bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

1 Comparison of IL-33 and IL-5 Family Mediated Activation of Human Eosinophils

2

3 Evelyn L. Angulo, Elizabeth M. McKernan, Paul S. Fichtinger, Sameer K. Mathur

4

5 Division of Allergy, Pulmonary and Critical Care, Department of Medicine, University of Wisconsin School

6 of Medicine and Public Health, Madison, WI 53792

7

8

9 Corresponding Author:

10 Evelyn L. Angulo, M.D.
11 University of Wisconsin School of Medicine and Public Health
12 Department of Medicine
13 Division of Allergy, Pulmonary and Critical Care
14 600 Highland Ave.
15 CSC 900
16 Madison, WI 53792
17 Email: eangulo@wisc.edu
18 Phone: (608) 262-2804
19 Fax: (608) 261-1005
20

21 Word Count: 3538

22 Key words: eosinophil, IL-33, IL-3, IL-5

23

24

25

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

26 ABSTRACT

27

28 Eosinophils are the prominent inflammatory cell involved in allergic asthma, atopic dermatitis,
29 eosinophilic esophagitis, and hypereosinophilic syndrome and are found in high numbers in local tissue
30 and/or circulating blood of affected patients. There is recent interest in a family of alarmins, including
31 TSLP, IL-25 and IL-33, that are epithelial-derived and released upon stimulation of epithelial cells. Several
32 genome wide association studies have found SNPs in genes encoding IL-33 to be risk factors for asthma.
33 In two studies examining the direct role of IL-33 in eosinophils, there were differences in eosinophil
34 responses. We sought to further characterize activation of eosinophils with IL-33 compared to activation
35 by other cytokines and chemokines. We assessed IL-33 stimulated adhesion, degranulation, chemotaxis
36 and cell surface protein expression in comparison to IL-3, IL-5, and eotaxin-1 on human eosinophils. Our
37 results demonstrate that IL-33 can produce as potent or more eosinophil activation than IL-3, IL-5 and
38 eotaxin-1. Thus, when considering specific cytokine targeting strategies, IL-33 will be important to
39 consider for modulating eosinophil function.

40

41

42

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

43 INTRODUCTION

44 Eosinophils are the prominent immune cells involved in allergic asthma, atopic dermatitis, eosinophilic
45 esophagitis, and hypereosinophilic syndrome and are found in high numbers in local tissue and/or
46 circulating blood of affected patients (1). In the tissue, eosinophils can release toxic granule contents
47 including Major Basic Protein (MBP), Eosinophil Derived Neurotoxin (EDN), and Eosinophil Peroxidase
48 (EPO), which may cause intended damage to the target in the case of parasitic infections, but can
49 inadvertently damage surrounding host tissue and trigger remodeling. In severe asthma, this can lead to
50 chronic inflammation of the airway resulting in long-term injury and remodeling. In addition, we and
51 others have demonstrated that eosinophils are pro-inflammatory cells signaling other immune cells
52 through cytokine release especially by driving and propagating the Th2 type immune response (2, 3).
53
54 IL-5 is a specific activator of eosinophils and is crucial to their development from bone marrow
55 progenitors. It increases adhesion, survival, and cytokine release as well as inhibits apoptosis. However,
56 treatment of asthmatic patients with anti-IL5 drug (Mepolizumab) fails to completely eliminate tissue
57 eosinophilia, though blood eosinophil numbers and asthma exacerbations are significantly reduced (4,
58 5). Thus, it is important to consider alternative eosinophil activators that may contribute to eosinophil-
59 mediated pathology in airway tissue.
60
61 There are other well-studied activators of eosinophils such as IL-3, GM-CSF, and TSLP causing varying
62 degrees of activation and involving different signaling kinetics (6). IL-3 and GM-CSF receptors share the
63 same β chain as IL-5, but IL-3 and GM-CSF have differential effects on eosinophils likely due to the
64 regulation of their specific α chain on eosinophils surface. Eosinophils from bronchial lavage (BAL) have
65 increased expression of IL-3Rα and GM-CSFRα and decreased expression of IL-5Rα compared to
66 circulating blood eosinophils (7). IL-5 added to eosinophils *in vitro* leads to up-regulation of IL-3Rα and

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

67 GM-CSFRα and down-regulation of IL-5Rα (8, 9). IL-3 more strongly induces eosinophil proteins
68 including CD48, CD13, and Semaphorin 7A than GMCSF and IL-5 (10, 11). When IL-3 is added along with
69 TNFα, mRNA for MMP-9 and Activin A are more strongly increased by transcription and mRNA stability
70 than with IL-5 and GMCSF stimulation (12, 13).
71
72 There is more recent interest in a family of alarmins, including TSLP, IL-25 and IL-33. The alarmins are
73 epithelial-derived, and released in response to a variety of triggers including epithelial trauma, allergic
74 inflammation, protease activity, and rhinovirus infection (14, 15). These cytokines are involved in the
75 pathophysiology of allergic diseases, including asthma and atopic dermatitis, through Th2 pathway
76 activation (16, 17). We and others have shown that TSLP can activate eosinophils leading to production
77 of Th2 cytokines, enhanced survival and degranulation (18, 19).
78
79 Several genome wide association studies have found SNPs in genes encoding IL-33 or its receptor ST2
80 (IL1R1) to be risk factors for eosinophilic asthma, early childhood onset asthma, and severe forms of the
81 asthma (20) Airway biopsies of asthmatics have higher IL-33 mRNA expression than healthy
82 subjects(21). In addition, Rhinovirus infection which is an important risk factor for asthma
83 exacerbations, increases IL-33 levels in nasal lavage of asthmatic patients (22). In two studies examining
84 the role of IL-33 in eosinophils, there were differences in eosinophil responses. Cherry et al.
85 demonstrated that IL-33 was as potent or more so than IL-5 in inducing adhesion and degranulation, but
86 did not evaluate chemotaxis (23). Suzukawa et al. demonstrated that IL-33 was more potent than IL-5 in
87 inducing adhesion, but not degranulation (24). In addition, CD11b(αM intergrin) was also shown to have
88 increased expression in the presence of IL-33, but no chemotactic effect was observed by Suzukawa et
89 al. We sought to further characterize activation of eosinophils with IL-33 compared to activation by
90 other cytokines and chemokines.

