# Pre-stimulation of Bone-marrow Derived Eosinophils with CCL24 Alters Responses to TLR Ligands and Helminth Extracts

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Abstract:     Eosinophil granulocytes are a hallmark of helminth infection, provide protection against helminth infections and elicit detrimental effects during allergy and asthma. Moreover, eosinophils are associated with diabetes, arthritis and sepsis. Thus, eosinophils have a broad range of implications, contributing to homeostasis as well as pathogenesis of various diseases. In the current study we used murine bone-marrow derived eosinophils (bmEos) to investigate the impact of eosinophil pre-stimulation with the chemoattractants CCL11 and CCL24 (eotaxin-1 and 2) on TLR2, TLR4 and filarial extract-induced eosinophil responses. Generation of bmEos consistently resulted in approximately 50 million bmEos from a single donor mouse and a purity and viability above 95%. Upon stimulation with CCL24, TLR2, TLR4, and filarial extract, bmEos released different quantities of IL-4, IL-6, CCL5, as well as CXCL1. CCL24 pre-stimulation partially affected those responses. Furthermore, CCL24 pre-stimulation of bmEos reduced the expression of the eotaxin receptor CCR3 independently of TLR2 stimulation. In contrast, expression of adhesion molecule ICAM-1 was increased by TLR2 stimulation, but not affected by CCL24 pre-stimulation. Hence, our results reveal an impact of CCL24 on bmEos activation. bmEos present a promising tool to study eosinophil responses that may help to further characterize their role in different immunological contexts and overcome the limitations given by the low eosinophil frequencies present in non-helminth-infected individuals.

## 1 INTRODUCTION

Eosinophil granulocytes are most famous for their involvement in the pathogenesis of allergies and asthma (Fulkerson and Rothenberg, 2013) as well as their characteristic expansion and protective effect during helminth infection (Gentil et al., 2014). However, eosinophils further support anti-bacterial responses, contribute to metabolic homeostasis and impact autoimmune diseases. Accordingly, eosinophils recognize pathogen associated molecular patterns and possess anti-bacterial functions due to the release of bactericidal NET like structures and phagocyte-recruiting chemokines and are discussed as potential marker for the severity of bacterial sepsis (Merino et al., 2012). Moreover, adipose tissue eosinophils help to maintain glucose and insulin tolerance by driving alternative macrophage activation via the release of IL-4 (Wu et al., 2011). Such a beneficial role of eosinophils was also described during inflammatory arthritis, which was mitigated by helminth-induced eosinophils (Chen et al., 2016).

Eosinophil granulocytes produce and detect numerous chemokines and cytokines and express pattern recognition receptors including toll-like-receptors (Rosenberg et al., 2013). In general, IL-5 is the main inducer of eosinophils and eotaxins that bind to the chemokine receptor CCR3 direct eosinophils to the site of inflammation. Thus, eosinophils are involved in a broad range of homeostatic and inflammatory conditions and essentially modulate immune responses and pathogenesis. We here provide evidence for the impact of the eotaxins CCL11 and CCL24 on subsequent TLR-induced and filarial extract-induced immune responses of bone-marrow derived eosinophils (bmEos), which may contribute to the diverse spectrum of eosinophil functions.

## 2 METHODS

### 2.1 Ethics Statement and Mice

BALB/c mice (Janvier, Saint Berthevin Cedex, France) were housed at the Institute for Medical Microbiology, Immunology and Parasitology of the University Hospital Bonn, Germany, with access to food and water ad libitum. All experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany and performed according to the European Union animal welfare guidelines.

### 2.2 In Vitro Eosinophil Differentiation from Bone-marrow

Eosinophils were differentiated from bone-marrow of naïve adult mice by stimulation with 100ng/ml stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (FLT3L) for four days followed by culture with 20ng/ml IL-5 for eight days (all Peprotech, Rocky Hill, USA, Fig. 1A) (Dyer et al., 2008). Cells were adjusted to $1x10^6$ cells/mL and incubated at 37°C and 5% $CO_2$. Half of the medium containing advanced RPMI 1640, 20% heat-inactivated fetal calf serum, 1M HEPES, 10.000 IU/mL penicillin, 10μg/mL streptomycin, 1X GlutaMAX™ (all Gibco® Technologies, Waltham, USA) was replaced every other day. Adherent cells were removed at day 8 and eosinophil purity was checked at day 12.

