonists that bind a ligand with high affinity and specificity acting like a molecular trap and inhibiting it from binding to its normal receptor. For example the dominant-negative inhibitors of TNF exert its anti-inflammatory effect by being a decoy receptor that binds to sTNF (38), while human IL-1 receptor antagonist is enhanced by type II IL-1 soluble receptor and inhibited by type I IL-1 soluble receptor (39). sCD160 released by mast cells (40), and NK cells can inhibit cell-mediated cytotoxicity (41). It was therefore important to assess what sCD48's function could be in comparison to its membrane bound counterpart.

Importantly we first detected binding between sCD48 and SEB. We reasoned that binding of SEB to sCD48 would possibly block its interaction with mCD48. Indeed, pre-incubation of SEB with sCD48 before its addition to the eosinophils significantly decreased EPO and IL-8 release. IL-8 release by eosinophils incubated with heat killed *S. aureus* pre-incubated with sCD48 was also found to be significantly reduced in comparison to *S. aureus* incubated with PBS (Minai-Fleminger, Y. et. al, unpublished data). Eosinophils migration towards sCD48 complexed SEB was also significantly reduced. Notably SEB was reported to induce T cell chemotaxis (42) but failed to induce neutrophil chemotaxis (43). Migration of eosinophils towards a *S. aureus* toxin is a new finding adding yet another interaction going on between this granulocyte and bacteria. These results altogether indicate that sCD48 interaction with SEB prevents its further binding to mCD48 and the receptor activation thus pointing to other blocking interactions that sCD48 could have towards other *S. aureus* toxins such as PGN and PtA, and towards 2B4. Importantly incubation of sCD48 with eosinophils did not influence

them indicating a potential therapeutic potential of this molecule in eosinophilic inflammation.

Next we evaluated whether SEB can induce sCD48 formation in SEB induced peritonitis that is associated with eosinophilia. At peritoneal eosinophils peak (48 hr.), significantly high levels of sCD48 were detected that were directly correlated to peritoneal eosinophils and lymphocytes count. This would indicate that these cells might be the main contributing source of the sCD48 in this model. sCD48 levels were correlated but to a lesser extent to granulocyte and mononuclear cells. It is noteworthy that sCD48 plasma/serum levels were directly correlated with absolute lymphocyte counts in lymphoproliferative disorders (21). The *in vivo* increased levels of sCD48 in peritonitis suggested that sCD48 might be playing a role in inflammation. In consequence, to validate the *in vitro* findings on sCD48 and the hypothesis on the inhibitory role of sCD48 we pre-treated mice with sCD48 and then induced the SEB peritonitis. This treatment caused a significant decrease in peritonitis as evident from decreased infiltrating total cells and eosinophils, decreased Lamp-1 expression and TNF- α levels and no splenomegaly. Notably, eosinophils were significantly decreased in sCD48 treated mice possibly because of decreased chemotaxis as was observed in human eosinophils. Interestingly in an ovalbumin/alum induced allergic peritonitis model sCD48 was also able to inhibit the allergic type inflammation in a similar manner to the one we observed in SEB model. This could be attributed to both sCD48 capturing SEB and preventing it from binding to eosinophils and to other effector cells but also to preventing the interaction between mCD48 and 2B4 that is involved in eosinophil chemotaxis, adhesion and activation (22, 44) *in vitro* and in the OVA/SEB induced atopic dermatitis model (22). Also in the 'Allergic Effector Unit' sCD48 binding to human eosinophils' 2B4 blocked the mast cells CD48 induced increased eosinophils survival (19).

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Although reports exist that soluble high-affinity receptor antagonists of variable beta chain of TCR could neutralize SEB (45) and a synthetic SEB antagonist can prevent animals from lethal shock (46), ours is the first report on native receptor soluble form acting as an antagonist. Therefore, we consider sCD48 as a pharmacological tool in eosinophils driven allergic and non-allergic inflammation concomitant with *S. aureus* bacterial infection. In conclusion, this work showed that sCD48 can be produced by eosinophils, and it as a potential anti-inflammatory activity in eosinophilic inflammation.

Author Contributions

RSG planned and performed all the experiments, analyzed the data and wrote the manuscript; FLS designed and directed the research, received the grant support, provided overall supervision and edited the manuscript.

Acknowledgements

The authors are extremely thankful to Dr. Uri Hershberg (Drexel University, Philadelphia, PA, USA) for his valuable suggestions and discussions regarding the sCD48 formation, Mansour Seaf (HUJI) for assistance during animal experiment, and Dr. Mats Johansson (University of Wisconsin-Madison, WI, USA) for discussions on eosinophil activation markers. Authors also acknowledge the helpful suggestions from Dr. Micha Ben-Zimra (HUJI) and Madelyn Segev for her help in proofreading the manuscript.

This work was supported by grants from MAARS EU 7th framework (number HEALTH-F2-2011-261366); Hebrew University of Jerusalem (Israel)-Drexel University (Philadelphia, PA, USA) Joint grant (number 0304917); Israel Science Foundation (grant number 213/05), and the Aimwell Charitable Trust (London, UK) to F Levi-Schaffer. RSG received the “PBC postdoctoral fellowship for Indian and Chinese students” from The Hebrew University of

Jerusalem, Israel. FLS is affiliated with the David R. Bloom Center of Pharmacy and the Adolph and Klara Brettler Center for Research in Molecular Pharmacology and Therapeutics at The Hebrew University of Jerusalem.

Conflict of Interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1. SEB activation of human eosinophils induces sCD48 formation. (A) Time course (5 min-18 hr.) of sCD48 formation (ELISA) from eosinophils incubated with medium or SEB; (B) Time course of mCD48 (flow cytometry) expression on SEB incubated eosinophils from the same 6 donors of panel 'A' shown as Δ MFI; (C) Time course of expression of the eosinophil activation marker CD11b (flow cytometry) on SEB incubated eosinophils shown as Δ MFI; (D) Linear regression analysis of CD11b expression on

eosinophils vs. sCD48 concentration in supernatants; **(E)** sCD48 formation from supernatants of PAF activated (18 hr.) eosinophils. All the data reported in this figure are from experiments done on eosinophils obtained from different donors. Data are the mean $\pm$ SD, n=6 (A), n=6 donors showing each one separately (B), and mean $\pm$ SD, n=3 (C–E). *, p<0.05. **, p<0.01.

