

Interacting mast cells and eosinophils acquire an enhanced activation state *in vitro*

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Abstract

Background: Mast cells (MCs) and eosinophils (Eos), the key effector cells in allergy, are abundantly co-localized particularly in the late and chronic stages of allergic inflammation. Recent evidence has outlined a specialized 'allergic effector unit' in which MCs and Eos communicate via both soluble mediators and physical contact. However, the functional impact of this bi-directional crosstalk on the cells' effector activities has not yet been revealed. We aimed to investigate whether MC/eosinophil interactions can influence the immediate and late activation phenotypes of these cells.

Methods: Human and murine MCs and Eos were co-cultured under various conditions for 1–2 h or 1–3 days, and in selected experiments cell–cell contact was blocked. Cell migration and mediator release were examined, and flow cytometry was applied to stain intracellular signaling molecules and surface receptors.

Results: Eosinophils enhanced basal MCs mediator release and co-stimulated IgE-activated MCs through physical contact involving CD48–2B4 interactions. Reciprocally, resting and IgE-stimulated MCs led to eosinophil migration and activation through a paracrine-dependent mechanism. Increased phosphorylation of activation-associated signaling molecules, and enhanced release of tumor necrosis factor α , was observed in long-term co-cultures. Eosinophils also showed enhanced expression of intercellular adhesion molecule 1, which depended on direct contact with MCs.

Conclusions: Our findings reveal a new role for MC/eosinophil interplay in augmenting short- and long-term activation in both cells, in a combined physical/paracrine manner. This enhanced functional activity may thus critically contribute to the perpetuation of the inflammatory response in allergic conditions.

For decades, mast cells (MCs) and eosinophils (Eos) have been regarded as the dominating cells in allergic inflammation (1–6). In hypersensitivity reactions, these two cells are mostly recognized for their individual roles in orchestrating the acute and late phases of the response: Allergen-induced activation of tissue-dwelling MCs through Fc ϵ RI leads to their immediate degranulation and release of preformed and newly synthesized mediators, while Eos are subsequently recruited and activated, inducing successive inflammatory events and, together with the MCs, also mediating tissue remodeling (1–6).

It is well accepted that late/chronic allergy involves both MCs and Eos, yet less is defined about the manner by which these two cells co-function to perpetuate the response. Consid-

ering their abundance and persistence in advanced inflammatory stages, MCs and Eos may cross-talk in a paracrine manner (7, 8). Moreover, we recently uncovered a novel specialized mode of communication between the two cells, via physical contact (9, 10): This interaction is stable, tight, and physiologically relevant, as high rates of MC-Eos coupling were detected in human and murine allergic disorders (9). Cell–cell contact, together with soluble growth factors, was found to mediate an MC-induced increase in Eos viability (9).

Similarly, it is conceivable that the ensemble of soluble/physical interactions between MCs and Eos, termed the Allergic Effector Unit (AEU) (9), affects also their effector activities by facilitating bi-directional exchange of regulatory

information and transfer of signals. Several plausible pathways could enable the cells to modulate each other's functions. Early studies have shown that mediators of MCs affect Eos, and vice versa (7, 8): Eos-derived major basic protein (MBP) can activate MCs in a non-IgE-dependent manner (11–13), and Eos peroxidase (EPO) is taken up by MCs (10, 14). On the other end, tryptase induces Eos influx and release of EPO, interleukin (IL)-6, and IL-8 (15–17). Eosinophils-stimulating properties are attributed to other MC products, that is, platelet activating factor (PAF) (18) and histamine (19). Mast cells might also promote Eos migration by releasing histamine, prostaglandin (PG) D₂, and eotaxin that can activate Eos via the H₄ receptor, the chemoattractant receptor-homologous molecule on Th2 (CRTH₂), and the chemokine receptor CCR3, respectively (19–22). Effector functions may also be regulated by direct MC-Eos interactions: Cell surface molecules implicated in the MC-Eos contact mechanism (i.e., the CD2-family molecule CD48 and its high-affinity ligand 2B4, the adhesion molecules DNAM-1, and Nectin-2) (9, 23–25) convey stimulatory signals in these cells (9, 23, 26, 27). In support of this, the activity of MCs, as well as Eos, is altered following physical contact with other immune cells (28–31).

In view of these diverse cell–cell communication mechanisms, the net outcome of a complex MC-Eos cross-talk in a physiological setting is still unknown. It remains to be seen whether such MC-Eos contacts can indeed enforce meaningful changes on cellular functionality and partake in shaping the allergic response in which they occur. Here, we explored these questions using a customized whole-cell co-culture system employing human/murine-derived MCs and Eos. Both cell types showed short- and long-term functional changes. These findings newly demonstrate that the MC-Eos AEU augments cellular effector functions and suggest that it has a role in intensifying and prolonging the late/chronic stages of allergy.

Methods

Cells and assays

Human cord blood-derived MCs (CBMCs), human peripheral blood Eos (pbEos), murine bone marrow MCs (BMMCs), and murine bone marrow Eos (BMEos) were prepared as described previously in (13, 23, 32–34) (Data S1). Mast cells-Eos conjugates were observed in 1 h multispectral-imaging flow cytometry (MIFC) experiments. In short-term co-culture activation experiments (ca. 1 h), release of β -hexosaminidase (β -hex), tryptase, and EPO was assessed by colorimetric assays, while phosphorylated intracellular proteins and lysosomal-associated membrane protein-1 (LAMP-1) were measured by flow cytometry. Eosinophils chemotaxis was examined in 1.5–3 h co-cultures by transmigration assays. In long-term co-cultures (1–3 days), cytokine release was analyzed by enzyme-linked immunosorbent assay (ELISA), and receptor expression and intracellular signaling were examined by flow cytometry. See Data S1, for detailed procedures.

Co-culture conditions

In most experiments, MC-Eos mixtures were predominantly at the 1 : 1 ratio (unless indicated otherwise). Control monocultures of either MCs or Eos were always set at the exact conditions as the respective co-cultures, to maintain equivalent concentrations and to allow for proper comparison between samples. In selected experiments, MCs and Eos were physically separated by 0.4- μ m transwell inserts (Greiner Bio-One, Frickenhausen, Germany). Other co-culture settings were optimized for every particular assay (Data S1).

Results

Eosinophils undergo chemotaxis toward resting and activated MCs

As MCs release factors that may induce Eos chemotaxis (20–22), we first examined whether, and to what extent, CBMCs would attract pbEos. In a transmigration assay, pbEos migrated significantly more toward IgE-activated CBMCs (a mean 45% of total cell migrating) than toward control medium (17%) or toward resting CBMCs (29%), indicating a chemotaxis effect rather than chemokinesis (Fig. 1A). Interestingly, the migration index in the MC-activated co-culture (showing 2.6-fold higher chemotaxis) was only slightly lower than that obtained by the Eos chemoattractant eotaxin-1, in which a 3.5-fold migration (60%) was found (Fig. 1A). Similar migration rates (47%) were detected also when CBMCs were stimulated through a non-IgE-dependent pathway, using compound 48/80 (Fig. 1A). In murine co-cultures consisting of C57BL/6 (B6)-derived cells, BMEos chemotaxis to dinitrophenyl (DNP)-activated BMMCs (pre-sensitized with IgE anti-DNP) was also significantly higher (69%), as compared to resting BMMCs (17%) or control wells (6%), and resembled migration levels induced by mouse eotaxin-1 (74%; Fig. 1B).