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

91 METHODS

92

93 Eosinophil purification

94 In an IRB approved protocol (UW-HS-IRB-2013-1570), adult volunteers (18-55 years) with allergic rhinitis
95 or mild atopic asthma were consented to provide peripheral blood samples (300 ml) in heparinized
96 syringes. Blood was diluted 1:1 with 1x HBSS (Corning), layered over 1.090g/mL Percoll, and spun at
97 2000rpm for 20 minutes. The mononuclear cell layer and Percoll layers were removed and the red blood
98 cell and granulocyte pellet was moved to a clean tube. Contaminating erythrocytes were removed
99 through two hypotonic lyses with ddH2O and divided into 200 million cell aliquots. The aliquots were
100 incubated at 4°C for 30 minutes with 200µL HBSS with 4% NCS (Sigma), 200µL of anti-CD16 beads, 15µL
101 of anti-CD14 and anti-CD3 beads, and 30µL of anti-glycophorin-A beads. The diluted cells were passed
102 through an Auto MACS (Miltenyi) to remove all of the CD16, CD14, CD3, and glycophorin-A positive cells.
103 Purified cells were 97% - 99.9% eosinophils with neutrophils and lymphocytes as contaminating cells.

104

105

106 Eosinophil adhesion

107 Eosinophil adhesion was assessed as previously described by measuring Eosinophil Peroxidase activity
108 (EPO) from adhered cells on 96 well plates (Immulon 4 HBX) stimulated with the respective cytokines for
109 30 min (25). Wells were coated with 10 ug/ml I-CAM1 (RnD Systems), 5ug/ml V-CAM1(RnD Systems) or
110 5ug/ml Periostin (RnD Systems). Wells were blocked with 0.1% gelatin in HBSS containing Ca^2+ and
111 Mg^2+ and washed with 3 times with HBSS. Cells, $1x10^4$ eosinophils, were added to each well along with
112 the respective cytokines (1ng/ml final concentration). After 30 min at 37°C, wells were washed 3x with
113 HBSS to remove non adherent cells. 100ul of HBSS was added to sample wells and 100ul with $1x10^4$
114 eosinophils were added to untreated wells to provide "Total" eosinophils for comparison. Eosinophil

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

115 peroxidase (EPO) activity was used to provide an index of eosinophil cell numbers. EPO substrate (1mN
116 H_2O_2, 1mM O-phenylenediamine (Sigma), and 0.1% Triton x-100 (Sigma) in Tris buffer, pH8.0) was added
117 to each well for 30 minutes at RT before stopping the reaction using 4M H_2SO_4. The colorimetric change
118 at 492nm was assessed with the BioTek Synergy HT plate reader and the % adherence was calculated
119 using: sample well OD 492/Total well OD 492 x100%.
120
121 Eosinophil degranulation
122 Eosinophil degranulation was assessed by performing ELISA for Eosinophil Derived Neurotoxin (EDN) on
123 cell free supernatants from eosinophils cultured for 4hrs with *N*-Formylmethionyl-leucyl-phenylalanine
124 (FMLP) (Calbiochem) or cytokines. Eosinophils in HBSS with 0.03% gelatin and Ca^2+/Mg^2+, 2.5 $x10^5$
125 /well, were stimulated with 100nM FMLP as a positive control and IL-5 (RnD Systems), IL-3 (RnD
126 Systems), and IL-33 (RnD Systems) at 1ng/ml final concentration for 4 hours at 37°C. A "Total" EDN
127 content well was created by adding $2.5x10^5$ cells directly to 0.1M HCL + 1% Triton x-100 to lyse the cells
128 and their granules. Cell free supernatants were collected and analyzed with EDN ELISA kit per
129 manufacturer's instructions (MBL).
130
131 Eosinophil chemotaxis
132 Eosinophil chemotaxis was measured with a 24-well plate and a 6.5mm thick transwell with 5µm
133 diameter pores. For each condition, 600µL of co-culture media and chemoattractant was added in
134 duplicates to the lower chamber. Eotaxin-1 (Promokine) was used at a concentration of 100ng/mL while
135 IL-3 (RnD Systems), IL-5 (RnD Systems), or IL-33 (RnD Systems) were added at a concentrations of
136 10ng/mL. The upper transwell was pre-wet using HBSS with 0.01% gelatin and Ca^2+/Mg^2+. Purified
137 eosinophils were also diluted in the HBSS with 0.01% gelatin and Ca^2+/Mg^2+ to a concentration of
138 3x10^6/mL. Each upper well of the transwell was aliquoted 100µL of the eosinophil solution and the

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

139 plate incubated at 37°C for one hour. In order to remove any cells stuck to the bottom of the transwell,
140 250mM EDTA (Boston Bioproducts) was added to the bottom well for 5 minutes at room temperature.
141 The transwell was then removed and 50µL of the solution from the bottom chamber was then diluted
142 1:1 with trypsin (0.4%, Gibco) and counted.
143
144
145 Flow cytometry
146 Eosinophils purified through AutoMACS separation were incubated with IL-3(10ng/mL, RnD Systems), IL-
147 5(10ng/mL, RnD Systems), IL-33(10ng/mL, RnD Systems), Eotaxin-1(100ng/mL, Promokine), in FBS and
148 1% RPMI for 4 hours at 37°C (0.5×10^6 cells/tube). Cells were incubated with antibody-fluorochrome
149 conjugates for 30 minutes at 4°C. Cells were washed, fixed with a paraformaldehyde solution, and
150 analyzed using the BD LSR Fortessa or BD LSR II (BD Biosciences). Antibodies used were: CD16 PE-CF594,
151 CD14 Alexa Fluor 488(BD Biosciences); CD18 PE-Cy7, CD11b PerCP Cy5.5, ICAM-1 PE (Biolegend); CD66b
152 PE-Cy7 (eBioscience); Ghost Dye™ Red 780 (Tonbo). Data was collected using FACSDivaTM Software (BD
153 Biosciences). Data was analyzed with Flow Jo Version 10 (Tree Star Inc.), with 75-150,000 events
154 captured. Experiments were performed with at least 7-9 replicates for each group. Appropriate
155 compensation controls were performed using antibodies and compensation control beads (Invitrogen™
156 Ebioscience™ UltraComp Ebeads™).
157
158
159 Statistics
160 Adhesion, degranulation, and chemotaxis data are expressed as mean ± SEM. One-way ANOVA test and
161 the Dunn's test or Student Newman Keul's test, if appropriate were used for pair-wise comparisons
162 (Sigmaplot 13.0, Systat Software). Differences were considered significant when $p < 0.05$.

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

163

164 For flow cytometry studies, MFI values were obtained from flow cytometry analysis software (FlowJo
165 10.2). MFI fold change values were normalized by performing $\log_{10}$ transformation of the ratio between
166 cytokine stimulated MFI values and unstimulated MFI values. Ratio-*t*-tests were performed on the log-
167 transformed data (RStudio Version 1.0.153). One-way ANOVA tests and the Dunn's test or Student
168 Newman Keul's test, if appropriate, were used for pair-wise comparisons of the log-transformed data
169 (SigmaPlot 13.0). Differences were considered significant when $p < 0.05$.