Figure 2. sCD48 from SEB activated human eosinophils is formed through a phospholipase mechanism. (A) sCD48 formation (ELISA) from eosinophils incubated with SEB (18 hr.) that were pre-incubated (30 min) with/without U73122 (1 μ M) or 1,10-PNT (10 μ M) (phospholipase–C and –D inhibitors respectively); **(B)** sCD48 formation and **(C)** mCD48 expression (flow cytometry) on eosinophils pre-incubated (30 min) with/without U73122 or 1,10-PNT and then incubated with SEB (18 hr.) shown as % Δ MFI. % Δ MFI was calculated by dividing Δ MFI of treated cells with Δ MFI of respective controls and multiplied by 100. All the data reported are from experiments done on eosinophils obtained from 4 different donors and are the mean $\pm$ SD, n=4 (A–C). *, p<0.05. See also Figure S1.

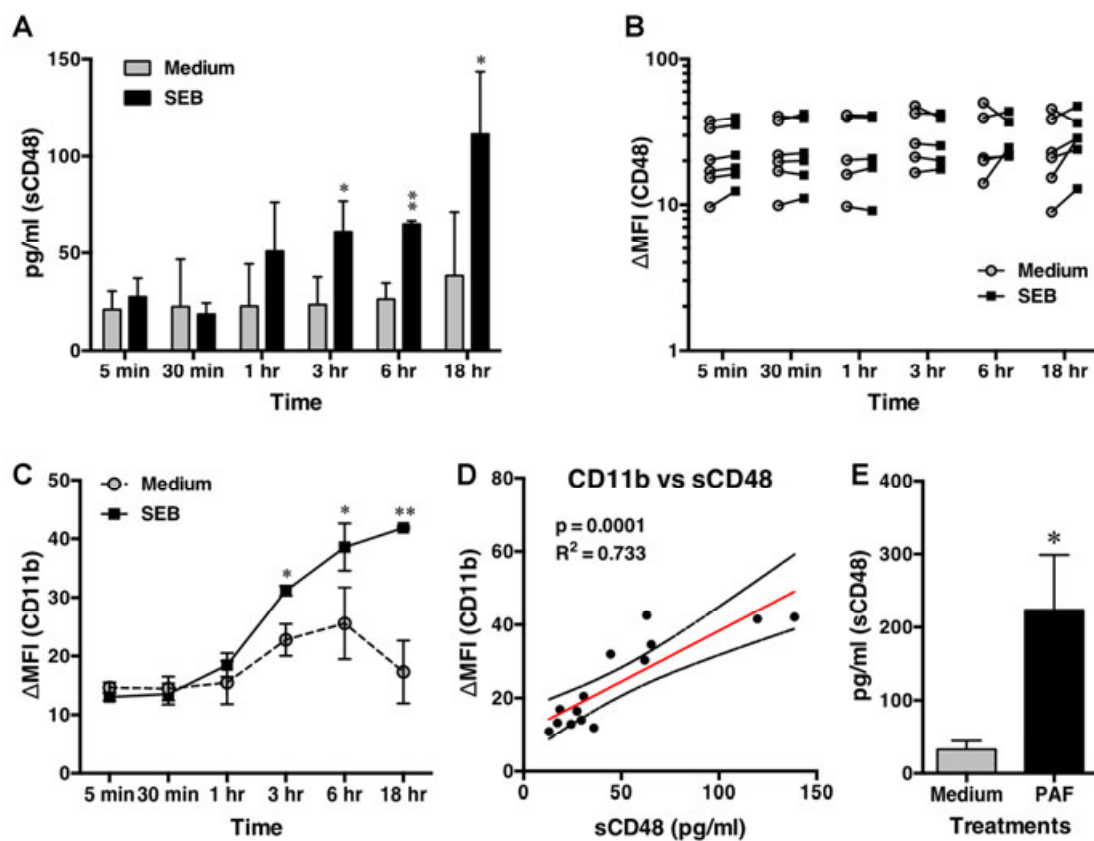
Figure 3. SEB activation of human eosinophils induces cellular trafficking and up-regulation of CD48 transcription. Confocal micrographs showing eosinophils stained with **(A)** anti-CD48 Ab (red); **(B)** anti-EDN Ab (green); **(C)** both anti-CD48 and anti-EDN Abs each channel separately and the merged one; **(D)** Alexa fluor® 647 and Alexa fluor® 488 (secondary Ab) as control. Nuclei were stained with DAPI (blue) and scale bar is 10 μ m. **(E)** sCD48 formation (ELISA) and **(F)** mCD48 expression (flow cytometry) on SEB activated (18 hr.) eosinophils, which were pre-incubated (30 min) with either brefeldin A or cycloheximide. (% Δ MFI was calculated as described in legend of **Figure 2**). Confocal micrographs showing eosinophils stained with **(G)** Alexa fluor® 647 (secondary Ab) as control. Eosinophils were stained for intracellular CD48 with anti-CD48 Ab after incubating