Eosinophils activate MCs in human/mouse co-cultures

Next, we explored the possibility that MC-Eos interactions (9, 10) affect the immediate functional capabilities of these cells. The activation phenotype of MCs under Eos regulation was first tested: CBMCs were mixed with pbEos at various cell/cell ratios (1 : 0.1–1 : 10) for 1 h, and their degranulation was assessed by β -hex release. Peripheral blood Eos increased the basal CBMC release by approximately 5%, particularly when Eos numbers equaled, or were more than, those of MCs (Fig. 2A). IgE-activated CBMCs co-cultured with increasing amounts of pbEos also demonstrated stronger degranulation, approximately 15% higher than that of IgE-stimulated CBMCs in monoculture (Fig. 2A). This co-stimulatory effect peaked at the 1 : 1 co-culture, yet was roughly 10% lower in the high MC/Eos ratios. This significant reduction indicates potential inhibiting processes taking place in Eos-dominating samples. The pbEos-induced boost in CBMC activation was evident also by elevated tryptase release in baseline and IgE-stimulated settings (1 : 1 co-cul-

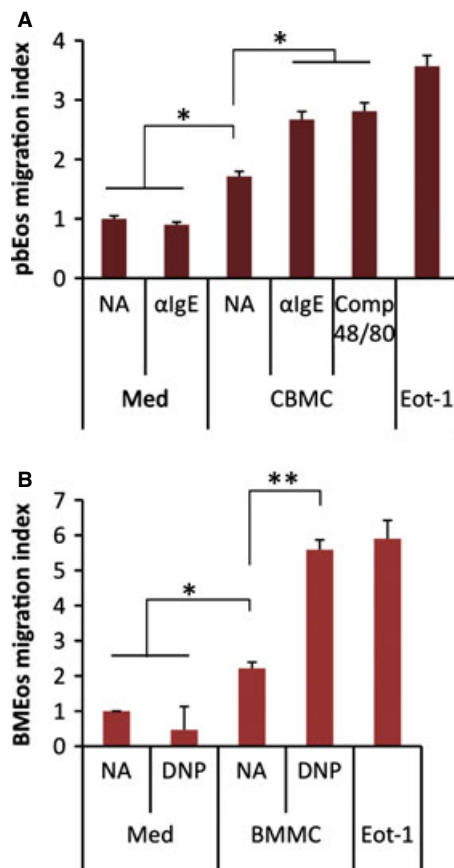


Figure 1 Eosinophil chemotaxis toward resting/activated mast cells. (A) Transmigration of peripheral blood eosinophils (pbEos) toward resting, IgE- or compound 48/80-activated cord blood-derived mast cells (CBMCs). (B) Transmigration of bone marrow eosinophils (BMEos) toward resting or IgE dinitrophenyl (DNP)-activated bone marrow mast cells (BMMCs). In both experiments, controls contain medium alone, with DNP, or with human/mouse eotaxin-1. Eos/MC ratios were 1 : 2. Chemotaxis is expressed as a migration index ($n = 3$, $*P < 0.05$, $**P < 0.005$). NA-not activated.

tures, Fig. 2B). Neither β -hex nor tryptase were detectable in samples containing Eos alone.

In view of this MC degranulation, we hypothesized that signaling molecules typically associated with the early phases of MC responses to stimuli (35) might be also upregulated in our co-cultures. To isolate the Eos contribution to MCs in the absence of IgE stimuli (Fig. 2A), we measured intracellular phosphorylation events solely in the resting CBMC-pbEos co-cultures (1 : 1), for up to 2 h following co-culture onset. No signaling events could be found at any of the early time points (Fig. 2C). However, in 2 h co-cultures, we detected a strong increase in phosphorylation of the cytoplasmic tyrosine kinase Syk, as well as one of its target proteins, linker of activated T cells (LAT) (as evident by a rise in their MFI levels over monocultured cells), suggestive of belated signaling events. Changes in phosphorylation of the Lyn kinase were overall insignificant.

To examine whether these Eos-induced changes in MC activation levels occur also in the mouse system, B6 BMMCs and BMEos were similarly co-cultured. Cell-cell interactions at acceptable frequencies were confirmed (Data S2 and Fig. S1). In 1 h co-cultures with BMEos (1 : 1), BMMCs indeed displayed augmented release of tryptase (Fig. 2D) and β -hex (data not shown). This higher degranulation of co-cultured BMMC was observed in resting conditions, as well as under IgE activation by suboptimal (<30 ng/ml) DNP concentrations (Fig. 2D), but not when BMMC were fully stimulated by high (>30 ng/ml) DNP levels (data not shown). Tryptase was undetectable in BMEos monocultures. BALB/c-derived co-cultures showed similar results (data not shown). Lysosomal-associated membrane protein-1, a molecule associated with BMMC activation (36), was increased in B6- or BALB/c-derived BMMCs already within 30 min of co-culture with BMEos (1 : 1) in resting conditions (Fig. 2E), supporting heightened degranulation in BMEos-stimulated BMMCs.

Eosinophils-induced MC activation requires cell-cell contact via CD48/2B4

We previously identified the interaction between CD48 (on MCs) and 2B4 (on Eos) as one pathway mediating direct MC-Eos contact and modulation of Eos viability in the human AEU (9). To understand whether the CD48-2B4 binding plays a role also in Eos-induced activation of MCs, short-term 1 h co-cultures (1 : 1 ratio) were carried out in receptor-blocking conditions. 2B4-neutralized PbEos lost their ability to augment IgE-activated CBMC degranulation (Fig. 3). Similar Ab neutralization of intercellular adhesion molecule (ICAM)-1 and Nectin-2 [two other Eos molecules that can bind MC receptors lymphocyte function-associated antigen (LFA)-1 and DNAM-1 (23, 24), respectively] also significantly reduced the activation of CBMCs in these co-cultures, albeit to a lower extent (Fig. 3). Blockade of the CD48/2B4 pathway similarly inhibited the BMEos-induced activation of BMMC in the murine co-cultures (see Data S3 and Fig. S2A,B).

Eosinophils are activated by MCs in human/murine co-cultures independently of CD48-2B4-contact

We next tested whether, in a reciprocal pathway, MC can activate Eos. Cord blood-derived MCs (1 h, 1 : 1 ratio) stimulated pbEos to release ca. three-fold more EPO, that is, an estimated 800 ng/ml (47% Eos degranulation) in co-cultures vs. the 317 ng/ml (18% basal release) in control monocultures (Fig. 4A). We observed this effect also across diverse cell/cell ratios (10). To assess whether the CD48-2B4 ligation mediates this increase, CD48 was preneutralized on CBMC. In contrast to the Eos co-stimulation of MCs (above), CD48 blockade did not impair CBMC activation of pbEos (Fig. 4A). Blockade of LFA-1 on CBMC was likewise ineffectual (Fig. 4A). These data indicate a lack of involvement of CD48/2B4 interactions in MC-induced Eos degranulation.

Higher EPO activity was also detected in BMEos co-cultured with BMMC (1 : 1, 1 h) as compared to BMEos alone (Fig. 4B, B6-derived cells). This activity (0.267 OD) corre-

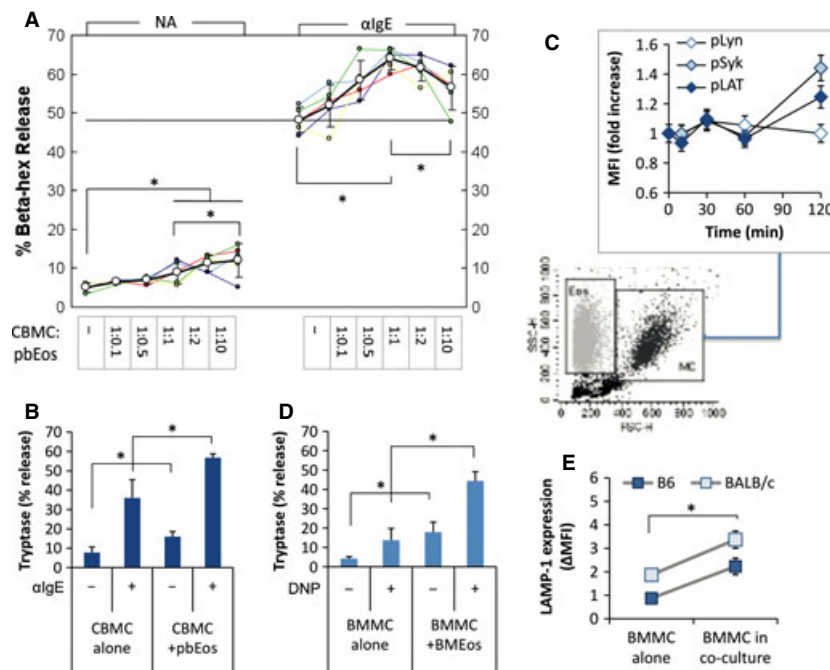


Figure 2 Eosinophil-induced activation of mast cells. (A) Relative β -hex release from resting/IgE-activated cord blood-derived mast cells (CBMCs) in 1 h co-cultures with peripheral blood eosinophils (pbEos) at different ratios ($n = 5$, $*P < 0.05$, different donors shown in colored lines, means $\pm$ O). (B) Tryptase release from resting/IgE-activated CBMCs in 1 h co-cultures with pbEos at a 1 : 1 ratio ($n = 5$, $*P < 0.05$). (C) Phosphorylated molecules in co-cultured

CBMCs (MFI fold-increase, $n = 3$, $*P < 0.05$). Tryptase release (D) and lysosomal-associated membrane protein (LAMP)-1 expression (Δ MFI) (E) in resting/activated bone marrow mast cells (BMMCs) co-cultured (1 : 1) with bone marrow eosinophils (BMEos) ($n = 3$, $*P < 0.05$). No β -hex/tryptase was released by Eos. Data shown vs. control monocultures. NA-not activated.