170

171

172

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

RESULTS

Eosinophil Adhesion

Percent adherence was measured by EPO activity from adhered cells (N=22). As shown in Fig 1, eosinophils were significantly more adherent to uncoated wells when stimulated with IL-33 than when stimulated with IL-3 ($p < 0.001$) or IL-5 ($p < 0.001$). Similarly, with ICAM-1 as a substrate, eosinophils stimulated with IL-33 were significantly more adherent then those stimulated with IL-3 ($p < 0.001$) or IL-5 ($p < 0.001$).

Fig 1. Eosinophils are more adherent to uncoated wells when stimulated with IL-33.
Eosinophil adhesion determined by EPO release from adherent cells cultured for 30min with cytokine stimulation, cytokine concentration 1ng/mL. Values shown are a percentage of optical density (OD) of "Total" wells containing 10x10e4 lysed eosinophils with control (no stimulation) subtracted. N=22 Experiments from different blood donors (n=22) with the exception of 3 donors that were repeated twice in which case, results were averaged from the 2 different experiments using their cells. Lines connect comparison groups with p-value denoting significant difference in pair-wise comparisons.

Eosinophil Degranulation

As shown in Fig 2, eosinophils stimulated with IL-33 for 4 hours induced more percent total EDN release than the positive control FMLP ($p = 0.04$, N=16). EDN release from IL-33 stimulated eosinophils was significantly higher than eosinophils stimulated with IL-3 ($p < 0.001$) and IL-5 ($p < 0.001$). IL-3 and IL-5 both induced significantly higher degranulation than our control ($p < 0.001$).

Fig 2. Eosinophils stimulated with IL-33 induce more percent total EDN release than positive control.

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

197 EDN release determined by ELISA for eosinophils cultured 4 hrs with cytokine stimulation, cytokine
198 concentration 1ng/mL (n=16). Values are expressed as % of "Total" well containing 0.5x10e6/well of
199 lysed eosinophils. Lines connect comparison groups with p-value denoting significant difference in pair-
200 wise comparisons.
201
202 **Eosinophil Chemotaxis**
203 As shown in Fig 3, Percent migration of eosinophils after stimulation using transwell chambers was
204 evaluated (N=8). Eotaxin-1 demonstrated significantly higher percent migration than did IL-33 ($p <$
205 0.001). IL-3($p < 0.001$) and IL-5 ($p = 0.005$) demonstrated significant increase in percent migration when
206 compared to IL-33. There was no significant difference in percent migration of eosinophils stimulated
207 with IL-33 when compared to control ($p = 0.28$). Compared to control, percent migration was
208 significantly increased with eotaxin-1 ($p < 0.001$).
209
210 **Fig 3. IL-33 stimulation does not result in increased eosinophil migration.**
211 Percent migration of eosinophils after stimulation using transwell chambers. Migration assessed after 1
212 hour after cytokine was placed in lower chamber, eotaxin-1 concentration 100ng/mL, IL-33 and other
213 cytokine concentrations 10ng/mL (n=8). Values are expressed as % migration of total cells in well
214 containing 0.3x10e6/well of eosinophils. Lines connect comparison groups with p-value denoting
215 significant difference in pair-wise comparisons.
216
217 **Eosinophil Expression of Cell Surface Markers**
218 As shown in Fig 4, eosinophil cell surface marker expression was assessed using flow cytometry.
219 We compared the change in cytokine stimulated expression for 4 hours versus baseline
220 expression (N=7-9). IL-33, IL-3, IL-5 and eotaxin-1 significantly increased expression of CD11b,

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

221 CD66b, and CD18 when compared to unstimulated control (with p-values < 0.05). ICAM-1
222 expression compared to unstimulated control, was significantly increased after incubation with
223 only IL-33, IL-3, IL-5 (with p-values < 0.001), but not eotaxin-1 (p = 0.3). In pairwise
224 comparisons, IL-33 stimulated eosinophils expressed significantly higher levels of CD11b (Fig
225 4A) and CD18 (Fig 4B) than eotaxin-1 (with p-values < 0.001) or IL-3 stimulated cells (with p-
226 values < 0.05). IL-33 stimulated cells demonstrated higher CD18 expression compared to IL-5 as
227 well (p=0.01). IL-33 stimulated eosinophils significantly increased expression of CD66b (Fig 4C)
228 and ICAM-1 (Fig 4D) when compared to eotaxin-1 (with p-values < 0.001) and IL-3 (with p-
229 values < 0.05), but not IL-5. When comparing IL-33 stimulated eosinophil surface marker
230 expression versus that of IL-3, IL-5 or eotaxin-1 at these concentrations, IL-33 stimulated
231 eosinophils demonstrated the highest fold change in marker expression. The 4hr stimulation
232 experiments did not result in statistically significant change for: N29, CD29, CD11a, CD11c,
233 CD54, CD23, CD40, CD44, CD41, CD62P (Data not shown).
234
235 **Fig 4. IL-33 induces significant changes in cell surface expression.**
236 Box plots of normalized fold change for A) CD11b, B) CD18, C) CD66b, D) ICAM-1 after 4 hour stimulation
237 with either IL-3, IL-5, IL-33, eotaxin-1 compared with unstimulated, represented by dotted line (n=7-8).
238 Lines connect comparison groups with p-value denoting significant difference in pair-wise comparisons.
239
240
241

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

242 DISCUSSION

243 Our results demonstrate that IL-33 is as or more potent than IL-3 and IL-5 in inducing eosinophil
244 adhesion and degranulation. In addition, eosinophil stimulation with IL-33 resulted in increased
245 expression of the cell surface markers CD11b, CD18, CD66b, and ICAM-1 in a manner comparable to or
246 higher than IL-3, IL-5, and eotaxin-1. Our study confirms the lack of chemotaxis from IL-33 stimulation.
247 Few studies have examined the comparative effects of IL-33 vs. IL-3, IL-5 or eotaxin-1 on eosinophils. To
248 our knowledge, increased eosinophil expression of CD18 and CD66b after IL-33 stimulation has not
249 previously been described.
250
251 IL-33 is a member of the IL-1 family of cytokines constitutively expressed in the nucleus of endothelial
252 and epithelial cells of mucosal membranes and fibroblasts and can be released in response to cell injury
253 (26). Characterized as an alarmin, IL-33 warns the immune system of barrier injury when cells
254 undergoing necrosis from infections or physical damage release their contents (27). In this case, the full-
255 length active form is released and IL-33 signaling can occur. However, if cells undergo programmed
256 death like apoptosis, the full length form is cleaved by caspases 3 and 7 and signaling is abrogated (28).
257 Proteases from environmental allergens have also been shown to cleave IL-33, which in turn leads to
258 downstream Th2 signaling and allergic inflammation (29). IL-33 is considered a Th2 type cytokine
259 signaling Th2 lymphocytes and ILC2s to release/produce cytokines such as IL-4, IL-5, IL-6, and IL-13
260 driving the type 2 immune response. Dendritic cells when stimulated with IL-33, release IL-6 and induce
261 production of IL-5 and IL-13 from naïve CD4+ T cells (30). Viral infections can also result in production of
262 Th2 cytokines. In addition, viral infections can lead to bronchial epithelial damage and trigger the release
263 of inflammatory mediators. In a study by Han et al., mice infected with rhinovirus were found to have
264 increased lung epithelial TSLP and IL-33 (31). The production of these two alarmins, in addition to the
265 presence of Th2 cytokines may result in increased eosinophil activity.