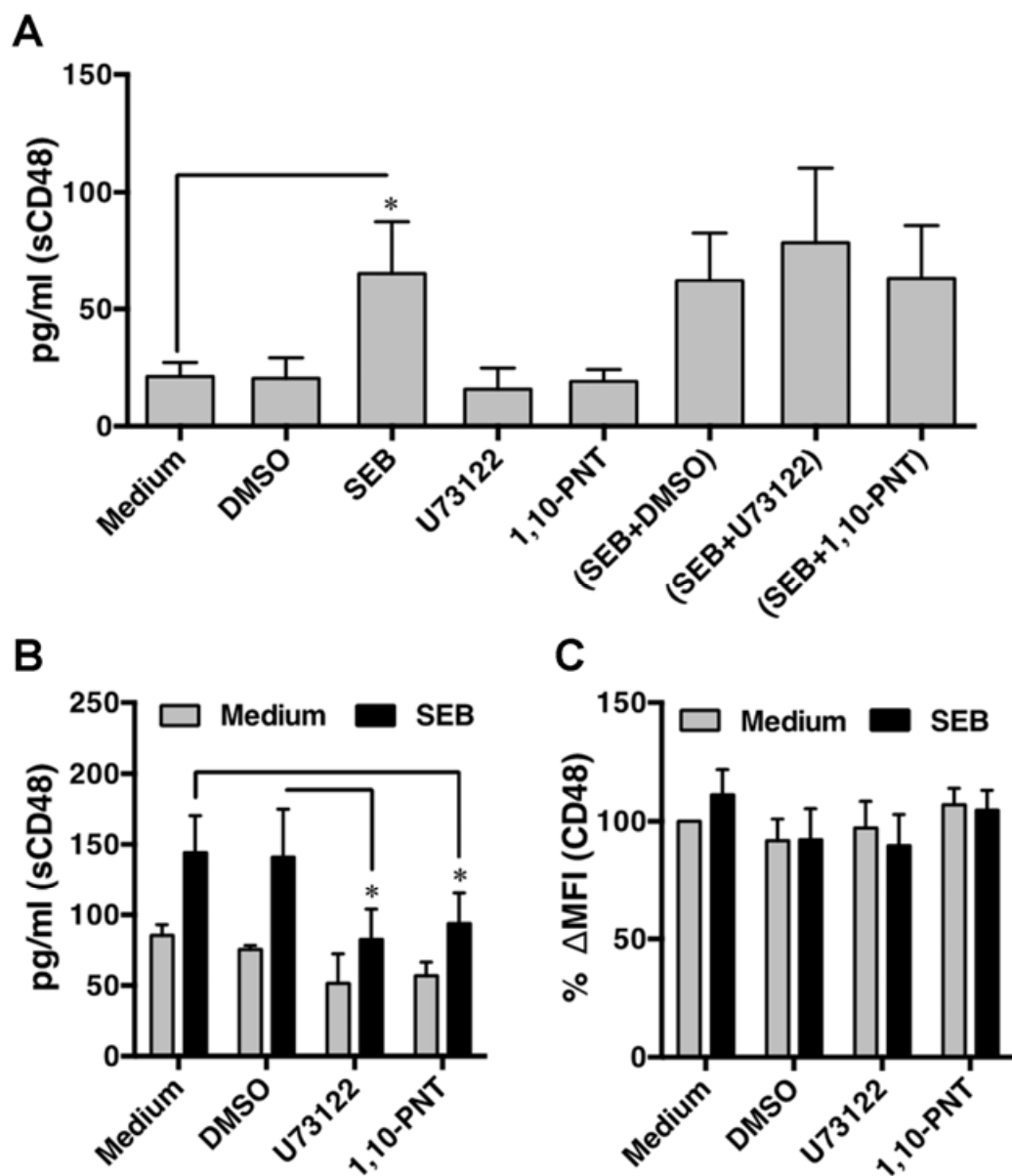
(18 hr.) with **(H)** medium only, **(I)** brefeldin A **(J)** SEB **(K)** brefeldin A (30 min) and then SEB (18 hr.). **(L)** Expression of CD48 (500 bp) in eosinophils incubated with medium or SEB by RT-PCR with beta-actin (200 bp) as the internal control. Lanes 1, 3, and 5 are from medium incubated eosinophils and lanes 2, 4 and 6 are from SEB incubated eosinophils (5 min, 3 hr. and 18 hr. respectively) from 3 different donors. Lane 'M' is 100 bp DNA marker. **(M)** Densitometry of the band intensities calculated and normalized (Image J) by beta-actin. Data reported in this figure are from experiments done on eosinophils obtained from different donors and represented as: representative of n=3 (A–D); mean $\pm$ SD, n=4 (E and F); representative of n=3 (G–K); three different donors (L); mean $\pm$ SD, (n=3). *, p<0.05. **, p<0.01. ***, p<0.001. See also supplementary Figure S2.

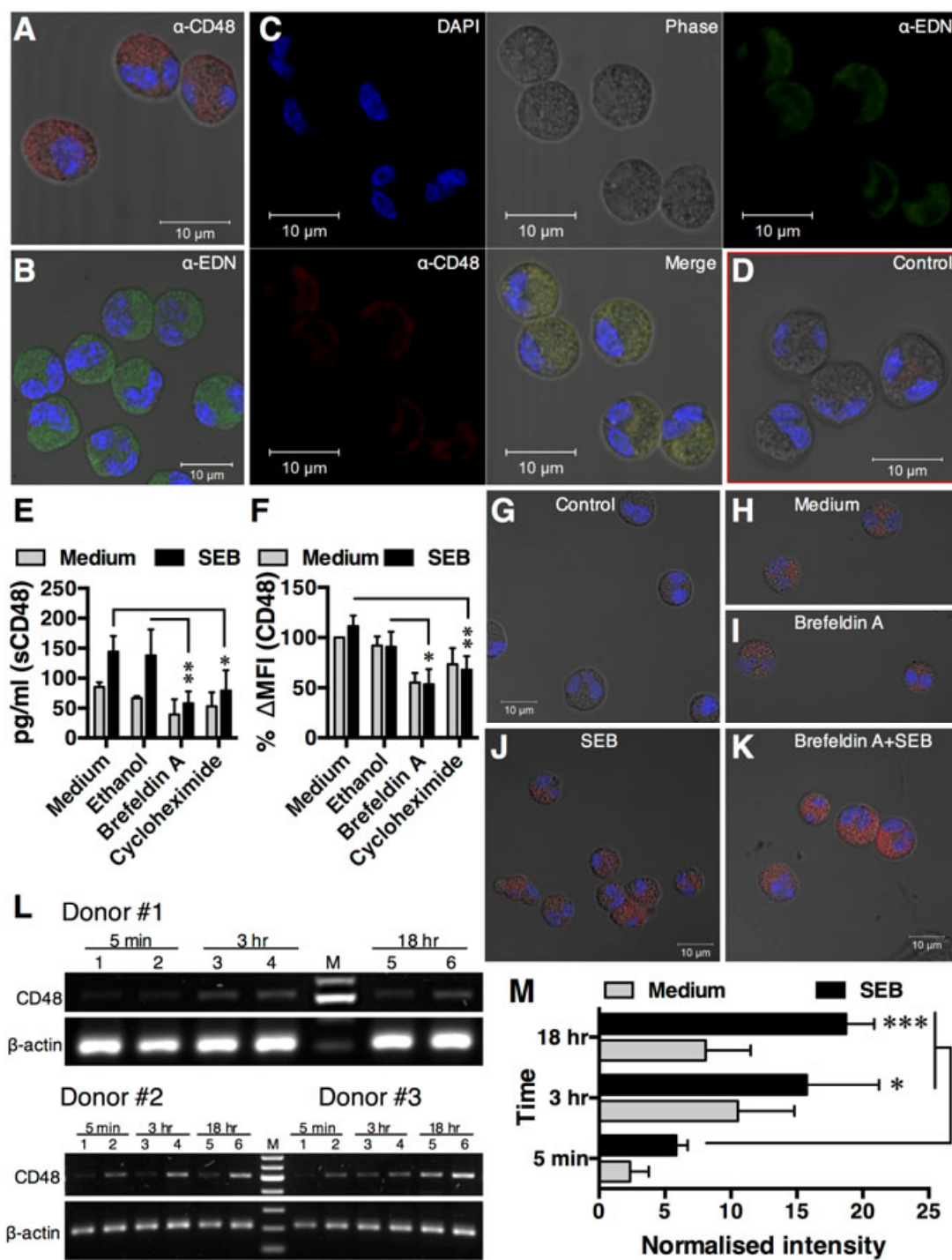
Figure 4. Anti-inflammatory effects of sCD48 *in vitro*. sCD48 binding to SEB was examined by sandwich ELISA, **(A)** sCD48-SEB complex was added to anti-SEB Ab coated ELISA plate and detected by anti-CD48 Ab and **(B)** sCD48 was added to SEB captured on anti-SEB Ab coated ELISA plate and detected by anti-CD48 Ab. **(C)** EPO release (colorimetric enzymatic assay) from eosinophils incubated (40 min.) with SEB that was pre-incubated with or without sCD48 (30 min); **(D)** IL-8 release (ELISA) from eosinophils incubated (18 hr.) with SEB that was pre-incubated with or without sCD48 (30 min). **(E)** Eosinophils chemotaxis towards eotaxin or SEB in a transmigration experiment. 'y' axis showing number of events counted in flow cytometer for 30 seconds at medium flow rate. Data reported in this figure are: the mean $\pm$ SD (for each experiment), n=3 (#1, #2 and #3) (A and B); from experiments done on eosinophils obtained from different donors (C–E), mean $\pm$ SD of normalized values, n=4 (C); mean $\pm$ SD of normalized values, n=5 (D); mean $\pm$ SD, n=3 (E). *, p<0.05. **, p<0.01 and ***, p<0.001. See also the Table S1 for absolute values of EPO and IL-8 release.