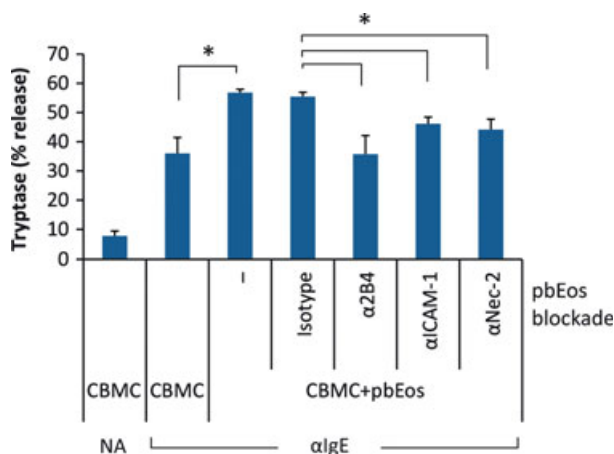


Figure 3 Involvement of the CD48-2B4 axis in eosinophil activation of mast cells. Relative tryptase release from resting/IgE-activated cord blood-derived mast cells (CBMCs) in 1 : 1 co-culture with peripheral blood eosinophils (pbEos), neutralized for 2B4, intercellular adhesion molecule (ICAM)-1, or Nectin-2 ($n = 3$, $*P < 0.05$). Control samples contain monocultures or co-cultures with isotype-treated cells. NA-not activated.

sponded to 660 ng/ml of EPO in co-culture supernatants (i.e., 39% Eos degranulation), an eight-fold increase over monocultured BMEos (0.105 OD; 4% Eos spontaneous release; 75 ng/ml EPO). Bone marrow MCs considerably stimulated BMEos (10% degranulation) even when reducing their amounts up to a quarter of BMEos numbers (Fig. 4B, 1 : 0.25 cell ratios). The MC-induced effect, however, was not further increased by IgE-stimulation: In 1 : 1 ratios, DNP-activated BMMCs (either B6- or BALB/c-derived) stimulated BMEos similarly to resting BMMCs (Fig. 4C). Of note, BMMC-induced BMEos stimulation appeared moderate compared to the potent PAF-induced stimulation that resulted in 82–96% degranulation (Fig. 4C). LAMP-1 upregulation verified that BMEos underwent degranulation (Fig. 4D). Of note, as in the human co-culture, CD48–2B4 contact was not implicated in BMMC activation of BMEos (Data S3 and Fig. S2C).

Mast cells and Eos maintain an activated phenotype in long-term co-cultures

As the AEU is long-lived (9), we sought to examine the long-term consequences of MC-Eos contact. An activated pheno-

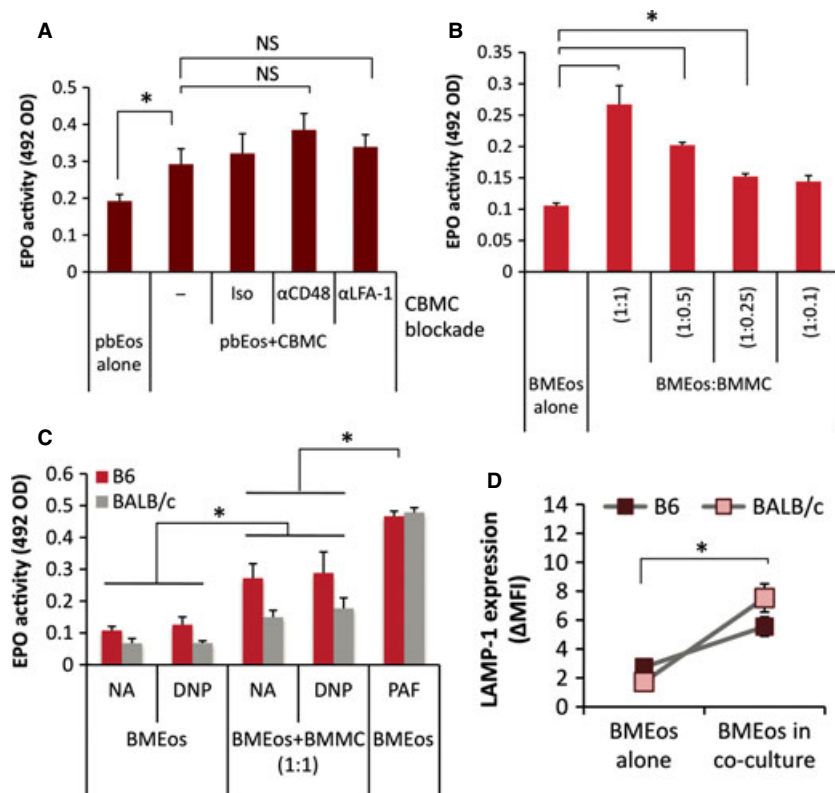


Figure 4 Mast cell-induced eosinophil activation independently of CD48/2B4. Eosinophil peroxidase (EPO) secretion from peripheral blood eosinophils (pbEos) co-cultured (1 : 1) with CD48/lymphocyte function-associated antigen (LFA)-1-neutralized cord blood-derived mast cells (CBMCs) (A), B6 bone marrow eosinophils (BMEos) co-cultured with bone marrow mast cells (BMMCs) at indicated ratios

(B), and BMEos co-cultured (1 : 1) with strain-matched resting/IgE-activated BMMCs (C). (D) Lysosomal-associated membrane protein (LAMP)-1 expression (ΔMFI) in (1 : 1) co-cultured BMEos. Data shown vs. untreated/platelet activating factor (PAF)-activated cells, or isotype-treated co-cultures ($n = 3$, $*P < 0.05$). NA-not activated. ns- not significant.

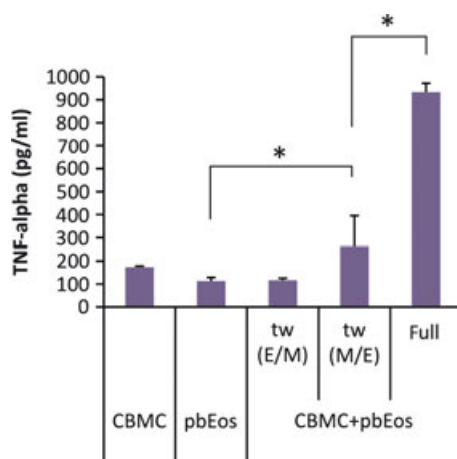


Figure 5 Sustained tumor necrosis factor-α (TNFα) release and activation of mast cells/eosinophils in long-term co-cultures. TNFα release in full-contact vs. transwell (tw) 3-day cord blood-derived mast cell (CBMC)/peripheral blood eosinophils (pbEos) co-cultures (1 : 1). Data are shown vs. monocultured cells ($n = 3$, $*P < 0.05$).

type persisted even in 3-day co-cultures (1 : 1), as shown by elevated tumor necrosis factor (TNF) α levels in co-culture supernatants (greater than eight-fold increase, Fig. 5). No other tested cytokine (IL-6, -8, -10) was modulated in co-culture (data not shown). The increased TNFα secretion, likely by both cells (2, 3), required physical cell-cell contact: Transwell-separated co-cultures yielded only two-fold higher TNFα levels, as observed only in samples where MCs were placed above Eos (Fig. 5).

We also explored surface receptor expression in long-term AEU: No changes were observed in AEU-mediating molecules (CD48, 2B4, DNAM-1, Nectin-2) following a 2-day co-culture (Fig. 6A). Of the screened adhesion molecules, ICAM-1 expression was strongly increased in co-cultured pbEos (Fig. 6A). This CBMC-stimulated elevation of pbEos ICAM-1 was apparent already after 1 day and increased in a time-dependent fashion throughout the 3-day co-culture (Fig. 6B). Interestingly, this effect required direct cell-cell contact because ICAM-1 was unaltered in pbEos within partial transwell-containing co-cultures (Fig. 6C, 48 h). These findings collectively show that long-term AEU interactions sustain MCs and Eos at a high activation level.