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

266

267 The alarmin family also includes TSLP and IL-25. We have previously demonstrated that TSLP promotes
268 eosinophil degranulation, and that its activity may be enhanced by the allergic cytokine milieu (18).
269 Elevated levels of IL-33 are found in bronchial tissue, and BAL fluid of asthmatics when compared to that
270 of controls (21, 32). This points to an important role for IL-33 in airway inflammation. The IL-33 and TSLP
271 pathways, may be a point of intersection where viral infections and allergic exposures combine to result
272 in increased eosinophilic inflammation and a heightened risk of exacerbation from the activation of
273 eosinophils.

274

275 The CD4+ Th2 cytokines IL-3 and IL-5 are produced during allergic inflammation. It has previously been
276 thought that eosinophil activation was mainly controlled by IL-3, IL-5 and GM-CSF. Eosinophil adhesion is
277 the one of the necessary steps in eosinophil migration to target tissues. We observed that eosinophils
278 were significantly more adherent in uncoated or ICAM-1 coated wells after 30 minute incubation with IL-
279 33 when compared to IL-3 and IL-5. This is similar than the findings of Cherry et al. and Suzukawa et al.
280 who both found a significant increase in eosinophil adhesion with IL-33 when compared to IL-5. Our
281 studies used different lengths of time for eosinophil incubation with stimulant than did the previously
282 described studies. In addition, for our adhesion experiments, we used a lower concentration of 1ng/ml
283 for each cytokine, as opposed to the 100ng/mL used in the Cherry et al. study and the 1-100ng/mL used
284 in Suzukawa et al.

285

286 In our studies, IL-33 induced degranulation of eosinophils significantly more than IL-3 and IL-5. This
287 differs to the results from Suzukawa et al. who also measured EDN after IL-33 incubation. However, our
288 work supports the finding of Cherry et al. that, IL-33 can induce degranulation to a level of at least that
289 of IL-5. One limitation of our experiments is that they were performed at single time points. Previous

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

290 work has shown that there can be differences in eosinophil activation at different time points. For
291 example, prolonged activation with IL-3 is more potent than IL-5 and GM-CSF in inducing eosinophil
292 expression of specific activation and adhesion molecules (33). This poses the question of whether IL-33
293 demonstrates similar potency when compared to IL-3, IL-5, or eotaxin-1 at time points not examined in
294 our study.
295
296 Our eosinophil chemotaxis studies did not demonstrate increased cell migration after IL-33 incubation.
297 These results are consistent with that of Suzukawa et al. who reported that IL-33 failed to attract
298 eosinophils in their migration study. As demonstrated in our results, IL-33 stimulation led to increased
299 expression of adhesion and migration molecules CD11b, CD18, CD66b and ICAM-1. ICAM-1 is a well
300 known adhesion molecule that binds to Mac-1 and LFA-1 on the surface of cells or endothelium, and has
301 been shown by Suzukawa et al. to increase after IL-33 incubation. Expression of these cell surface
302 molecules was increased with IL-33 to a similar or higher level than IL-3, IL-5 and eotaxin-1. It is possible
303 that IL-33 participates in priming eosinophils to expresses these markers, but does not directly lead to
304 eosinophil migration. Of note, our flow cytometry results revealed changes in expression that are
305 consistent with prior data. For example, others have demonstrated that eosinophils express increased
306 ICAM-1 after incubation with IL-33 (34, 35). Various studies have demonstrated increased expression of
307 CD11b, CD18, CD66b and ICAM-1 in response to IL-5, IL-3, or GM-CSF, although direct comparisons with
308 IL-33 induced expression were not performed for all of these cell surface markers (33, 36).
309
310 CD11b (αM integrin) and CD18 (β2 integrin) form the Mac-1 (macrophage integrin, also known as
311 complement receptor 3, CR3) complex. It has previously been reported that Mac-1 plays a key role in
312 eosinophil degranulation and adhesion (37). IL-33 has been demonstrated to increase CD11b expression
313 to levels comparable to that of IL-5 (24), and we have extended these observations to demonstrate that

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

314 the CD18 dimer partner is also upregulated. CD66b (CEACAM8) is GPI anchored glycoprotein, which has
315 been associated with CD11b. Yoon et al. demonstrated that CD66b cross linking by monoclonal
316 antibody, or galectin-3 led to CD11b clustering on the eosinophil cell surface, and promoted
317 degranulation (36). Therefore, the IL-33 mediated increased CD66b and CD11b likely play a role in
318 enhanced eosinophil degranulation.
319
320 These eosinophil cell surface molecules have been studied in a number of inflammatory diseases.
321 Sputum eosinophils of asthma patients have been shown to have up-regulated CD66b and CD11b (38). A
322 study of peripheral eosinophils of asthma patients in response to segmental antigen challenge
323 demonstrated increases in the CD11b and CD18 post-challenge (39). CD66b has been found to be
324 elevated on the surface of peripheral eosinophils in untreated eosinophilic esophagitis patients when
325 compared to healthy controls. Other inflammatory disorders such as rheumatoid arthritis have
326 demonstrated that CD66b, CD11b, and CD18 are down-regulated by glucocorticoid use. These studies
327 highlight that these cell surface markers likely play an important role in mediating inflammation. Our
328 results indicate that IL-33 increased the expression of CD66b, CD18, and CD11b to the level of or more
329 so than IL-3, IL-5 and eotaxin-1. A representative sample of $CD11b^{high}CD18^{high}$ and $CD11b^{high}CD66b^{high}$
330 percent positive eosinophils from our stimulation experiments is shown in Table 1. IL-33 induced as high
331 or higher percent-positive cells exhibiting these markers. Therefore, it is plausible that IL-33 leads to
332 potent eosinophil activation through its effects on CD66b, and thereby the Mac-1 complex.
333
334 Table 1. Representative Sample of Double "High" Percent Positive Eosinophils

	Unstimulated	IL-3	IL-5	IL-33	Eotaxin-1
CD11bhighCD66bhigh	27.80%	63.50%	89.20%	92.60%	56.60%
CD11bhighCD18high	41.30%	81.10%	97.70%	97.80%	86.60%

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

335 The above table contains double "high" population percentages for the markers listed. These
336 percentages were obtained by gating on the unstimulated median fluorescence intensity and observing
337 the increase in double "high" percentage shown in a single representative sample.
338

339 We recently described relative abundance of proteins of the peripheral blood eosinophil proteome (40).
340 In comparing protein content of IL-5, IL-3 and IL-33 receptor molecules, IL-5Rα was the highest, with the
341 relative abundance of IL-3Rα and ST2 being 15.6% and 28.1% respectively. This demonstrates that
342 despite less relative abundance of the ST2 receptor when compared to IL-5Rα, IL-33 is still capable of
343 inducing comparable or higher eosinophil adhesion, degranulation and cell surface markers when
344 compared to IL-5.