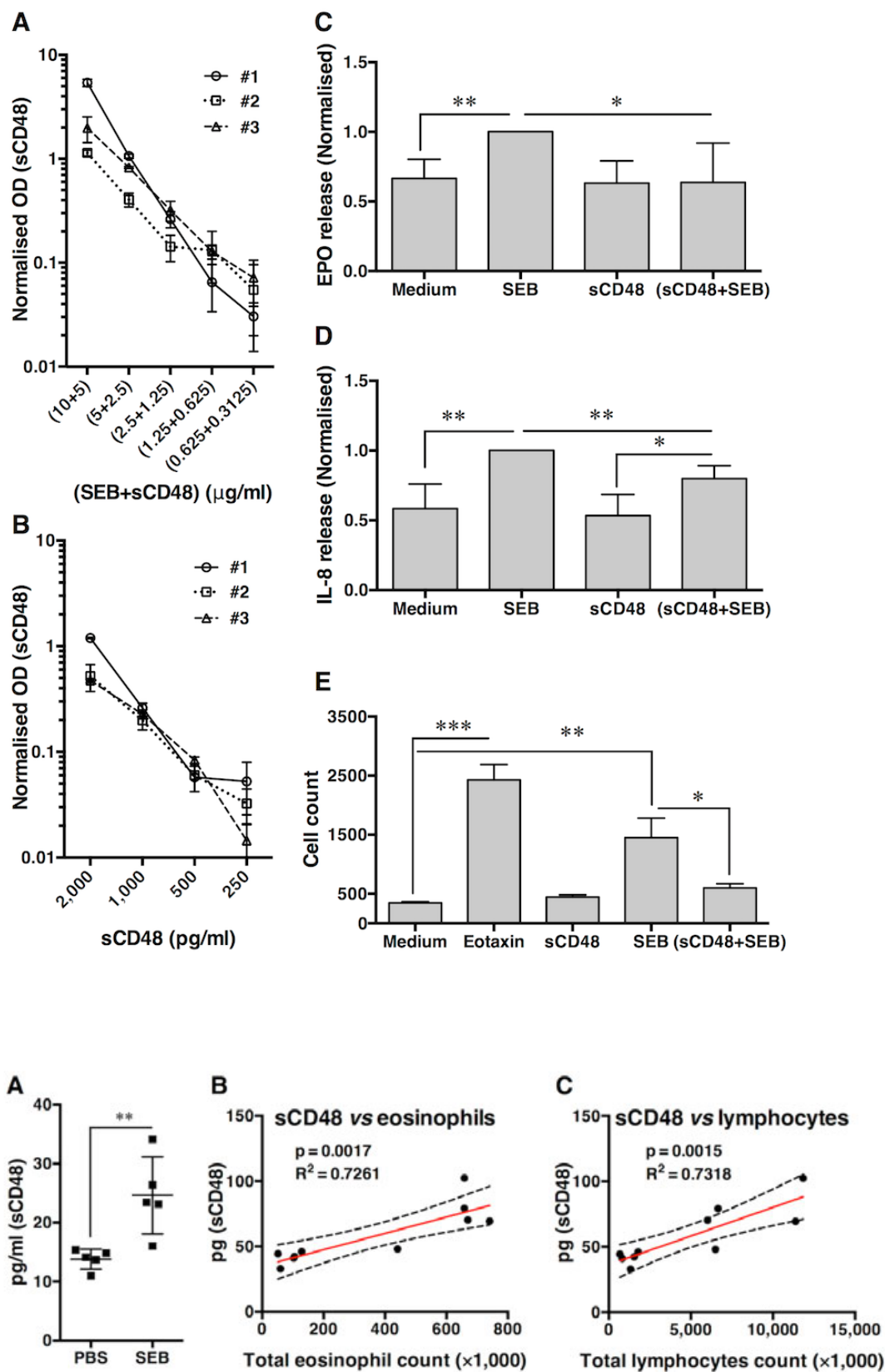
Figure 5. SEB induces sCD48 formation in mouse peritonitis. (A) sCD48 in peritoneal lavages from SEB-induced mouse peritonitis at 48 hr. (B) Linear regression analyses of sCD48 with total eosinophils count and (C) with total lymphocytes counts in peritoneum. The data are the mean $\pm$ SD of each mice group (5 mice/group). **, $p < 0.01$. See also Figure S3.