### 2.3 In Vitro Stimulation of Bone-marrow Derived Eosinophils

$1x10^6$ bmEos were pre-stimulated for 24 hours with 100ng/mL CCL11 or CCL24 (both Peprotech, Rocky Hill, USA) in eosinophil growth medium. Subsequently, cells were re-stimulated for 24 h with 200ng/mL lipopolysaccharide (LPS) ultrapure, 500ng/mL Pam3CSK4 (P3C) (both InvivoGen, San Diego, USA) or 25μg/mL *Litomosoides sigmodontis* crude adult worm extract (LsAg). LsAg was prepared as previously described (Gentil et al., 2014).

### 2.4 Flow Cytometry, Fluorescence Microscopy and Enzyme Linked Immunosorbent Assay

After blocking with PBS containing 1% bovine serum albumin and 0.1% rat IgG (Sigma-Aldrich, St. Louis, USA) for 30 min, bmEos were washed and stained with combinations of anti-SiglecF AL647 (BD Pharmingen, San Diego, USA), anti-CD54/ICAM-1 AL488, and anti-CD193/CCR3 PE (both BioLegend, San Diego, USA). Data were acquired using a BD FACS Canto (BD Bioscience, San Jose, USA) and analyzed by FlowJo v10 software (Tree Star, Ashland, USA).

For confocal fluorescence microscopy bmEos were fixed with 3% formaldehyde fixative solution for 20 min on 15 mm glass slides (P+W Medizintechnik, Berlin, Germany) and stained with rabbit anti- eosinophil cationic protein (ECP) (Biorbyt Ltd, Cambridge, UK) for 1 h followed by 1 h staining with goat anti-rabbit FITC (Invitrogen, Waltham, USA) and anti-SiglecF AL647. DAPI was stained for 10 min (Sigma-Aldrich, Steinheim, Germany). Z-stack pictures were taken with the Zeiss LSM 710 and the ZEN 2.3 software (both Carl Zeiss AG, Oberkochen, Germany).

Cytokine and chemokine concentrations were determined from supernatants by ELISA according to kit protocols (IL-6 and TNFα (eBioscience); CXCL1 and CCL5 (R&D, Minneapolis, USA) using a SpectraMAX 190 system and SoftMax Pro 6.5 software (Molecular Devices, Sunnyvale, USA).

### 2.5 Statistical Analysis

Statistical analysis was performed using Prism GraphPad 5.01 (GraphPad Software, San Diego, USA). Statistical significance was tested by Kruskal-Wallis test followed by Dunn's Multiple Comparison post hoc test. Significance is defined as p value < 0.05 and error bars represent means ± SEM.

## 3 RESULTS

### 3.1 Generation of Murine Bone-marrow Derived Eosinophils

Flow cytometric analysis of in vitro generated bmEos revealed a 98% purity of SiglecF⁺CCR3⁺ cells (Fig. 1A, B). H&E staining as well as fluorescence microscopy using anti-ECP, anti-SiglecF and DAPI confirmed that bmEos had the typical eosinophil appearance with eosin-stained granule, U-shaped nucleus and contained ECP (Fig. 1C, D). The viability of bmEos was analyzed by Annexin V and propidium iodide staining after twelve days of culture and was consistently above 95% (Fig. 1E). In general, 50-80 million bmEos

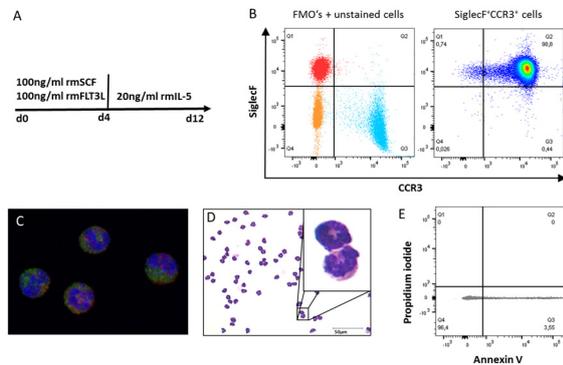were obtained from one single donor mouse (data not shown).