345

346 In this paper, we identify that IL-33 exerts its effects on eosinophils, resulting in as potent or more
347 adhesion, degranulation, and cell surface marker expression when compared to IL-3, IL-5, or eotaxin-1.
348 IL-33 as an alarmin has the ability to set off a number of down-stream effects that lead to a skewing
349 toward the Th2 pathway and the development of allergic inflammation. The fact that IL-33 has as potent
350 effects on eosinophils strengthens the argument for use of this molecule as a therapeutic target and
351 broadens our knowledge in regards to how these pathways may be intersecting to produce
352 inflammation. Anti-IL-33 biologics are currently in clinical trials for asthma and other atopic conditions
353 and will be expected to significantly impact eosinophil function in allergic disorders.

354

355 ACKNOWLEDGEMENTS

356 We thank the nurse coordinators for recruiting patients and providing samples for this study. We also
357 thank the patients who volunteered their time and participated in our study.

358

359
360
361

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

362 REFERENCES

363 1. Bochner BS. The eosinophil: For better or worse, in sickness and in health. Annals of allergy,
364 asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology.
365 2018;121(2):150-5.
366 2. Jacobsen EA, Taranova AG, Lee NA, Lee JJ. Eosinophils: Singularly destructive effector cells or
367 purveyors of immunoregulation? Journal of Allergy and Clinical Immunology. 2007;119(6):1313-20.
368 3. Liu LY, Mathur SK, Sedgwick JB, Jarjour NN, Busse WW, Kelly EAB. Human airway and peripheral
369 blood eosinophils enhance Th1 and Th2 cytokine secretion. Allergy. 2006;61:589-97.
370 4. Cabon Y, Molinari N, Marin G, Vachier I, Gamez AS, Chanez P, et al. Comparison of anti-
371 interleukin-5 therapies in patients with severe asthma: global and indirect meta-analyses of randomized
372 placebo-controlled trials. Clinical and experimental allergy : journal of the British Society for Allergy and
373 Clinical Immunology. 2017;47(1):129-38.
374 5. Kelly EA, Esnault S, Liu LY, Evans MD, Johansson MW, Mathur S, et al. Mepolizumab Attenuates
375 Airway Eosinophil Numbers, but Not Their Functional Phenotype in Asthma. Am J Respir Crit Care Med.
376 2017.
377 6. McBrien CN, Menzies-Gow A. The Biology of Eosinophils and Their Role in Asthma. Frontiers in
378 medicine. 2017;4:93.
379 7. Bates ME, Liu LY, Esnault S, Stout BA, Fonkem E, Kung V, et al. Expression of interleukin-5-and
380 granulocyte macrophage-colony-stimulating factor-responsive genes in blood and airway eosinophils.
381 American Journal of Respiratory Cell and Molecular Biology. 2004;30(5):736-43.
382 8. Liu LY, Sedgwick JB, Bates ME, Vrtis RF, Gern JE, Kita H, et al. Decreased expression of membrane
383 IL-5 receptor alpha on human eosinophils: II. IL-5 down-modulates its receptor via a proteinase-
384 mediated process. Journal of Immunology. 2002;169(11):6459-66.
385 9. Gregory B, Kirchem A, Phipps S, Gevaert P, Pridgeon C, Rankin SM, et al. Differential regulation
386 of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and
387 GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate
388 IL-3 receptor alpha expression. J Immunol. 2003;170(11):5359-66.
389 10. Esnault S, Johansson MW, Kelly EA, Koenderman L, Mosher DF, Jarjour NN. IL-3 up-regulates and
390 activates human eosinophil CD32 and alphaMbeta2 integrin causing degranulation. Clinical and
391 experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2017;47(4):488-
392 98.
393 11. Esnault S, Kelly EA, Johansson MW, Liu LY, Han ST, Akhtar M, et al. Semaphorin 7A is expressed
394 on airway eosinophils and upregulated by IL-5 family cytokines. Clinical immunology (Orlando, Fla).
395 2014;150(1):90-100.
396 12. Kelly EA, Esnault S, Johnson SH, Liu LY, Malter JS, Burnham ME, et al. Human eosinophil activin A
397 synthesis and mRNA stabilization are induced by the combination of IL-3 plus TNF. Immunology and cell
398 biology. 2016;94(7):701-8.
399 13. Kelly EA, Liu LY, Esnault S, Quinchia Johnson BH, Jarjour NN. Potent synergistic effect of IL-3 and
400 TNF on matrix metalloproteinase 9 generation by human eosinophils. Cytokine. 2012;58(2):199-206.
401 14. Han H, Roan F, Ziegler SF. The atopic march: current insights into skin barrier dysfunction and
402 epithelial cell-derived cytokines. Immunol Rev. 2017;278(1):116-30.
403 15. Mitchell PD, O'Byrne PM. Epithelial-Derived Cytokines in Asthma. Chest. 2017;151(6):1338-44.
404 16. Al-Sajee D, Oliveria JP, Sehmi R, Gauvreau GM. Antialarmins for treatment of asthma: future
405 perspectives. Current opinion in pulmonary medicine. 2018;24(1):32-41.
406 17. Ziegler SF, Artis D. Sensing the outside world: TSLP regulates barrier immunity. Nat Immunol.
407 2010;11(4):289-93.