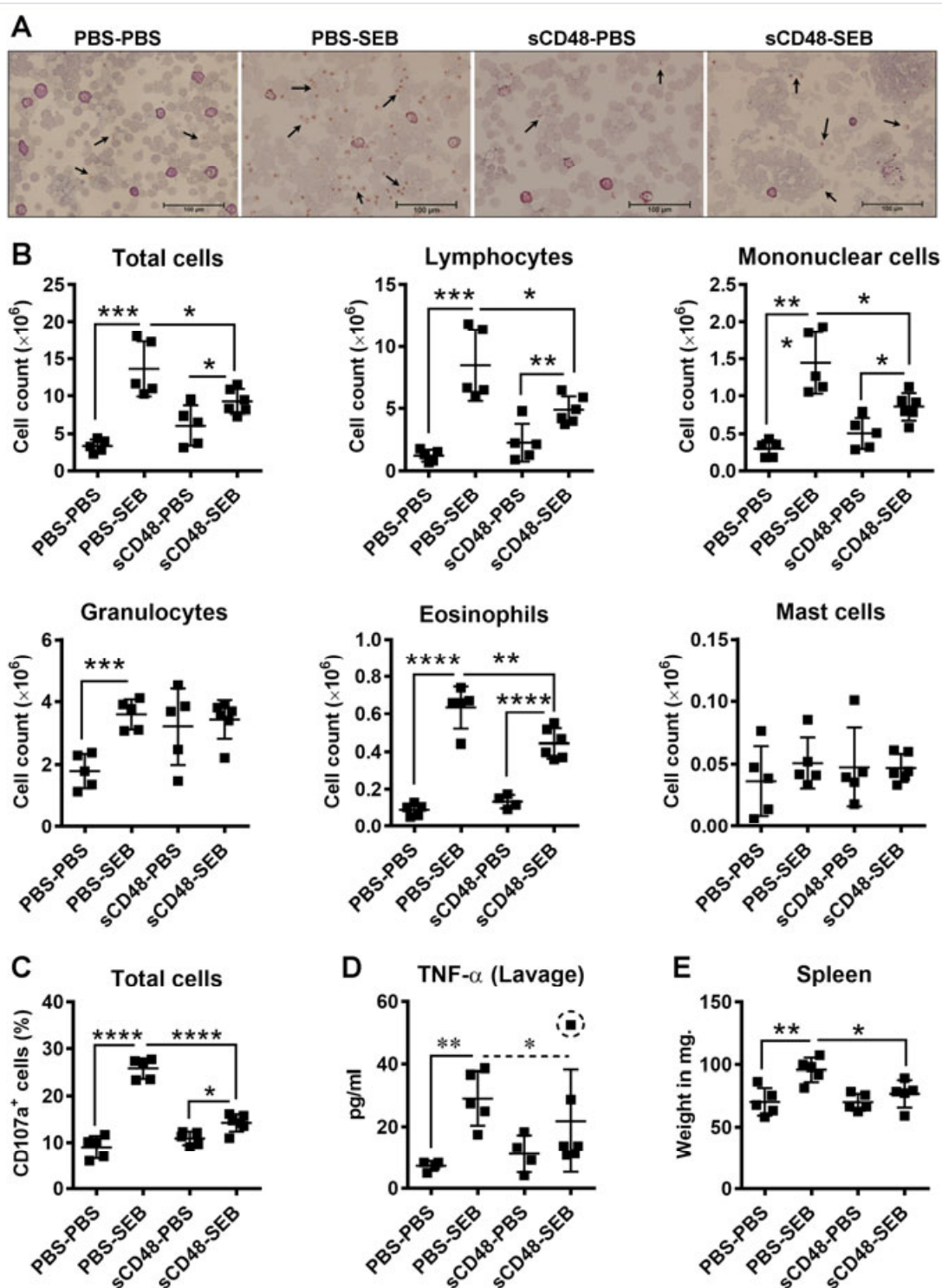
Figure 6. Anti-inflammatory effect of sCD48 on SEB-induced mouse peritonitis. sCD48 was injected (2.5 μ g/ mouse, ip.) 30 min. before SEB (1 μ g/mouse, ip.) injection and 48 hr. after mice were sacrificed and peritoneal lavages were analyzed for different parameters. (A) Representative image of cytopsin slides of peritoneal cells from each mice group (PBS-PBS, PBS-SEB, sCD48-PBS and sCD48-SEB) with eosinophils indicated by arrows, scale bar =100 μ m; (B) Absolute cell counts of total cells and different cell populations (lymphocytes, mononuclear cells, granulocytes, eosinophils and mast cells) in peritoneal lavage; (C) percentage of CD107a positive total cells; (D) TNF- α levels (ELISA), the solid box surrounded by dotted circle (in sCD48-SEB group) was a likely outlier identified by 'Grubbs' method in prism software and hence excluded from the analysis and (E) Spleen weight. The data are the mean $\pm$ SD of each mice group (5 mice/group), representative of $n=3$. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ and ****, $p < 0.0001$. See also Figure S4.











Supplemental Methods

Human peripheral blood eosinophils isolation

Eosinophils were purified as described previously ([1](#)), from the peripheral blood of asymptomatic mildly atopic volunteers (blood eosinophil levels 5–12 %). Written informed consent was obtained from all volunteers according to the guidelines of the Hadassah-Hebrew University Human Experimentation Helsinki Committee. “The Committee on Research Involving Human Subjects of The Hebrew University-Hadassah Medical School” approved the Helsinki request number ‘18-3.11.06’ for studies involving human subjects. Peripheral blood (150 ml) was collected from each donor and eosinophils were purified by negative selection using anti-CD16 Ab and anti-CD3 Ab magnetic beads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and the MACS system, at a purity of ≥ 99 % (Kimura staining), with a viability of ≥ 98 % (trypan blue staining). The cells were re-suspended (2×10^6 cells/ml) in culture medium (RPMI 1640 medium, 10 % heat-inactivated foetal bovine serum (FBS), and penicillin-streptomycin solution (100 U/ml) (Biological Industries, Beit Haemek, Israel)). Recombinant human GM-CSF (5 ng/ml; Peprotech, Rocky Hill, NJ) was added for cell survival in *in vitro* experiments. Before each experiment we checked eosinophil viability by staining them by Trypan blue and counting under the microscope or by Propidium iodide staining following assessment in flow cytometry. Eosinophil viability was always found to be $\geq 95\%$ unless otherwise mentioned.

Eosinophil activation by SEB and assessment of sCD48 formation, expression of mCD48 and CD11b

Freshly isolated eosinophils were activated by *S. aureus* enterotoxin-B (SEB) for 5 min, 30 min., 1 hr., 3 hr., 6 hr. and 18 hr. and the supernatant was analyzed for sCD48 formation by ELISA and cells for expression of mCD48 and CD11b by flow cytometry. In brief

eosinophils were washed three times in culture medium, seeded (2×10^5 /100 μ l/well in 96-well 'U' bottom plates (Nunc, Roskilde, Denmark)) and incubated with/without SEB (10 μ g/ml, S4881, Sigma-Aldrich, Rehovot, Israel) at 37 °C, 5 % CO₂ for 5 min, 30 min, 1 hr., 3 hr., 6 hr. and 18 hr. to examine the kinetics of sCD48 formation. Supernatants were collected by centrifugation, kept at -20 °C and examined for sCD48 by ELISA (DEIA237, Creative Diagnostics NY, USA) as per manufacturer's instructions. Cells were washed, blocked with 5 % goat serum (30 min, 4 °C) and stained with FITC conjugated anti-human CD48 Ab (clone MEM-102, AbD Serotec, Kidlington, UK) and APC conjugated CD11b (clone CBRM1/5, eBioscience, San Diego, CA, USA) or matched isotype control Ab (FITC-mouse IgG1 and APC- mouse IgG1, kappa). Cells were acquired by BD FACSCalibur and CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). The specific expression of the receptors on cell surface was calculated by subtracting the histogram geometric mean values of the isotype Ab staining from specific Ab staining and denoted as Δ MFI. In some instances the Δ MFI of CD48 in SEB incubated eosinophils was divided by Δ MFI of CD48 of respective controls (medium incubated eosinophils) of the same experiments, then multiplied by 100 and shown as % Δ MFI.