Figure 1: In vitro differentiation of bone-marrow derived eosinophil granulocytes. Bone-marrow from tibiae and femur of 6 week-old BALB/c mice were stimulated with 100ng/ml recombinant mouse SCF and recombinant mouse FLT3L for four days followed by eight day stimulation with recombinant IL-5 (A). Analysis of the purity of SiglecF+CCR3+ eosinophils by flow cytometry on day 12 (B). Fluorescence microscopy of eosinophils stained with anti-SiglecF (red), anti-ECP (green) and DAPI (blue) (C) and H&E staining of differentiated eosinophils (D). Viability of differentiated eosinophils as determined by Annexin V and propidium iodide staining via flow cytometry (E).

## 3.2 CCL24 Modulates Cytokine and Chemokine Release by Bone-marrow Derived Eosinophils

Since eosinophils are predominantly recruited by the chemokines CCL11 and CCL24, we investigated their role on bmEos activation in vitro. BmEos were stimulated for 24h with the filarial extract LsAg, the TLR4 agonist LPS and the TLR1/2 agonist P3C, in the presence or absence of CCL11 or CCL24 pre-stimulation. IL-4 release by bmEos was not induced by CCL11, LPS, P3C or LsAg stimulation alone, but tended to be increased upon stimulation with CCL24. Pre-stimulation of bmEos with CCL24 before LPS and P3C re-stimulation resulted in a significantly increased release of IL-4 compared to LPS- and P3C-only stimulated controls. Similarly, CCL24 pre-stimulation significantly increased LsAg-induced IL-4 release compared to unstimulated controls (p<0.05). LPS and P3C potently induced IL-6 and CCL5/RANTES by bmEos (Fig. 2B, C). While pre-stimulation with CCL11 had no impact on subsequent LsAg-, LPS- or P3C-induced IL-6, CCL5 and CXCL1 release by bmEos, CCL24 pre-stimulation reduced P3C-

stimulated CCL5 production and increased by trend LsAg-induced IL-6 release (Fig. 2B, C). CXCL1 release was significantly induced by P3C but none of the other stimulations alone (Fig. 2D). However, pre-stimulation with CCL24 led to a significantly increased CXCL1 release upon LPS re-stimulation (Fig. 2D). Those results indicate that CCL24 pre-stimulation affects bmEos responses to subsequent stimuli.
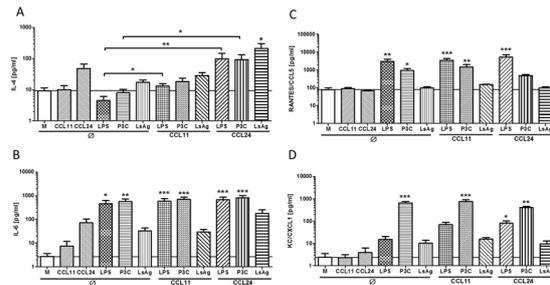


Figure 2: CCL24 modulates cytokine and chemokine release by bone-marrow derived eosinophils. Concentrations of IL-4 (A), IL-6 (B), RANTES/CCL5 (C), and KC/CXCL1 (D) in the supernatant after a total of 48h stimulation. Cells were untreated or pre-stimulated with CCL11 or CCL24 for 24 hours followed by re-stimulation with LPS, Pam3CSK4 (P3C) or crude *Litosomoides sigmodontis* adult worm extract (LsAg) for additional 24 hours. Data of two independent and pooled in vitro experiments with 7 replicates are shown. Data is presented as mean + SEM and analyzed for statistical significance using Kruskal-Wallis test followed by Dunn's posthoc test (*p<0.05, **p<0.01, ***p<0.01).