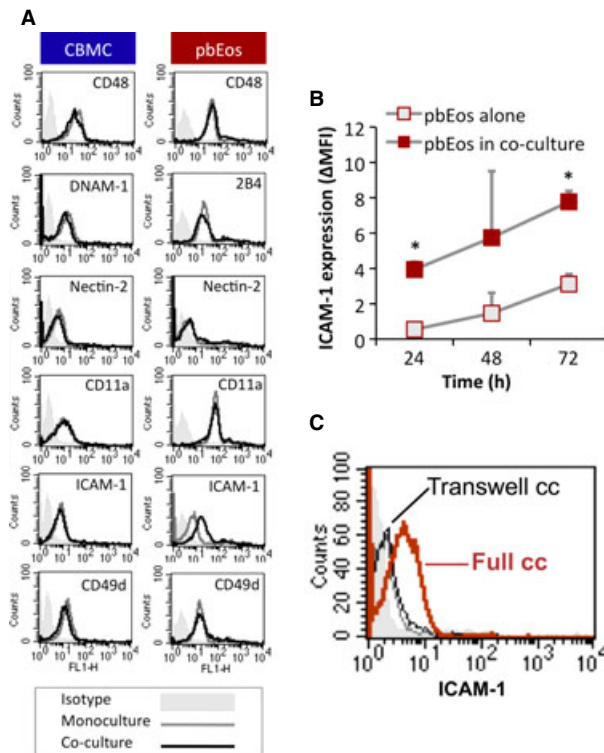


Figure 6 Intercellular adhesion molecule (ICAM)-1 expression on eosinophils in long-term mast cell containing co-cultures. (A) Expression of cell surface molecules in co-cultured cord blood-derived MCs (CBMCs) and peripheral blood eosinophils (pbEos) (2 days, 1 : 1, representative of $n = 4$). (B) ICAM-1 expression (Δ MFI) in 1–3 day co-cultured pbEos ($n = 3$, $*P < 0.05$). (C) ICAM-1 expression in pbEos in full-contact vs. transwell co-cultures (2 days, 1 : 1, representative of $n = 4$). Data are shown vs. monocultured cells, and over isotype-staining controls.

Discussion

In this work, we have begun to define the outcomes of the AEU (9) on MC/Eos effector activities, gaining initial insight into the ability of this unique interface to shape the functions of these key cells in an advanced inflammatory response. Although soluble MC/Eos communication has long been suggested (7, 8), hitherto the evidence for such interactions was largely indirect, as most studies investigated the effect of purified MC/Eos granule constituents, or cell lysates/sonicates, in single-cell cultures. Thus, our findings are the first demonstration that MCs modulate Eos effector functions, and vice versa, using a whole-cell co-culture strategy in both human/mouse systems.

Our observation that Eos migrate toward activated MCs emulates the signals stemming from MCs immediately after allergen exposure, igniting Eos tissue influx (2, 3). Moreover, augmented degranulation of co-cultured MCs and Eos was observed in cell/cell ratios that are physiologically feasible in acute or progressive inflammatory states: Eos activated MCs already at a 1 : 1 ratio, characteristic of initial Eos infiltration states, while MCs induced Eos degranulation even in an

Eos-dominating setting (1 : 0.25 ratio), typical in later stages. However, despite the fact that MCs can activate Eos (and vice versa) by a number of distinct mediators (7, 8), our data do not show overt activation levels; this suggests that the different individual pathways may not necessarily synergize in the ‘whole-cell’ context. Indeed, this is a very reasonable finding: It is plausible that the cells’ mediators, when present in lower, more physiologically relevant concentrations than those used in the purified systems in the past, actually exert a lower activation effect. Another possible explanation for the subtle readouts is that Eos proteolytic factors (also likely released in co-culture) may have degraded MC mediators in the whole-cell system, and vice versa. Finally, we cannot exclude the possibility that inhibition of the cells also occurs, because MC activation was slightly reduced when Eos were ten-fold more frequent. Yet, the collective bi-cellular activation in our whole-cell setting is still very notable.

Interestingly, the activation phenotypes were boosted by the AEU under both resting and IgE-activated conditions. Although pbEos activation of resting CBMCs was subtle as compared to IgE stimulation, pbEos could still further activate IgE-stimulated CBMCs. The contribution of BMEos to BMDC degranulation under IgE-stimulation was more pronounced, because partial IgE-activation by suboptimal DNP was employed in these settings. Eosinophils signals could be additive to IgE stimuli, especially because their mediators (i.e., MBP) are known MC stimulants (13). Eosinophils could also reduce the threshold of MC responsiveness to IgE, that is, conveying co-stimulatory signals integrating into IgE-mediated pathways. Indeed, phosphorylation events (heightened Syk and LAT) in the resting MCs under co-culture suggest that Eos alone ignite activation-associated signaling, albeit perhaps slower and milder than potent IgE stimuli. Taken together, bi-directional MC-Eos activation is likely of complex nature, potentially spanning across a wide time window of inflammation and varying depending on the allergen presence and cellular density. This may allow to maintain a continuously stimulated response throughout the dynamic allergic response.

The CD2 molecules CD48 and 2B4 contribute to immediate activation in MC-Eos co-cultures. Both molecules participate in stimulatory/co-stimulatory signaling in lymphocytes and natural killer (NK) cells (25, 37–40). CD48, a murine asthma signature gene (25, 41) and a potential allergy biomarker in humans (25, 42), is a glycoposphatidylinositol-anchored molecule co-stimulating IgE-dependent secretion of MC inflammatory cytokines (Levi-Schaffer, unpublished data) and mediating MC bacterial uptake (43, 44). Bone marrow MCs lacking glycoposphatidylinositol molecules possess weakened FcεRI signaling and impaired degranulation under IgE stimuli (45). Our data provide further evidence for a co-activatory role for CD48, because 2B4-blocked Eos (that could not ligate CD48) were unable to augment IgE-stimulated human/mouse MCs. In view of the MC-Eos conjugation frequencies in our co-culture snapshots [ca. 1–5%, (9)], a multiphase effect may occur: The MC-Eos couples, activated via 2B4–CD48 contact, degranulate and release mediators, which may then induce further bi-directional activation of other cells which have not yet interacted with each other.

In contrast, the opposite route of MC-induced Eos degranulation did not need CD48–2B4 contact (as CD48-blocked MC properly activated Eos). This was an unexpected finding, because 2B4 ligation has been shown to provoke human pbEos degranulation (27). This discrepancy might be explained by the fact that 2B4 stimulation in the early study was achieved by mAb cross-linking, while the ligand in our system was MC-expressed CD48, the latter possibly less potent. Alternatively, it may be that CD48–2B4 signaling does contribute to MC activation of Eos, yet other soluble/physical mechanisms compensate for its absence in the neutralized co-cultures, ultimately activating Eos despite the blockade. Notwithstanding, a working CD48–2B4 axis is probably important for other late Eos functions: MC induction of Eos survival (9) is partially attributed to 2B4-ligation. Our finding that CD48/2B4 expression is unhindered in long-term co-cultured MCs and Eos also supports this. Collectively, this demonstrates a prominent, yet nonsymmetrical, role for the CD48–2B4 pair in boosting immediate/chronic functions within interplaying MCs and Eos. Specific targeting of the AEU by CD48/2B4 inhibitors may thus be of benefit in allergy.

Other pathways may contribute to MC–Eos bi-directional activation. Mast cells mediators can stimulate Eos, and vice versa (7, 8). Leukotrienes (LTs) produced by activated MCs/Eos may also interact with the cells, as both express LT receptors (1, 3). Beyond the CD48–2B4 axis, other surface adhesion molecules and/or integrins with capacity to boost and extend immunological synapse signals could also partake in the MC–Eos contact-dependent interplay: The binding of MCs DNAM-1 to Eos Nectin-2, for example, has already been implicated in Eos-augmented degranulation of MCs (23), as observed also herein. A similar finding surfaced concerning the ICAM-1-involved pathway, as its blockade on pbEos reduced their ability to co-stimulate IgE-activated CBMCs. Bearing in mind that ICAM-1 augments cytokine-induced activation in Eos (46, 47), it is possible that ICAM-1-neutralized pbEos underwent poorer activation in co-culture, which in turn limited their capability to activate MCs. In this context, it is worth mentioning that ICAM-1/LFA-1 interactions also facilitate T-cell induction of MC degranulation in comparable IgE-involved settings (30, 48, 49).

As MCs and Eos persist in the inflamed tissue for significant periods, during which late-phase/chronic responses surface, AEU long-term phenomena also warranted our investigation. As expected, and in continuance to the boosted activation phenotypes observed in the short-term, late co-cultures demonstrated persisting pro-inflammatory effects. This involved increased TNF α secretion, likely released from both cells (1, 3), in a mechanism necessitating physical MC–Eos binding. Interestingly, TNF α is largely involved in MC induction of recruitment, proliferation and activation of lymphocytes, both *in vitro* and in allergic airway inflammation (50, 51). Thus, by raising TNF α levels, the AEU might have an active part in shaping the characteristic Th2-profile in allergy.