Cleavage of mCD48 from eosinophils by phosphatidylinositol-specific phospholipase-C (PI-PLC)

Freshly purified eosinophils (2×10^5 /100 μ l/well) were incubated with PI-PLC (25, 50 and 100 mU/ml, P5542, Sigma-Aldrich, Rehovot, Israel) for 1 hr. or 18 hr. After incubation cells and supernatants were collected, cells were examined for mCD48 expression by flow cytometry and supernatants for sCD48 by ELISA as above.

Immunofluorescence

Freshly isolated eosinophils were adhered on to Poly-L-lysine (P8920, Sigma-Aldrich, Rehovot, Israel) coated glass coverslips, fixed with paraformaldehyde solution (4 % in PBS, 20 min at 37 °C) and permeabilized with 0.2 % Triton X-100 (T8532, Sigma-Aldrich, Rehovot, Israel). Cells were blocked with 2 % BSA and 10 % goat serum and probed with mouse anti-human CD48 Ab (clone BJ40, BioLegend, San Diego, CA, USA) and/or mouse anti-human EDN/ECP Ab (a kind gift from Prof. G. Gleich, University of Utah, Salt Lake City, UT, USA) to stain the eosinophil granules and to determine the co-localization of CD48 followed by fluorochrome labelled secondary Ab (alexa flour 647 and/or 488, Life-technologies, Carlsbad, CA, USA) and DAPI 1:5000 and acquired and analyzed by confocal microscopy (ZEISS LSM710, Germany) for the expression of CD48.

RT-PCR from SEB activated eosinophils

A two-step semi-quantitative RT-PCR method was used to follow CD48 gene expression in eosinophils activated with SEB. Eosinophils (2×10^6 /ml) were incubated with or without SEB (10 µg/ml) for 5 min, 3 hr. and 18 hr.; total RNA was isolated using TRI Reagent® (T9424, Sigma-Aldrich, Rehovot, Israel) and 1 µg of total RNA was used for cDNA synthesis with qScript™ cDNA synthesis kit (95047 Quanta Biosciences, Gaithersburg, MD, USA) according to manufacturer's instructions. PCR was performed in 25 or 50 µl reaction mixture containing 1-2 µl of cDNA as template and CD48 specific forward primer 5'-GGCTCTGGAATTGCTACT-3' and reverse primer 5'-CTGTTCTGGAGCTCCTTTG-3' using ThermoPrime 2x ReddyMix PCR Master Mix (Thermo Scientific, Mevasseret Zion, Israel) according to manufacture's instructions. Housekeeping gene β-actin was used as PCR control and the amplified products were analyzed by electrophoresis on 1.0 % agarose gels and documented on ChemiDoc™ XRS+ System with Image Lab software (Bio-Rad

Laboratories, Inc., Hercules, California, USA). Band intensities were measured by Image J and results were normalized with β -actin amplification.

sCD48-SEB binding assays

sCD48 binding to SEB was analyzed by sandwich ELISA using anti-SEB mAb (1 μ g/ml, overnight at 4 °C) (Clone S222, Abcam, Cambridge, UK) as capture Ab and biotin labelled anti-CD48 Ab from human CD48 ELISA kit (DEIA237, Creative Diagnostics NY, USA) as detection Ab. In brief ELISA plates (Maxisorp Nunc, Roskilde, Denmark) were coated with capture Ab (1 μ g/ml) in carbonate buffer pH 9.6 and incubated overnight at 4 °C. The plates were then washed (0.05 % Tween 20 in PBS) and blocked with 5 % skimmed milk in PBS (1 hr. at RT). Plates were washed and SEB (10 μ g/ml) was added and incubated 2 hr. at RT followed by washing and human recombinant CD48 incubation (2000 – 250 pg/ml, 2 hr., RT). In another set of experiments SEB (10 μ g/ml) was incubated with sCD48 (5 μ g/ml) 30 min. at 37 °C and then added to anti-SEB capture Ab coated wells (2 hr. at RT). Plates were then washed and biotinylated anti-CD48 Ab (1 μ g/ml, 1 hr., RT) was added. The color development was monitored by the addition of streptavidin HRP (1 hr., RT) followed by TMB One Component Microwell Substrate, solution (SouthernBiotech Birmingham, AL, USA). Absorbance was measured at 650 nm (unstopped) or 450 nm (stopped by 2 M H₂SO₄) with BioTek instruments (Power wave xs; Winooski, VT, USA). OD values were normalized by blank and plotted against sCD48 concentrations.

Eosinophil transmigration assay

Eosinophils transmigration assay was carried out as previously described ([2](#)). In brief chemotaxis of human eosinophils towards SEB was monitored using polycarbonate membrane Transwell inserts (8.0-micron), placed in 24-well insert-containing polystyrene plates (Costar, Corning, NY, USA). Lower chambers were first loaded with 600 μ l of

medium containing controls or treatments; medium only as negative control, eotaxin (10 ng/ml) (recombinant human eotaxin, CHM-256, Prospec, Ness-Ziona, Israel) as positive control, SEB (10 µg/ml) and SEB (10 µg/ml) pre-incubated with sCD48 (5 µg/ml). Then eosinophils were loaded (100 µl) in the upper chambers in all the wells. The plate was immediately put into and incubated in a humidified incubator (37 °C, 5 % CO₂) for 90 min. After incubation inserts were gently removed, the cell suspensions in the lower chamber were collected in two different tubes and total events were counted for 60 sec (two times from each sample) at medium flow rate by BD FACSCalibur and CellQuest software. Data are reported as mean ±SD events counted in 60 seconds from lower chamber.

SEB-induced mouse peritonitis

Peritonitis in C57BL/6J (WT), female, 6-8 weeks old mice was induced as reported previously (3). In brief, mice were injected ip. with SEB (1 µg/200 µl PBS/mice) or PBS (200 µl/mice). After 48 hr. mice were sacrificed by CO₂ inhalation and peritoneal lavages were performed with PBS (3 ml ice-cold PBS was injected and ~90-95 % injected volume was recovered) and infiltrating total cells and eosinophils were analyzed by flow cytometry (S3 Fig) and sCD48 by ELISA in peritoneal lavages.

