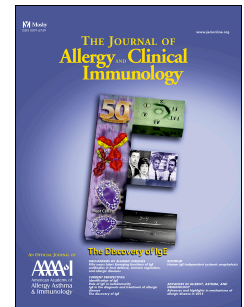


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Letter to the Editor, JACI

IL-33 induces functional CCR7 expression in human mast cells

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39

40 **1078 words**

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43

44 **Short summary**

45 Functional CCR7 expression—which enables Erk 1/2 phosphorylation, chemotaxis
46 and enhancement of cytokine production upon exposure to CCR7 ligands—was
47 induced in human mast cells, strongly by IL-33 and weakly by anti-IgE Ab
48 stimulation.

49

50 **Key words** human mast cell; IL-33; asthma; CCR7; chemotaxis; tertiary lymphoid
51 structures; migration.

52

53 **Abbreviations:**

54 CCL19: chemokine (C-C motif) ligand 19

55 CCL21: chemokine (C-C motif) ligand 21

56 CCR7: chemokine (C-C motif) receptor 7

57 IL-33: interleukin-33

58 MAPK: mitogen-activated protein kinases

59 NF-κB: nuclear factor-kappa B

60 TLS: tertiary lymphoid structures

61 TSLP: thymic stromal lymphopoietin

62

63 *To the Editor:*

64 The pathogenesis of allergic diseases is characterized by type 2 inflammation, which
65 involves several tissue-resident cells and immune cells, including mast cells. Recent
66 studies demonstrated that not only acquired but also innate immune responses play
67 critical roles in the development of type 2 inflammation. Mast cells play key roles in
68 induction of type 2 inflammation through both acquired and innate immune responses.
69 Those responses include degranulation, chemical mediator release and T_H2 cytokine
70 production initiated by IgE-crosslinking, and leukotriene synthesis and T_H2 cytokine
71 production mediated by epithelium-derived cytokines (IL-33 and thymic stromal
72 lymphopoietin (TSLP)).¹

73 In chronic type 2 inflammatory sites, such as asthmatic lungs or nasal polyps of
74 chronic rhinosinusitis, tertiary lymphoid structures (TLS) are formed. TLS reportedly
75 facilitate close interactions among different immune cell types and contribute to local
76 IgE production or persistence of airway inflammation and remodeling. As a result,
77 formation of TLS can be a therapeutic target for allergic diseases.² Mast cells are
78 reportedly localized in TLS.³ However, it remains unclear how mast cells are recruited
79 to TLS, because resting human cultured mast cells do not express CCR7, the receptor
80 for chemokines (CCL19 and CCL21) critical for TLS formation.⁴

81 To clarify the mechanisms by which human mast cells migrate into TLS, we used
82 microarray analysis to comprehensively assess the gene expression profiles of
83 chemokine receptors in human mast cells following exposure to IL-33 (a typical innate
84 stimulant), in two independent experiments with three separate donors, and exposure to
85 anti-IgE Ab (a typical acquired stimulation), in one experiment with three separate
86 donors. Detailed information regarding the methods is described in the methods section

in this article's Online Repository at www.jacionline.org. Consistent with previous studies, we found upregulation of IL-13, IL-5 and amphiregulin by IL-33 stimulation and upregulation of IL-13 by anti-IgE Ab stimulation. (See **Table E1** in this article's Online Repository at www.jacionline.org.) **Table E2** and **E3** in this article's Online repository at www.jacionline.org summarize the upregulated gene expression in IL-33- and anti-IgE Ab-stimulated human mast cells. The numbers of genes in these tables are shown in **Fig. E2**. IL-33 and anti-IgE stimulations induced almost equivalent numbers of genes in mast cells.

Among 19 analyzed chemokine receptor genes, only CCR7 mRNA expression was dramatically upregulated in human mast cells in response to IL-33 stimulation (**Table 1**), and this microarray result was confirmed by qPCR analysis. This upregulation was significantly inhibited by dexamethasone, but FK506 had no effect (**Fig 1A**). While the raw signal was 20-fold lower than that induced by IL-33 stimulation, anti-IgE Ab stimulation nevertheless induced statistically significant CCR7 mRNA upregulation.

To investigate whether the CCR7 on IL-33-stimulated mast cells was functional, we performed western blotting analysis for phosphorylation of two mitogen-activated protein kinases (MAPK), Erk 1/2 and p38, which are downstream signaling molecules of CCR7.⁵ We found strong phosphorylation of both molecules upon exposure to CCR7 ligands (CCL19 and CCL21) in IL-33-stimulated human mast cells, but not in non-stimulated mast cells (**Fig. 1B**). Additionally, CCR7-neutralizing antibody inhibited CCR7-mediated Erk 1/2 phosphorylation (**Fig. 1C**).