In long-term co-cultures, MCs also raised ICAM-1 expression on Eos. Similar rising levels of the Eos adhesion molecule CD11b were seen following interaction with fibroblasts (52) or endothelial cells (53). Interestingly, here, the MC-

augmented Eos ICAM-1 effect required cell–cell contact, yet the mechanism could entail also paracrine communication between the cells: As histamine elevates ICAM-1 on Eos (19), immediate MC degranulation events in co-culture may contribute to this effect. Notwithstanding, TNF α , released in the long-term MC–Eos co-cultures, can also increase Eos ICAM-1 (54). Communication via this proinflammatory cytokine likely occurs downstream of the critical cell–cell interactions, because transwell MC–Eos co-cultures failed to produce high TNF α concentrations. Moreover, considering that ICAM-1 signaling is linked to prolonged Eos survival (47), the rise in this molecule's levels on co-cultured Eos may be an additional mechanism underlying the MC-induced maintenance of these cells in the long term (9). Concomitantly, this elevated Eos ICAM-1 expression, in tandem with CD48–2B4 binding, could serve to fortify the MC–Eos adhesion within late inflammation phases [as in the case of NK adhesion (55, 56)]. Altogether, these mechanisms could underlie the continuous occupation of active Eos within the inflamed tissue.

The above-described pathways and others, whether via soluble or physical factors, presumably integrate to additively or synergistically enhance MC/Eos activation, forming a 'vicious cycle'. Thus, our results support the notion of a unique self-perpetuating MC/Eos AEU contributing to the persistence of allergic reactions. We speculate that, compared to potent allergen-induced activation, the AEU has a more subtle stimulatory ability, fine-tuning effector activities of MCs/Eos in the heat of active inflammation but also during late/chronic phases in which both cells are abundant. Co-stimulatory signals reduce the critical threshold of cellular activation in the immunological synapse (57–59). By analogy, co-stimulation within the AEU may be exactly what is necessary for an activatory outcome, particularly when allergen stimuli are lacking, or when MCs are temporarily desensitized to allergens. As we have mentioned, inhibiting outcomes are also plausible, as MCs and Eos have inhibitory surface molecules (60) and immunoregulatory properties (1). If such negative signals exist in the AEU, they may be initially overpowered by stimulatory pathways yet come to play later within allergic resolution (61, 62). Future studies should reveal the AEU effects on MC/Eos responsiveness in diverse conditions.

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Author contributions

M.E. designed the research, performed all experiments, analyzed data, and wrote the manuscript; I.B. performed

activation experiments; A.H.N.B.E helped to assemble the co-culture system; D.M. provided cord blood samples; and F.L.S. designed the research, received the grant supports, provided overall supervision, analyzed the data, and edited the manuscript.

Conflict of interest

The authors have no financial relationships to disclose.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supporting Methods.

Data S2. Visualization of the contact between BMMCs and BMEos and measurement of their interaction frequencies *in vitro*.

Data S3. Involvement of CD48-2B4 signaling in BMMC/BMEos bi-directional stimulation.

Figure S1. Physical contact between mouse-derived MCs and Eos *in vitro*.

Figure S2. Receptor expression in mature BMMC and BMEos cultures.

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Supplemental Information

Interacting Mast Cells and Eosinophils Acquire an Enhanced Activation State *in vitro*

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Section A. Supporting Methods*Cell purification and culturing*

Human MCs were prepared from umbilical cord blood of healthy donors according to a previously described method (1-2). Briefly, fresh cord blood diluted in Hank's solution was loaded on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged (350g, 25 min). Mononuclear cells were washed twice and resuspended in MEM α medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 μ g/ml ribonucleases (all purchased from Biological Industries, Israel), and containing recombinant human stem cell factor (SCF; a gift of BioVitrum AB, Stockholm, Sweden; 100 ng/ml) and human IL-6 (Peprotech, Rocky Hill, NJ; 10 ng/ml). Prostaglandin E₂ (PGE₂; Sigma-Aldrich, Israel; 0.3 μ M) was also added to this media regularly, in order to prevent cultivation of possible monocytic contaminants (3). Cells were maintained in a humidified incubator (37°, 5% CO₂) with media replaced on a weekly basis. Following a 6-8 week culture period, the cord blood-derived MCs (CBMCs) were used following examination for maturity by acidic toluidine blue staining (>90%), and for viability by Trypan blue exclusion (>95%). Non-MCs were usually of non-adherent nature, and monocyte-like cells were rarely observed in CBMC cultures.

For purification of human peripheral blood Eos (pbEos), blood donations of mildly atopic non-treated volunteers (with Eos counts of 5%-15%) were

processed as detailed previously (4). Donors were usually asymptomatic and did not require medication. Briefly, samples (150 ml) were left to sediment for 1h in 6% Dextran (Sigma-Aldrich, Israel). Leukocytes were centrifuged on Ficoll-Hypaque (700g, 25 min), and neutrophils and lymphocytes were tagged in the granulocyte-enriched pellet using anti-CD16 and anti-CD3 micromagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), respectively. The pbEos were purified by negative selection via magnetic cell sorting, and the resulting cell suspension was 95-100% pure for Eos (as determined by Kimura staining) and >98% viable (Trypan blue exclusion). Cells were washed at least twice, resuspended in supplemented MEM α media, and used immediately.

Mouse MCs and Eos were cultured from bone marrow (BM) progenitors, as described previously (5-6). BM was obtained from femurs of female C57BL/6 (B6) and BALB/c mice (6-8 weeks old), purchased from Harlan Laboratories and housed under specific pathogen-free conditions. To yield BM-derived MCs (BMMCs), progenitors were placed in RPMI 1640 media (Gibco BRL, Rockville, Maryland) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1X nonessential amino acids (Biological Industries, Israel), and containing recombinant murine SCF and IL-3 (both from PeproTech, Rocky Hill, NJ; 20 ng/ml). Media was replenished on a weekly basis, and cells were cultured for 4-5 weeks. To obtain BM-derived Eos (BMEos), cells were placed in RPMI 1640

media supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, 1X nonessential amino acids, 2mM glutamine (all from Biological Industries, Israel), and 50 mM β-mercaptoethanol (Invitrogen). The media, which was replaced every other day, contained recombinant murine SCF and Fms-like tyrosine kinase 3 (FLT3) ligand (both from PeproTech, Rocky Hill, NJ; 100 ng/ml) from days 0–4, and recombinant mouse IL-5 (R&D Systems, Israel; 10 ng/ml) from day 5 and on. Cells were cultured for at least 12 days, and were not used beyond day 21. Prior to experimentation, the BM-derived cells were assessed for >95% viability (by Trypan blue exclusion) and for >90% maturity by acidic toluidine blue staining (BMMC), Giemsa-staining in cytopsin preparations (BMEos), and expression of characteristic surface markers (cKit and FcεRI in BMMC; Siglec-F and CCR3 in BMEos) by flow cytometry.

All human blood samples were collected following ethical approval of the Hadassah-Hebrew University Human Experimentation Helsinki Committee, and written informed consent was obtained according to its guidelines. Experiments involving murine cells were approved by the Animal Experimentation Ethics Committee of the Hebrew University of Jerusalem.

Transmigration assays

Chemotaxis of pbEos and BMEos towards diverse stimuli was measured using 8.0- and 5.0-micron polycarbonate membrane Transwell inserts,

respectively, that were placed in 24-well insert-containing polystyrene plates (Costar, Corning, NY) similar to prior studies (5, 7-8). Lower chambers were first loaded with 600 μ l of the negative controls (media alone or with anti-IgE stimuli), positive controls (the migration stimulants human eotaxin-1, Peprotech, Rocky Hill, NJ; 30 ng/ml, or mouse eotaxin-1, R&D Systems, Israel; 300 ng/ml), or MC-suspensions. In the latter, IgE-sensitized CBMCs or BMMCs (prepared as detailed for mediator assays, see below) were placed at a 2-fold higher cellular amount as the Eos, in order to achieve a 1:2 Eos:MC ratio. In activated samples, stimulation was induced by 5 μ g/ml rabbit anti-IgE in CBMCs, or 30 ng/ml dinitrophenyl (DNP) in BMMCs, and selected samples of CBMCs were also activated by compound 48/80 (Sigma Aldrich, Israel; 1 μ g/ml) in an IgE-independent manner. The plates were immediately put in a humidified incubator (37°, 5% CO₂) for 30 min, to allow the relevant MCs to undergo activation. Subsequently, Eos (resuspended in their media at 10⁶ cells/ml) were loaded (100 μ l) in the upper chambers in all wells. To ensure no significant unidirectional migration of Eos, chemokinesis was evaluated by assessing random Eos migration in control media (i.e. in absence of any stimulant). Additional controls containing eotaxin-1 in both upper and lower chambers (i.e. in absence of a gradient of the stimuli), as applied previously (5, 7-8), confirmed chemokinesis as negligible. The plates were placed in the incubator for 1.5h (pbEos) or 3h (BMEos). After gently removing the inserts, the cell suspensions in the lower chamber were collected and processed by flow cytometry. To enable quantification of solely pbEos populations (thus disregarding CBMCs in co-culture), only FSC^{low}/SSC^{high} events were counted. For BMEos isolation, we counted only

Siglec-F-positive events following a staining procedure applied to the samples (see flow cytometry). Data are reported as a migration index, expressing the ratio of the number of cells migrating in response to stimuli, relative to the number of cells migrating in response to media alone (8).