## 3.3 CCL24 Pre-Stimulation Reduces the Expression of CCR3

Since CCL24 pre-stimulation and P3C stimulation induced bmEos activation, the impact of CCL24 pre-stimulation on the expression of CCR3 and ICAM-1 were investigated. The expression of CCR3 significantly decreased upon CCL24 pre-stimulation and was not altered by TLR2 stimulation (Fig. 3A, B). In contrast, ICAM-1 expression was increased by P3C stimulation, but not altered by CCL24 pre-stimulation (Fig. 3C, D). These results indicate that bmEos react upon TLR activation with an increased ICAM1 expression, which may facilitate their tissue migration and reduce the expression of the CCR for the major eosinophil recruiting factors after pre-stimulation with CCL24.
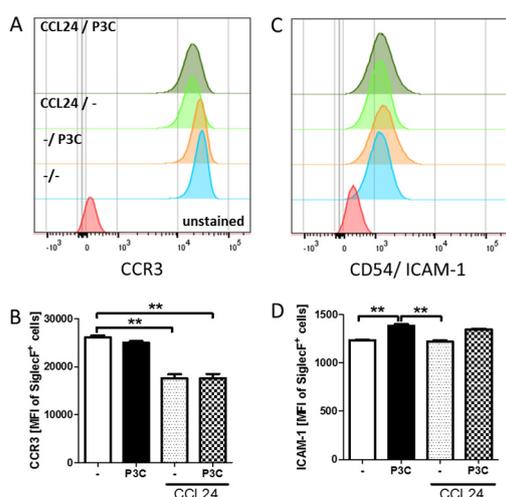
Figure 3: Treatment with CCL24 modulates CCR3 expression. Bone-marrow derived eosinophils were pre-stimulated with CCL24 for 24h followed by six hours re-stimulation with Pam3CSK4 (P3C). Histograms and MFI of CCR3 (A, B) and CD54/ICAM-1 (C, D) SiglecF⁺ eosinophils are shown. Data of two independent and pooled in vitro experiments with 6 replicates are shown and are presented as mean + SEM and analyzed for statistical significance using Kruskal-Wallis followed by Dunn's post hoc test (*p<0.05, **p<0.01, ***p<0.01).

## 4 DISCUSSION AND CONCLUSION

In this study we describe the in vitro generation of bmEos and the impact of bmEos pre-stimulation with CCL11/CCL24 on cytokine and chemokine release in response to TLR ligands and LsAg. The stimuli chosen for this study induced different cytokine/chemokine pattern from bmEos, with P3C and LPS triggering CCL5 and IL-6 release, P3C inducing CXCL1 production and CCL24 the release of IL-4. Interestingly, LsAg-induced cytokine/chemokine release by bmEos was only present after pre-stimulation with CCL24, resulting in increased IL-6 and IL-4 release. CCL24 pre-stimulation also increased IL-4 responses after re-stimulation with P3C and LPS. Such an effect by eotaxin to induce the release of preformed IL-4 was also observed for human eosinophils that was additionally enhanced by IL-5 (Bandeira-Melo et al., 2001). This indicates that in the context of increased CCL24 concentrations, as they may occur during type 2 inducing helminth infections, eosinophils may be more prone to support type 2 immune responses independent on the stimulus, which may render them more efficient for protection against filarial infections (Gentil et al., 2014). However, pre-stimulation with CCL24 also triggered the release of pro-inflammatory mediators like CXCL1 upon LPS re-stimulation and IL-6 after LsAg re-stimulation and bmEos responded to TLR2 and TLR4 stimuli by the release of IL-6. Those results suggest that bmEos may also support anti-bacterial responses by triggering neutrophil recruitment via CXCL1 and acute phase responses, which can be in part enhanced by CCL24 pre-stimulation. BmEos further reacted upon TLR2 activation with an increased ICAM1 expression, which may increase cell contact with other leucocytes and promote inflammation (Czech et al., 1993). In contrast, CCR3 expression of BmEos was reduced by CCL24 treatment independently of TLR2 stimulation suggesting two independent mechanisms of eosinophil migration and eosinophil activation (Humbles et al., 2002). In summary, our data demonstrate that bmEos possess characteristics that are known from ex vivo isolated eosinophils and indicate that CCL24 pre-treatment modulates eosinophil responses.

## SOURCES OF FUNDING

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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