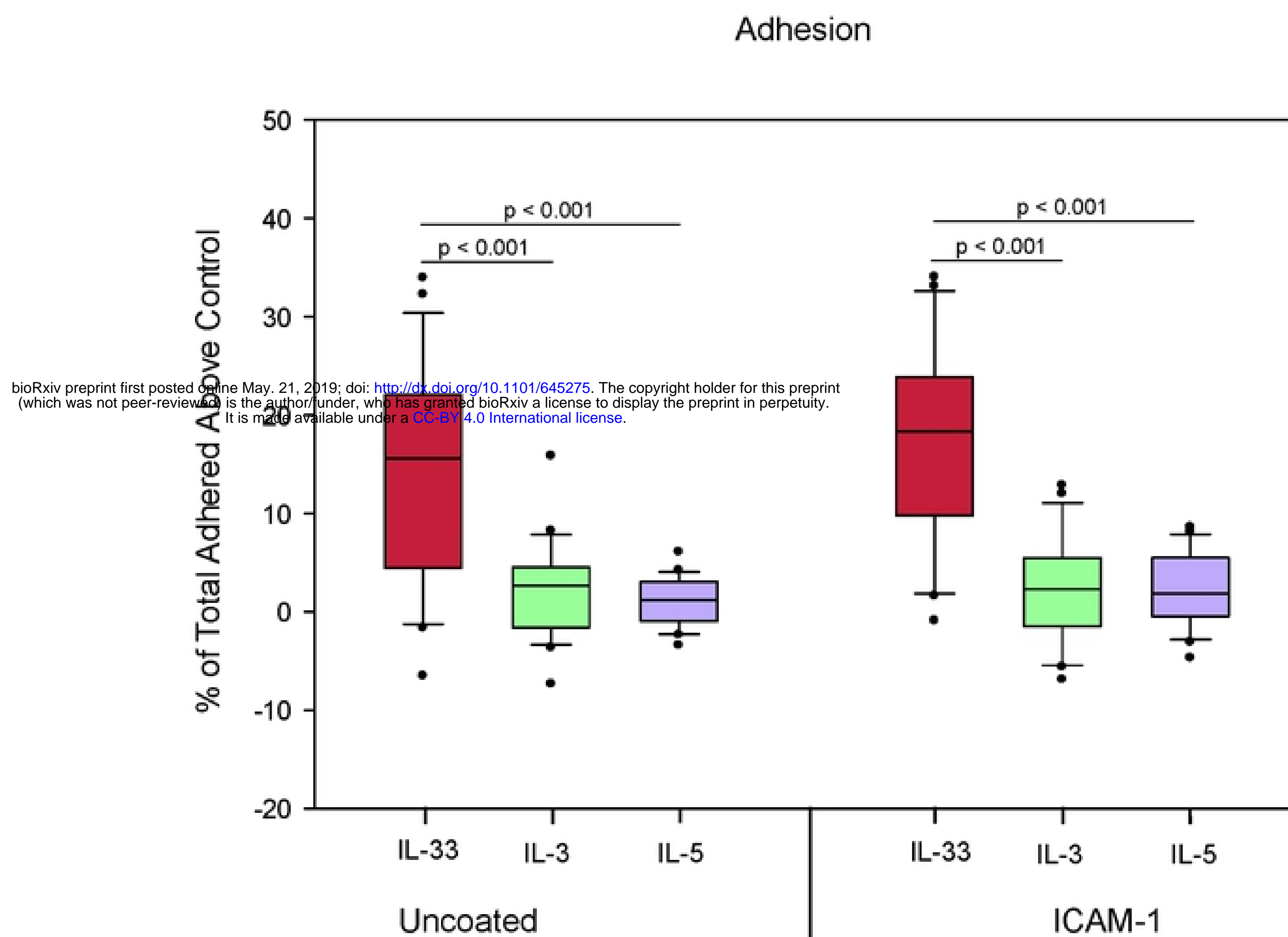
bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

408 18. Cook EB, Stahl JL, Schwantes EA, Koziol-White CJ, Bertics PJ, Graziano FM, et al. Thymic Stromal
409 Lymphopoietin Directly Activates Eosinophil Degranulation. The Journal of Allergy and Clinical
410 Immunology. 2010;125(2):AB133.
411 19. Wong CK, Hu S, Cheung PFY, Lam CW. TSLP Induces Chemotactic and Pro-survival Effects in
412 Eosinophils: Implications in Allergic Inflammation. American Journal of Respiratory Cell and Molecular
413 Biology. 2010;43(3):305-15.
414 20. Jones BL, Rosenwasser LJ. Linkage and Genetic Association in Severe Asthma. Immunol Allergy
415 Clin North Am. 2016;36(3):439-47.
416 21. Prefontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, et al. Increased IL-33
417 expression by epithelial cells in bronchial asthma. J Allergy Clin Immunol. 2010;125(3):752-4.
418 22. Jackson DJ, Makrinioti H, Rana BM, Shamji BW, Trujillo-Torralbo MB, Footitt J, et al. IL-33-
419 dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. Am J Respir Crit
420 Care Med. 2014;190(12):1373-82.
421 23. Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H. A novel IL-1 family cytokine, IL-33, potently
422 activates human eosinophils. J Allergy Clin Immunol. 2008;121(6):1484-90.
423 24. Suzukawa M, Koketsu R, Iikura M, Nakae S, Matsumoto K, Nagase H, et al. Interleukin-33
424 enhances adhesion, CD11b expression and survival in human eosinophils. Laboratory investigation; a
425 journal of technical methods and pathology. 2008;88(11):1245-53.
426 25. Sedgwick JB, Calhoun WJ, Vrtis RF, Bates ME, Mcallister PK, Busse WW. Comparison of Airway
427 and Blood Eosinophil Function After Invivo Antigen Challenge. Journal of Immunology.
428 1992;149(11):3710-8.
429 26. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the
430 nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? PLoS One. 2008;3(10):e3331.
431 27. Mehraj V, Ponte R, Routy JP. The Dynamic Role of the IL-33/ST2 Axis in Chronic Viral-infections:
432 Alarming and Adjuvanting the Immune Response. EBioMedicine. 2016;9:37-44.
433 28. Luthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, et al. Suppression of
434 interleukin-33 bioactivity through proteolysis by apoptotic caspases. Immunity. 2009;31(1):84-98.
435 29. Cayrol C, Duval A, Schmitt P, Roga S, Camus M, Stella A, et al. Environmental allergens induce
436 allergic inflammation through proteolytic maturation of IL-33. Nat Immunol. 2018;19(4):375-85.
437 30. Rank MA, Kobayashi T, Kozaki H, Bartemes KR, Squillace DL, Kita H. IL-33-activated dendritic cells
438 induce an atypical TH2-type response. J Allergy Clin Immunol. 2009;123(5):1047-54.
439 31. Han M, Rajput C, Hong JY, Lei J, Hinde JL, Wu Q, et al. The Innate Cytokines IL-25, IL-33, and TSLP
440 Cooperate in the Induction of Type 2 Innate Lymphoid Cell Expansion and Mucous Metaplasia in
441 Rhinovirus-Infected Immature Mice. J Immunol. 2017;199(4):1308-18.
442 32. Li Y, Wang W, Lv Z, Chen Y, Huang K, Corrigan CJ, et al. Elevated Expression of IL-33 and TSLP in
443 the Airways of Human Asthmatics In Vivo: A Potential Biomarker of Severe Refractory Disease. J
444 Immunol. 2018;200(7):2253-62.
445 33. Esnault S, Kelly EA. Essential Mechanisms of Differential Activation of Eosinophils by IL-3
446 Compared to GM-CSF and IL-5. Crit Rev Immunol. 2016;36(5):429-44.
447 34. Chow JYS, Wong CK, Cheung PFY, Lam CWK. Intracellular signaling mechanisms regulating the
448 activation of human eosinophils by the novel Th2 cytokine IL-33: implications for allergic inflammation.
449 Cellular And Molecular Immunology. 2009;7:26.
450 35. Wong C-K, Leung KM-L, Qiu H-N, Chow JY-S, Choi AOK, Lam CW-K. Activation of Eosinophils
451 Interacting with Dermal Fibroblasts by Pruritogenic Cytokine IL-31 and Alarmin IL-33: Implications in
452 Atopic Dermatitis. PLOS ONE. 2012;7(1):e29815.
453 36. Yoon J, Terada A, Kita H. CD66b regulates adhesion and activation of human eosinophils. J
454 Immunol. 2007;179(12):8454-62.

bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

455 37. Horie S, Kita H. CD11b/CD18 (Mac-1) is required for degranulation of human eosinophils induced
456 by human recombinant granulocyte-macrophage colony-stimulating factor and platelet-activating
457 factor. J Immunol. 1994;152(11):5457-67.
458 38. Tak T, Hilvering B, Tesselaar K, Koenderman L. Similar activation state of neutrophils in sputum
459 of asthma patients irrespective of sputum eosinophilia. Clin Exp Immunol. 2015;182(2):204-12.
460 39. Johansson MW, Gunderson KA, Kelly EA, Denlinger LC, Jarjour NN, Mosher DF. Anti-IL-5
461 attenuates activation and surface density of β(2) -integrins on circulating eosinophils after segmental
462 antigen challenge. Clin Exp Allergy. 2013;43(3):292-303.
463 40. Wilkerson EM, Johansson MW, Hebert AS, Westphall MS, Mathur SK, Jarjour NN, et al. The
464 Peripheral Blood Eosinophil Proteome. Journal of Proteome Research. 2016;15(5):1524-33.
465

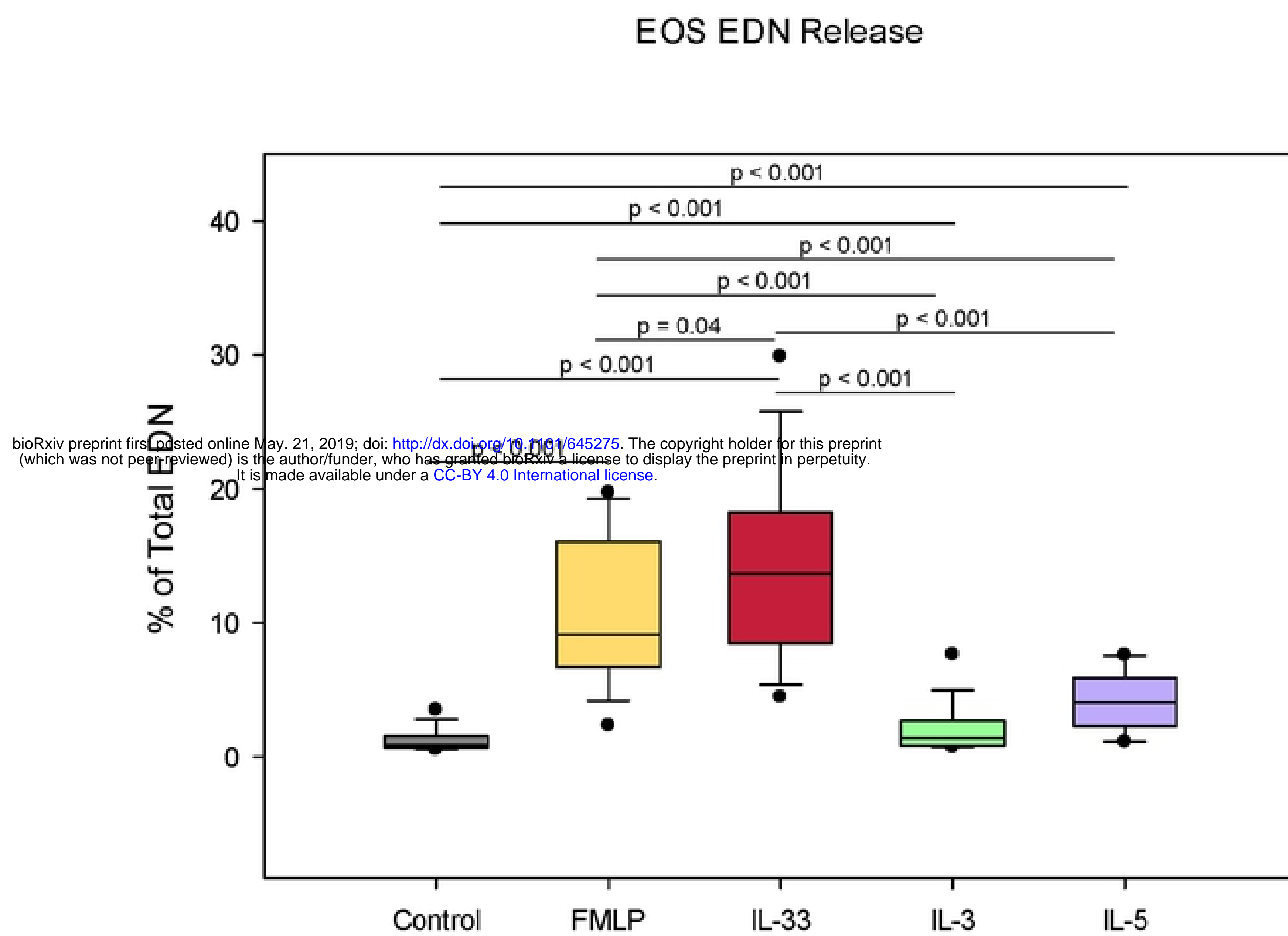
Figure 1.



bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

Figure 1

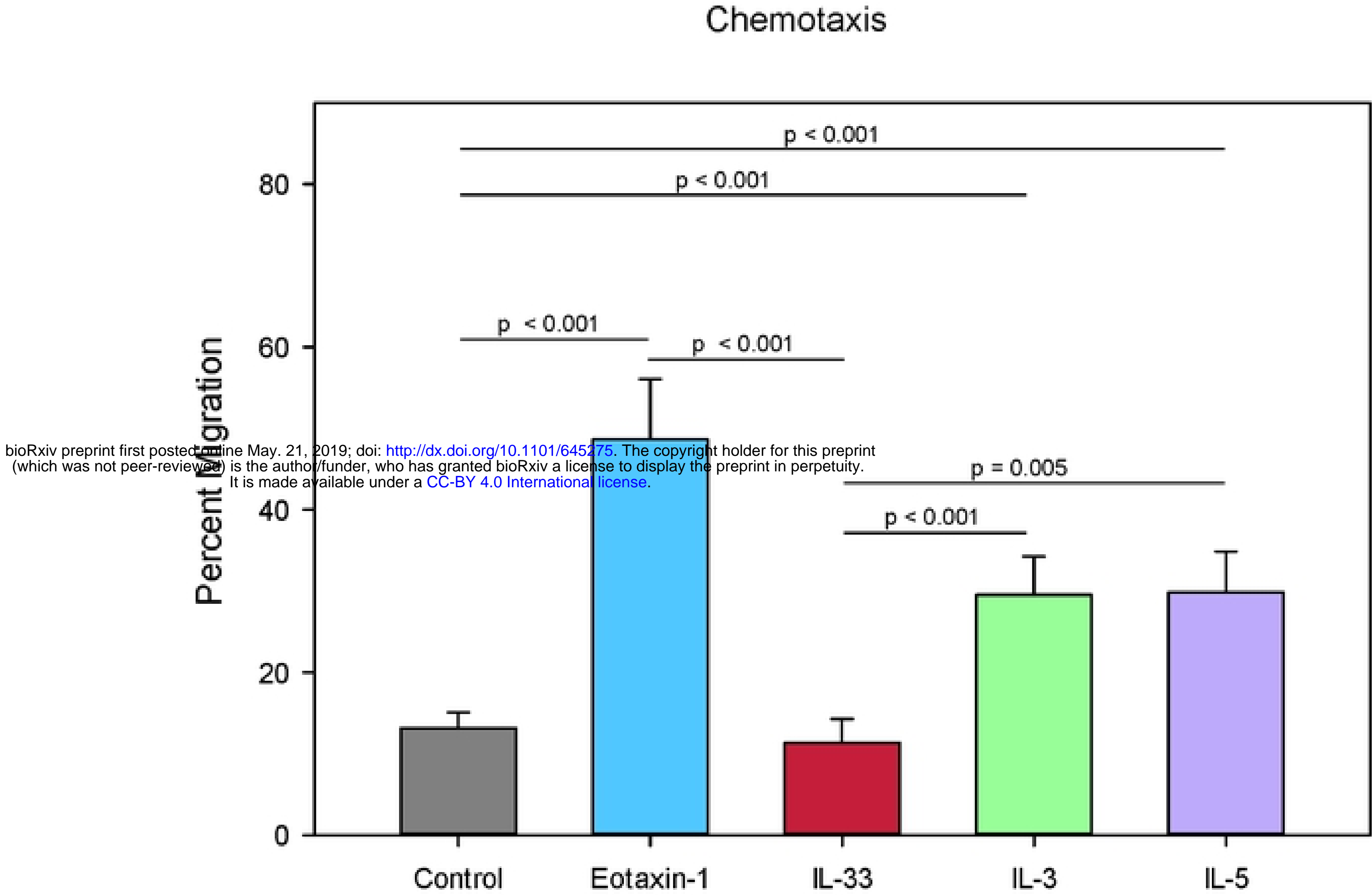
Figure 2.



bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

Figure 2

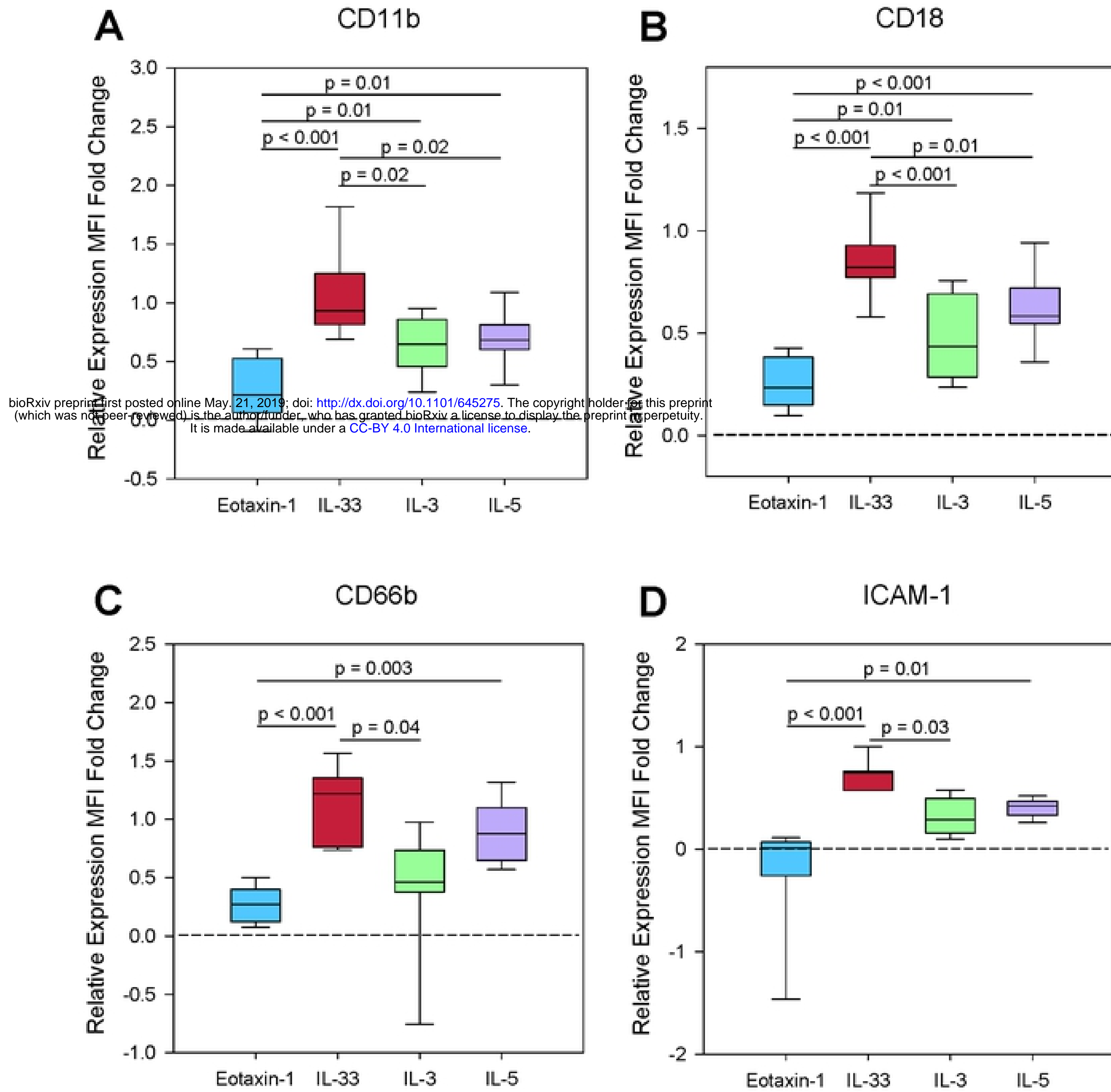
Figure 3.



bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

Figure 3

Figure 4.



bioRxiv preprint first posted online May. 21, 2019; doi: http://dx.doi.org/10.1101/645275. The copyright holder for this preprint (which was not peer-reviewed) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a CC-BY 4.0 International license.

Figure 4