In another set of experiments to check the role of sCD48 *in vivo*, recombinant murine CD48 (sCD48; Icosagen AS, Estonia) (2.5 µg/200 µl/mice) or PBS (200 µl/mice) were injected ip., and 30 min after SEB (1 µg/200 µl/mice) or PBS (200 µl/mice) was injected ip. Mice were sacrificed 48 hr. after second ip. injection (SEB or PBS) and peritoneal lavages were performed as above; then serum and spleens were collected. Spleen weight was recorded. Lavages were centrifuged (7 min, 300 g, 4 °C) and supernatants were saved (-20 °C). Cells were re-suspended in PBS containing 0.1 % BSA and 5 % goat serum (333 µl/ml recovered lavage) and 75 µl of the cell suspension was diluted up to 200 µl and total events were recorded in BD FACSCalibur for 30 seconds at medium flow rate to count the total cell

numbers. Granulocytes, mononuclear cells and lymphocytes were identified by scatter properties and their absolute numbers and percentage were evaluated by specific gating (Fig. S4A). To determine specific cell populations and receptor expression, cells were then incubated with 2 µg/mL of either the specific conjugated Ab [Siglec-f (clone E50-2440, BD Biosciences, New Jersey, USA) and CCR3 (clone REA122, Miltenyi Biotec, Shoham Israel) for eosinophils (Fig. S4B); IgE (clone MEB-38, Biolegend, San Diego, USA) and c-Kit (clone 2B8, eBioscience, San Diego, USA) for mast cells (Fig. S4C); Lamp-1/CD107a (clone eBio1D4B, eBioscience, San Diego, USA) for cell activation] or the appropriate isotype-matched control Rat IgG1, kappa (Biolegend), Rat IgG2a kappa and Rat IgG2b kappa (eBioscience), clone REA293 (Miltenyi Biotec), and analyzed by flow cytometry as above. Ten µl of re-suspended cells from each sample was diluted to 100 µl and cytopspined onto slides (5 min, 1250 rpm, RT). The cells were then fixed and stained using a Giemsa modified preparation (Diff Quik, Sigma Aldrich, Rehovot, Israel). Slides were examined and images were acquired by light microscopy using Nikon Ti microscope (Nikon Corporation, Japan). sCD48 and TNF-α were quantitated in peritoneal lavages and serum by commercially available mouse CD48 ELISA kit (K4-003-096, Icosagen AS, Estonia) and mouse TNF-α ELISA kit (900-K54 PeproTech Asia, Rehovot, Israel) respectively according to the manufacturers' instructions.

Statistical analysis

All experiments were done at least three times unless otherwise stated. Experiments on human eosinophils were repeated always on the cells obtained from different donors. *In vitro* experiments were done always in triplicates and *in vivo* with 5 mice in each treatment group. The data were analyzed using GraphPad Prism software and statistical significance was determined using a two-tail unpaired Student's "t-test" between two groups and a "p" value of <0.05 was considered significant.

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Supplementary Figures and Legends

Supplementary Figure S1

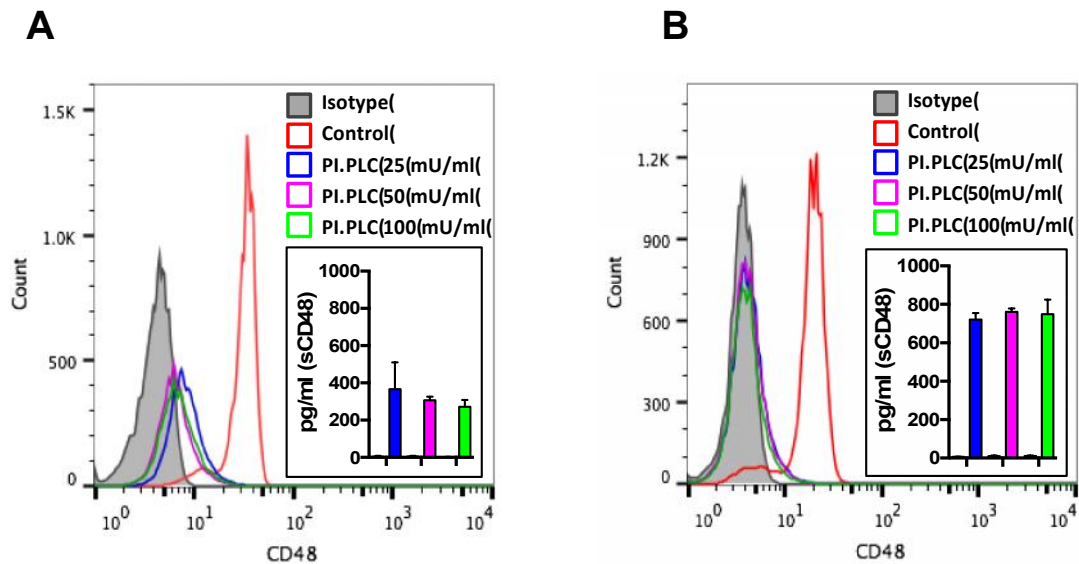


Figure S1. Cleavage of mCD48 from eosinophils by phosphatidylinositol-specific phospholipase-C (PI-PLC). (A) mCD48 expression (histogram plot) on eosinophils incubated with PI-PLC (25, 50 and 100 mU/ml) or control for 1 hr., and (B) for 18 hr. inset box (bar graph) in each histogram plot showing sCD48 in supernatants from same cells. Key – gray shaded histogram: eosinophils stained with isotype matched Ab, red line: cells incubated without PI-PLC as control, blue line: cells incubated with 25 mU/ml of PI-PLC, magenta line: cells incubated with 50 mU/ml of PI-PLC and green line: cells incubated with 100 mU/ml PI-PLC. The same color key was followed for the inset bar graph of sCD48 in each panel. The data are representative of n=3.