MC activation systems

For activation of CBMCs, human myeloma IgE (Calbiochem-Merck, Schwalbach, Germany; 1 $\mu\text{g/ml}$) was used for sensitizing the cells for 4-5 days in a humidified incubator (37°, 5% CO_2). Subsequently, cells were washed twice and resuspended in cold Tyrode's gelatin buffer (9). For BMBC activation, cells were sensitized for 24h with mouse IgE anti-DNP at a concentration of 0.5 $\mu\text{g/mL}$ (Sigma-Aldrich, Israel), washed twice and resuspended with cold Tyrode's buffer (10). Human and mouse MCs were seeded in 96-well (Nunc) plates (50 $\mu\text{l/well}$, at a final concentration of 4×10^6 cells/ml). In co-culture wells an additional 50 μl of the human/mouse Eos suspension (4×10^6 cells/ml) was placed, while MC monocultures were completed by 50 μl buffer. Thus, all samples contained a final 100 μl volume per well. In co-cultures with varied cell-cell ratios, the concentration of the inserted Eos was determined based on the indicated ratio, and only the Eos (but not MC) numbers differed between samples, to enable adequate comparison between activation levels in monocultured and co-cultured MCs. In receptor-neutralizing experiments in the human system, pbEos were first incubated in MEM α media containing 1% FcR blocking reagent (Miltenyi Biotech, Germany) and 10% heat-inactivated fetal bovine serum for 10 min,

on ice. The cells were then washed once, and treated (for 20 min on ice) with a specific Ab. Blocking Abs included the following: mouse mAb to human 2B4 (clone C1.7, eBioscience; 1 μ g/ml), intercellular adhesion molecule (ICAM)-1 (CD54; Biolegend, San Diego CA; 1 μ g/ml), and Nectin-2 (mouse hybridoma, a gift of Dr. A. Moretta, Genoa, Italy; a 1:10 dilution). Control cells were pretreated with a matched isotype Ab (mouse IgG1, R&D Systems, Israel; 1 μ g/ml). For receptor neutralization in the mouse system, BMEos were incubated in RPMI media with hamster mAb to mouse CD48 (clone HM48-1, Biolegend, San Diego CA; 1 μ g/ml) and/or rat mAb to mouse 2B4 (clone C1.9, eBiosciences; 1 μ g/ml), or treated with control isotype Abs (hamster and rat IgG, eBiosciences; 1 μ g/ml each). Following Ab-blockade, the cells were washed twice, resuspended and added to the indicated co-cultures.

In relevant samples, a rabbit anti-human IgE Ab (Dakocytomation; 5 μ g/ml) or dinitrophenylated human serum albumin (DNP-HSA; Sigma-Aldrich, Israel; a suboptimal concentration of 5 ng/ml) was added respectively. The stimulant was inserted 5-10 min after co-culture onset, to allow for effective engagement and initiation of cross-talk between the two cells. Plates were kept in a humidified incubator for 1h, until analysis of mediator release (see below) and lysosomal-associated membrane protein (LAMP)-1 expression (see below).

Eos activation systems

In experiments assessing activation of pbEos and BMEos, cells were washed twice in PBS containing 10% heat-inactivated fetal bovine serum, and seeded in 96-well (Nunc) plates (50 μ l/well in a 4×10^6 cells/ml concentration). In co-culture wells an additional 50 μ l of the human/mouse MC suspension (4×10^6 cells/ml) was placed, while Eos monocultures were completed by 50 μ l buffer. Thus, all samples contained a final 100 μ l volume per well. In co-cultures with varied cell-cell ratios, the concentration of the inserted MCs was determined based on the indicated ratio, and only the MCs (but not Eos) numbers differed between samples, to enable adequate comparison between activation levels in monocultured and co-cultured Eos. In receptor-neutralizing experiments in the human system, CBMCs were first incubated in MEM α media containing 1% FcR blocking reagent (Miltenyi Biotech, Germany) and 10% heat-inactivated fetal bovine serum for 10 min, on ice. The cells were washed once, and then treated (for 20 min on ice) with the specific Abs against CD48 (clone MEM108, Biolegend, San Diego CA; 1 μ g/ml) or lymphocyte function-associated antigen (LFA)-1 (CD11a; Biolegend, San Diego CA; 1 μ g/ml), or a matched isotype control Ab (mouse IgG1, R&D Systems, Israel; 1 μ g/ml). For neutralizing receptors in the mouse system, BMMC were incubated in RPMI media with hamster mAb to mouse CD48 (clone HM48-1, Biolegend, San Diego CA; 1 μ g/ml) and/or rat mAb to mouse 2B4 (clone C1.9, eBiosciences; 1 μ g/ml), or treated with control isotype Abs (hamster and rat IgG, eBiosciences; 1 μ g/ml each). Following Ab-blockade, the cells were washed twice, resuspended and added to the indicated co-cultures.

In relevant samples, PAF (Sigma-Aldrich, Israel) was added at 5 μ M, 5-10 min after co-culture onset. In mouse samples also containing sensitized BMMCs, DNP was added 5-10 min after starting the co-culture, as detailed above. Activation was initiated by placing the samples in a humidified incubator (37°, 5% CO₂) for 1h, until analysis of mediator release (see below) and LAMP-1 expression (see below).

Mediator and cytokine release assays

Secretion of MC mediators was evaluated by previously described chromogenic assays for β -hexosaminidase (β -hex) and tryptase (9, 11). Activation was terminated by two-step centrifugation of the plate at 250g (4°C) and separation of supernatants from cells. The cellular fraction was lysed for 15 min in Tyrode's solution containing 0.1% Triton X-100 (Sigma-Aldrich, Israel). Both fractions were snap-frozen for further analysis. For measuring release of pre-formed β -hex, 18 μ l of a citric acid- based solution containing the substrate *p*-nitrophenyl-*N*-acetyl- β -d-glucopyranoside (Sigma-Aldrich, Israel; 8 mM) was mixed with 42 μ l of the indicated sample for 2h, and the reaction was stopped with 120 μ l of ice-cold glycine buffer (0.2M, pH=10.7]. Optical density (OD) was measured at 405 nm in an ELISA plate reader. For obtaining tryptase levels, the activity of this enzyme was assessed by mixing 48 μ l of the indicated sample with 2 μ l of a specific substrate solution containing *N*-*p*-tosyl-gly-pro-lys-*p*-nitroanilide (Sigma Aldrich, Israel; 25 mM) in 100% dimethyl sulfoxide. The 96-well plate was incubated at 37°C until chromogenesis, and OD was measured at 410 nm. After

subtracting background readings in empty wells, the OD values were used for calculating the percentage release of β -Hex and tryptase relative to total cell content, as given by the formula $100 \times [\text{supernatant} / (\text{lysate} + \text{supernatant})]$.

For detecting EPO release, we used a previously developed enzymatic assay (12) applicable for both human and mouse-derived Eos (5, 12): Cell activation was terminated by placing the samples on ice for 5 min. A reagent containing the peroxidase substrate o-phenylenediamine (Sigma-Aldrich, Israel; 0.8 ml, 5 mM) was added to Tris buffer (4ml, 1M, pH=8), water (5.2 ml), and 30% H_2O_2 (1.25 μl), and the solution was added to the samples (100 μl /well) at the end of activation. Sulfuric acid (2M, 100 μl /well) was used for stopping the reaction, and OD was measured at 492 nm. Data are reported following subtraction of background readings in empty wells. To assess EPO concentrations, we used purified human EPO (generously provided by Dr. G. J. Gleich, University of Utah) to generate a standard curve in each experiment. The extent of Eos degranulation was also assessed in each experiment: Based on the OD values in the tested samples, and the values in separate Eos lysates (prepared by incubating the same amount of cells in similar PBS volumes containing 0.1% Triton X-100), we estimated the percentage release of EPO relative to total cell content, as given by the formula $100 \times [\text{supernatant} / (\text{lysate})]$.

To assess tumor necrosis factor (TNF) α levels, CBMCs and pbEos were co-cultured at 1:1 (total 2×10^6 cells/ml) in MEM α media containing 100 ng/ml SCF and 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-

CSF; Peprotech, Rocky Hill, NJ), alongside their respective monoculture controls, in 24-well (Nunc) plates for 3 days. In selected co-cultures, 0.4-micron transwell inserts (Greiner Bio-One, Frickenhausen, Germany) were placed to prevent physical contact, where MCs were in the upper chamber and Eos in the lower, or vice versa to test both possibilities. At the end-point, samples were centrifuged at 250g (in 4°C), and supernatants were separated from cells and frozen at -30°C. TNF α was measured in thawed supernatants using a commercial ELISA kit (Peprotech, Rocky Hill, NJ), where samples were evaluated in triplicates, and OD was measured at 405 nm in an ELISA microplate reader. The lower detection limit was 32 pg/ml.

Intracellular molecule staining by flow cytometry

CBMCs and pbEos (1:1, a total 2×10^6 cells/ml concentration) were mixed in MEM α media containing SCF and GM-CSF (100 ng/ml and 20 ng/ml, respectively), in 24-well (Nunc) plates, for up to 2h. Control monocultures were held at similar conditions. At the stated time points, samples were washed with cold HBA buffer (0.1% bovine serum albumin in Hanks' solution) and fixed for 20 min (2% formaldehyde in cold HBA). Permeabilization was then carried out for 20 min using an ice-cold 90% methanol solution, followed by a blocking stage for 15 min in HBA containing 5% goat serum, and one wash in HBA. Cells were then stained for 1h using rabbit anti-human phospho-syk (ZAP70), phospho-LAT, and phospho-Lyn Abs (Cell Signaling, Beverly, MA; 1:100 dilution) or a rabbit IgG control Ab (Jackson Laboratories, West Grove, PA), followed by a 1h

incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ab (Jackson Laboratories, West Grove, PA). Each Ab-incubation step was followed by two washes in HBA. Data were acquired in a BD Biosciences FACScalibur and analyzed using CellQuest software. For examination of separate staining in each cell population, human MC and Eos were gated by their different physical parameters (pbEos being FSC^{low}/SSC^{high} as compared to CBMCs). Data are presented as the fold-increase in mean fluorescence intensity (MFI) between compared samples.

Surface molecule staining by flow cytometry

CBMCs and pbEos (1:1, a total 2×10^6 cells/ml concentration) were mixed in MEM α media containing SCF and GM-CSF (100 ng/ml and 20 ng/ml, respectively), in 24-well (Nunc) plates, for 1, 2, and 3 days. Control monocultures were held at similar conditions, and in selected co-cultures, 0.4-micron transwell inserts (Greiner Bio-One, Frickenhausen, Germany) were placed to prevent physical contact. At the indicated time points, samples (cultures and co-cultures) were washed with cold HBA buffer (0.1% bovine serum albumin in Hanks' solution), and blocked for 15 min in a 5% goat serum HBA buffer in order to eliminate non-specific binding. An indirect 2-step immunofluorescence protocol was used for staining CBMCs and pbEos: samples were incubated for 1h on ice in the presence of the primary mouse anti-human Abs against CD48, ICAM-1, CD11a, CD49d (Biolegend, San Diego CA; 1:100 dilution), 2B4 (C1.7, eBiosciences; 1:100), DNAM-1 and

Nectin-2 (mouse hybridomas provided by Dr. A. Moretta, Genoa, Italy; 1:10), as well as the relevant isotype control Abs. Secondary antibody incubations were carried out for 1h using a FITC-conjugated goat anti-mouse Ab (Jackson Laboratories, West Grove, PA; 1:300 dilution). Cells were washed twice in HBA after each Ab-incubation. For separate analysis of each cell population, CBMCs and pbEos were gated by their different physical parameters (Eos being FSC^{low}/SSC^{high} as compared to MCs). Data were acquired in a FACScalibur and analyzed with CellQuest. Selected data are presented as Δ of MFI, representing the difference between the specific Ab staining and the isotype-matched control Ab.

To stain murine BMMC and BMEos, mature cells (<week 4 in BMMCs; >week 2 in BMEos) were washed twice in cold HBA and blocked in a 5% goat serum HBA (15 min) to eliminate non-specific binding. Cells were then labeled in direct immunofluorescence single-staining using the following reagents: phycoerythrin (PE)-conjugated rat anti-mouse Siglec-F (BD Pharmingen, Israel), PE-hamster anti-mouse CD48 (HM48-1, Biolegend, San Diego CA), FITC-conjugated rat anti-mouse Fc ϵ RI (eBiosciences), FITC-rat anti-mouse CCR3 (R&D Systems, Israel), FITC-rat anti-mouse cKit (CD117; eBiosciences), and FITC-rat anti-mouse 2B4 (eBiosciences). Relevant isotype control Abs were used appropriately. In the mouse activation experiments (see described above), monocultured/co-cultured BMMC and BMEos were obtained following the activation time point, washed and blocked as above, and subsequently double-stained with a PE-conjugated rat anti-mouse Ab against LAMP-1 (CD107a; eBiosciences) and allophycocyanin (APC)-

conjugated rat anti-mouse cKit (CD117; eBiosciences). The latter Ab allowed for effective gating of cKit⁺ BMMCs from cKit⁻ BMEos. In the mouse transmigration studies (see described above), monocultured/co-cultured BMMC and BMEos were obtained following the activation time point, washed and blocked as above, and subsequently stained by a PE-conjugated rat anti-mouse Siglec-F Ab, for effective gating of Siglec-F⁺ BMEos from Siglec-F-BMMC. All above Abs for the murine system were applied at manufacturer-recommended dilutions in an HBA buffer, and the incubation was followed by two washes in HBA. Data were acquired in a FACScalibur and analyzed with CellQuest software. Data are presented as Δ MFI.

Multispectral imaging flow cytometry (MIFC)

Visualization and imaging of BMMC-BMEos conjugation were carried out as in our earlier studies (13), based on a protocol calibrated for investigating immunological synapses (14). BMMC were pre-labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Invitrogen; 1 μ M) according to the manufacturers instructions, washed extensively, and mixed (1:1) with BMEos to a total of 10^7 cells/ml, in supplemented RPMI media. Control monocultures were held at similar conditions. Samples were briefly microfuged, incubated for up to 30 min in a humidified incubator (37°, 5% CO₂), and then fixed with a paraformaldehyde solution (4% in PBS). Data were acquired by an MIFC instrument (AMNIS Inc., Seattle WA) collecting 4000 cells per sample, and results were analyzed

with IDEAS 3.0 software. Cell-cell conjugates were gated as low nuclear aspect ratio events (14), and aggregates as high-area events. CFSE-intensity was used for distinguishing conjugate populations: CFSE^{high} pairs were homotypic BMMC-BMMC couples, CFSE-negative pairs were homotypic BMEos-BMEos couples, and CFSE^{low} events were heterotypic BMMC-BMEos couples. Each gated population was individually observed for side-scatter and bright-field parameters, in order to eliminate cell debris and irrelevant events.

Statistical analysis

All experiments were carried out at least three times, and data is reported as mean $\pm$ -SDs. Data analysis was performed by paired Student's t-test assuming equal variances, where P values <.05 were considered significant.

Section B. *Visualization of the contact between BMMCs and BMEos and measurement of their interaction frequencies in vitro*

To examine whether BMMCs and BMEos interact in a similar manner as we had first characterized in our human co-culture system (13), the two cell-types were co-cultured at a 1:1 ratio for a brief period and examined by MIFC.

Similar to our earlier study, the mouse derived cells coupled (Fig. E1A, first two images) at notable frequencies (Fig. E1B), where heterogenous MC-Eos conjugates were significantly more abundant than the homotypic interactions in each cell type. Multiple-cell aggregates were also seen (Fig. E1A, last two images), and the rate of such complexes was also higher in the co-cultures than in the control monocultures (Fig. E1C).

Of note, comparable interaction frequencies have been found in other studies of cell-cell coupling and aggregation using MIFC (see for example (15)). Yet in our previous examination of human co-cultures (13), dynamic follow-up of these MC-Eos co-cultures by microscopy over 15 min revealed many cells interacting and re-interacting (data not shown), suggesting that the actual contact frequencies are higher than these MIFC-produced “snapshots”.

Considering that these interaction levels reflect a resting unprimed setting, binding frequencies in inflamed tissues likely exceed these values (as we have initially observed in chronically diseased murine tissues, (13)).