Supplementary Figure S2

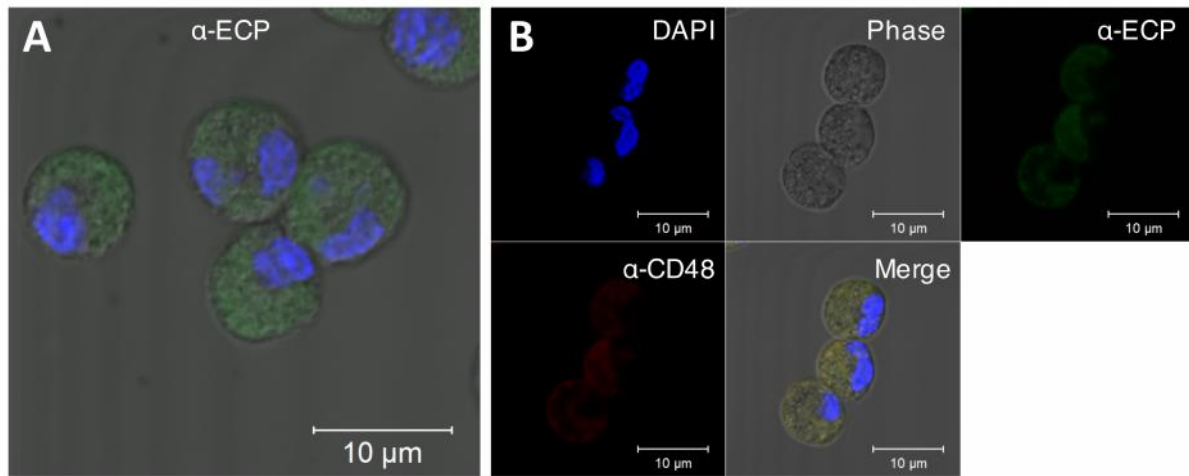


Figure S2. Confocal micrographs showing intracellular CD48 is colocalized with eosinophil granule protein ECP. Eosinophils stained with (A) anti-ECP Ab (B) both anti-ECP and anti-CD48 as each channel separately and labeled and also the merged one. Nuclei were stained with DAPI (blue) and scale bar is 10 μ m. Data are representative of n=3 experiments done on eosinophils obtained from different donors.

Supplementary Figure S3

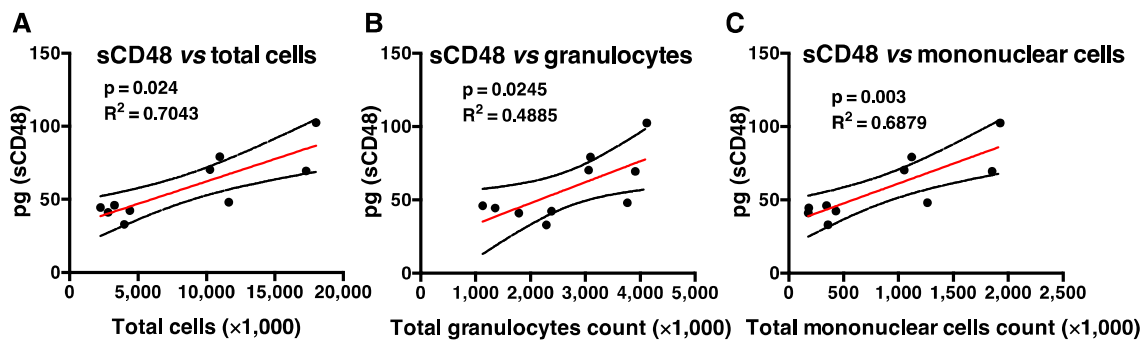


Figure S3. sCD48 correlation with absolute counts of infiltrated cells present in peritoneum. Linear regression analysis of sCD48 with infiltrated (A) total cells, (B) total granulocytes and (C) total mononuclear cells in the peritoneum showing correlation.

Supplementary Figure S4

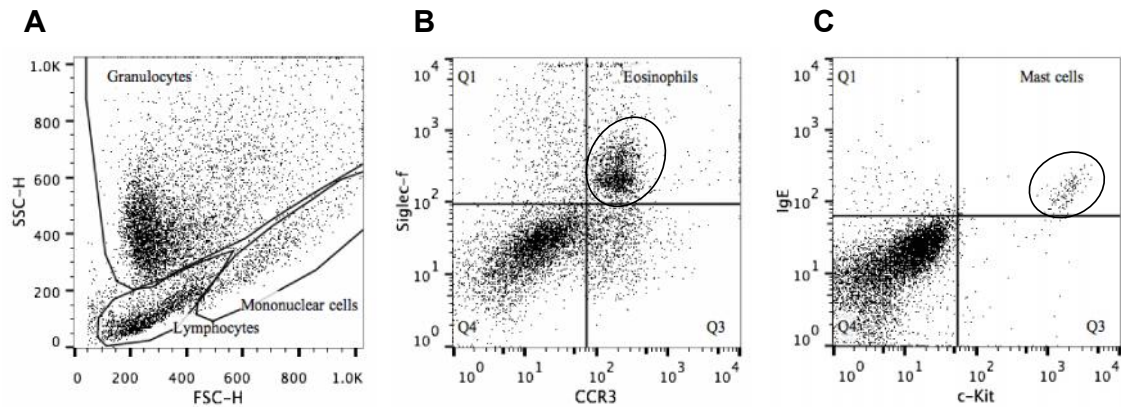


Figure S4. Physical parameters and gating strategy to identify different cell populations by flow cytometry. (A) FSC and SSC parameters to identify the granulocytes, lymphocytes and mononuclear cells, **(B)** Eosinophils were identified as Siglec-F and CCR3 double positive and **(C)** mast cells were identified by IgE and C-kit double positive.

Supplementary Table S1**Table S1. EPO and IL-8 release from SEB incubated eosinophils**

Donor	Released EPO (ng/ml) (40 min)			
	Medium incubated	SEB incubated	sCD48 incubated	(sCD48+SEB) incubated
#1	230.60 ± 12.49	312.60 ± 7.21	223.27 ± 8.33	307.27 ± 22.30
#2	480.00 ± 25.57	643.33 ± 19.50	530.00 ± 17.71	220.00 ± 7.07
#3	732.22 ± 18.36	1037.78 ± 35.59	530.00 ± 8.82	767.78 ± 28.28
#4	153.33 ± 6.81	331.00 ± 24.04	160.67 ± 7.07	162.00 ± 10.58
Released IL-8 (pg/ml) (18 hr.)				
#1	126.67 ± 10.50	313.67 ± 24.34	102.33 ± 12.06	220.67 ± 37.87
#2	219.24 ± 12.01	252.00 ± 24.27	131.67 ± 10.97	215.33 ± 15.57
#3	282.89 ± 16.46	483.67 ± 66.37	366.00 ± 22.52	375.00 ± 18.03
#4	137.00 ± 4.00	237.00 ± 6.10	220.33 ± 6.86	125.67 ± 5.14
#5	272.16 ± 5.77	555.67 ± 9.87	296.33 ± 6.11	407.00 ± 18.00

Eosinophils from different donors were incubated with SEB that was pre-incubated with or without sCD48: absolute values are given from each of the donors.