Section C. *Involvement of CD48-2B4 signaling in BMMC/BMEos bi-directional stimulation*

In the human co-cultures, we found a role for the CD48-2B4 interaction between MCs and Eos in mediating Eos-induced stimulation of MCs, but not vice versa (see above). Reciprocal activation of MCs and Eos was found also in mouse co-cultures (see above). Thus, we investigated whether the CD48-2B4 contact mechanism is equally influential in the mouse system. We first examined the B6-derived BMEos and BMMCs for their characteristic receptors, as well as for CD48 and 2B4. BMEos uniquely expressed CCR3 and Siglec-F, whereas BMMC uniquely displayed c-Kit and FcεRI. Of note, FcεRI was not found on BMEos, in agreement with past studies in human and mouse Eos of diverse sources (16-18). Interestingly, both BMMCs and BMEos significantly expressed CD48 and 2B4 (Fig. E2A). In light of this, we reasoned that bi-directional CD48-2B4 ligation between MCs and Eos, i.e. via two possible routes, can exist. BMEos were therefore pretreated with Abs against both 2B4 and CD48, resulting in significant, but only partial, impairment of their BMMC-stimulating function (Fig. E2B). On the other hand, blockade of the CD48 and 2B4 receptors on BMMCs did not hinder the high EPO release detected in BMEos co-cultured with these BMMCs (Fig. E2C). These data confirm the findings in the human setting (see above), whereby the CD48-2B4 axis appears an important player in Eos-induced activation of MCs, but not in MC-activation of Eos.

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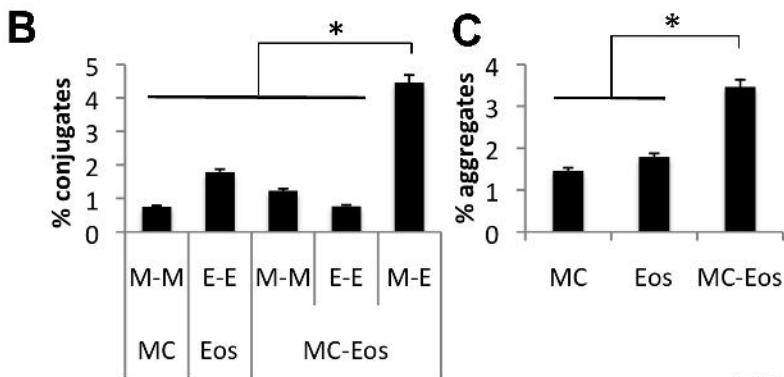
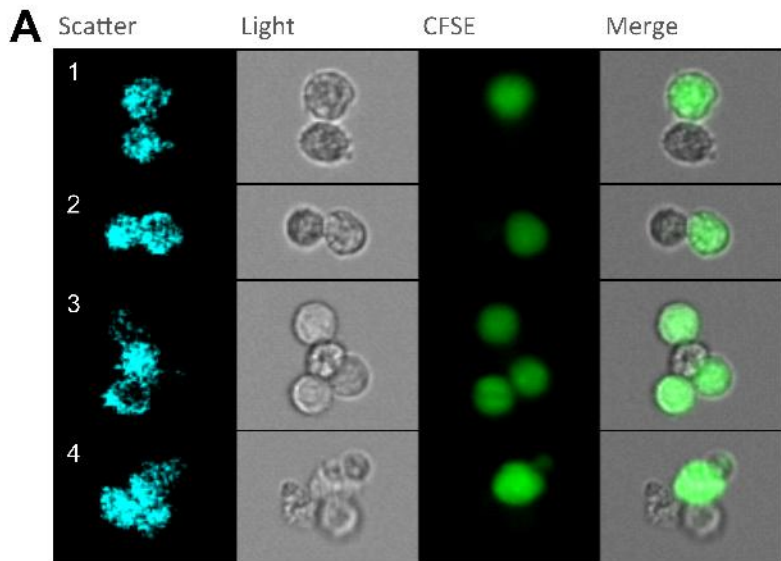
Figure Legends

Fig. E1. Physical contact between mouse-derived MCs and Eos *in vitro*. (A)

Interactions between unlabeled BMEos and CFSE-labeled BMMCs, analyzed in MIFC (representative examples of n=3). Homotypic/heterotypic frequencies of cellular couples (B) and aggregates/complexes (C) in 5 min (1:1 ratio) BMMC-BMEos co-cultures, vs. monocultures of these cells alone (n=3, * $P < .05$).

Fig. E2. Receptor expression in mature BMMC and BMEos cultures. (A)

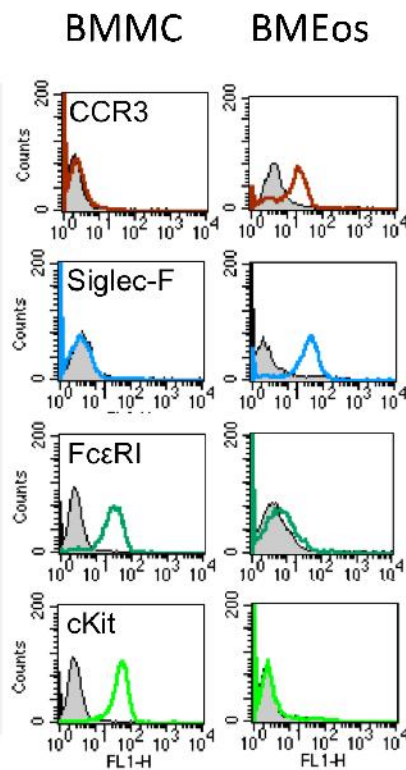
Levels of characteristic markers of mature BMMCs (cKit, FcεRI) and mature BMEos (Siglec-F, CCR3), and expression of CD2 molecules (CD48 and 2B4) in these cell types, as detected by flow cytometry (representative of n=7). (B) Relative tryptase release from resting/IgE (DNP)-activated BMMCs co-cultured (1:1) with CD48/2B4-blocked BMEos (n=3, * $P < .05$). (C) EPO release in BMEos co-cultured (1:1) with CD48/2B4 pre-neutralized BMMCs (n=3, * $P < .05$). Control samples contain monocultures or co-cultures with isotype-treated cells. NA-not activated. ns- non-significant.



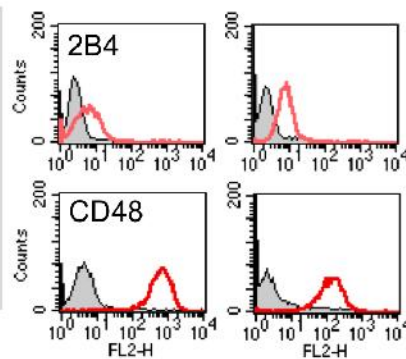
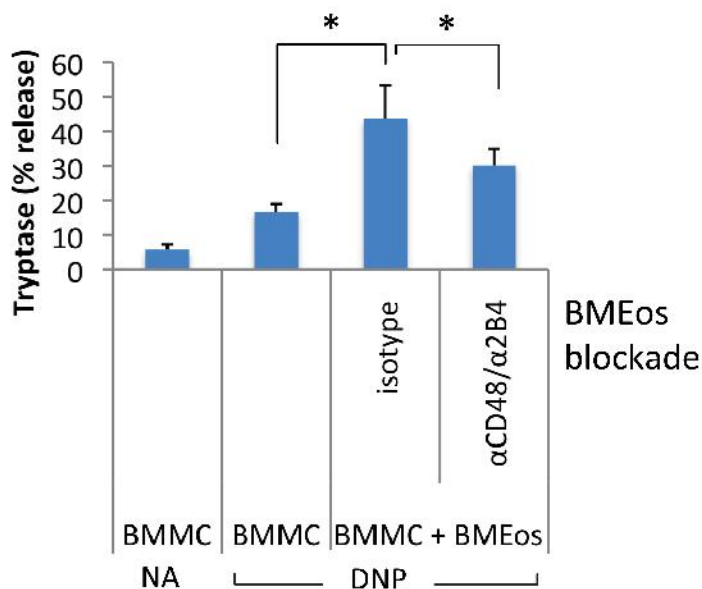
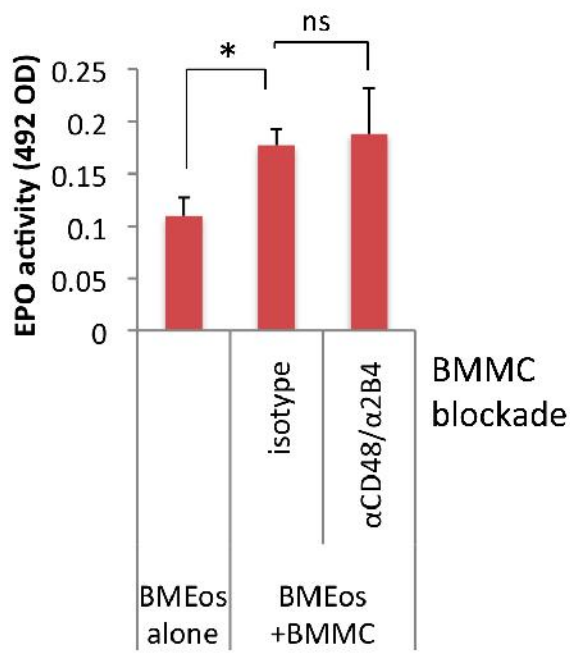
E1

A

Cell markers



CD2 markers

**B****C****E2**