We performed a conventional chemotaxis assay to examine chemotaxis of human mast cells toward CCR7 ligands. IL-33-stimulated human mast cells showed statistically significant chemotaxis toward a CCR7 ligand, CCL21, whereas

111 non-stimulated mast cells did not (**Fig. 1D**). This migration was suppressed by adding
112 CCL21 to the upper chamber, indicating that CCR7-expressing human mast cells
113 exhibited significant chemotaxis toward CCL21. On the other hand, another CCR7
114 ligand, CCL19, showed a similar tendency for induction but did not reach statistical
115 significance, presumably because of its lower potency for chemotaxis compared with
116 that of CCL21. (See **Fig. E1A** in this article's Online repository at www.jacionline.org)

117 Finally, when we added CCR7 ligands to IL-33-stimulated mast cells, 2-fold
118 upregulation of 4 genes (MYRIP, IL10, IL13 and SYTL4) was observed, confirming
119 functional expression of CCR7 in IL-33-treated human mast cells. (See **Table E5** in this
120 article's Online Repository at www.jacionline.org)

121 Because IL-33 is highly expressed in innate-related type 2 inflammatory sites,
122 including asthmatic lungs and nasal polyps,⁶ we used IL-33 as a typical innate stimulant.
123 In this study, we demonstrated that IL-33 induced functional CCR7 expression in
124 human mast cells. This upregulation is particularly meaningful, because CCR7 was the
125 most-upregulated gene among all IL-33-activated genes (**Table E2**). These findings
126 suggest that both mast cell migration toward CCR7 ligand-expressing TLS and
127 enhancement of mediator production through CCR7 signaling are the main results of
128 IL-33's effects on mast cells. Indeed, mast cell-T cell proximity was observed in a
129 murine model of IL-33-induced type 2 inflammation.⁷ Based on our data, a typical
130 acquired stimulant (anti-IgE Ab) induced statistically significant, but 20-fold lower,
131 CCR7 expression, suggesting that *in vivo* mast cell migration to TLS is induced mainly
132 by innate stimulation.

133 Regarding the upstream signaling for CCR7 induction upon IL-33 stimulation, four
134 nuclear factor-kappa B (NF- κ B)/activator protein 1 (AP-1) binding sites have been

135 identified in the CCR7 promotor region.⁹ To clarify the upstream signaling pathway to
136 CCR7 by IL-33 stimulation, we performed *in silico* pathway analysis (See Online
137 Repository Methods, **Fig. E1C1** and **E1C2** in this article's Online Repository at
138 www.jacionline.org.) and found that induction of CCR7 by IL-33 is likely to be
139 mediated by NF- κ B, but not by NF-AT (**Fig. E1C3**), which was consistent with a
140 previous study that IL-33 induced NF- κ B phosphorylation in mouse mast cells.

141 This study has several limitations. First, we did not measure the CCR7 protein level
142 in IL-33-activated human mast cells. However, our results from the chemotaxis assay,
143 Erk 1/2 phosphorylation assay and microarray analysis upon exposure to CCR7 ligands
144 indicated that CCR7 protein must be expressed in IL-33-activated human mast cells.
145 Second, we stimulated mast cells with either IL-33 or anti-IgE Ab alone, but not in
146 combination. A recent study suggested that combined IL-33 and IgE-mediated
147 stimulation may have some crosstalk that enhances their signals.¹ This should be further
148 investigated in the future. Third, it remains unclear whether connective tissue mast cells
149 show similar results or not, since we studied only mucosal-type cultured human mast
150 cells. Finally, we think that the 100 ng/ml IL-33 used in this study was relatively high,
151 but probably not extremely high, and CCR7 upregulation on mast cells may occur in
152 local tissue sites.

153 In conclusion, we demonstrated that functional CCR7 expression—which may
154 enable CCR7-expressing human mast cells to migrate to T-cell-rich TLS having CCR7
155 ligands and to enhance mediator production—was strongly induced in human mast cells
156 by IL-33 (typical innate stimulation), and more weakly by anti-IgE Ab (acquired
157 stimulation). (see **Fig E1D** in this article's Online Repository at www.jacionline.org).

158 These results contribute to elucidation of the pathogenesis of allergic diseases and a

159 novel therapeutic target of asthma exacerbation following virus infection.

160

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168

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REFERENCES

1. Joulia R, L'Faqihi FE, Valitutti S, Espinosa E. IL-33 fine tunes mast cell degranulation and chemokine production at the single-cell level. *J Allergy Clin Immunol* 2017; 140:497-509.
2. Pitzalis C, Jones GW, Bombardieri M, Jones SA. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nat Rev Immunol* 2014; 14:447-62.
3. Larocca LM, Maggiano N, Capelli A, Bevilacqua P, Ruscito P, Maurizi M, et al. Immunopathology of nasal polyps: an immunohistochemical approach. *Ann Allergy* 1989; 63:508-12.
4. Nakajima T, Iikura M, Okayama Y, Matsumoto K, Uchiyama C, Shirakawa T, et al. Identification of granulocyte subtype-selective receptors and ion channels by using a high-density oligonucleotide probe array. *J Allergy Clin Immunol* 2004; 113:528-35.
5. Riol-Blanco L, Sanchez-Sanchez N, Torres A, Tejedor A, Narumiya S, Corbi AL, et al. The chemokine receptor CCR7 activates in dendritic cells two signaling modules that independently regulate chemotaxis and migratory speed. *J Immunol* 2005; 174:4070-80.
6. Prefontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, et al. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* 2010; 125:752-4.
7. Morita H, Arae K, Unno H, Miyauchi K, Toyama S, Nambu A, et al. An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity* 2015; 43:175-86.
8. Guest IC, Sell S. Bronchial lesions of mouse model of asthma are preceded by immune complex vasculitis and induced bronchial associated lymphoid tissue (iBALT). *Lab Invest* 2015; 95:886-902.
9. Mburu YK, Egloff AM, Walker WH, Wang L, Seethala RR, van Waes C, et al. Chemokine receptor 7 (CCR7) gene expression is regulated by NF-kappaB and activator protein 1 (AP1) in metastatic squamous cell carcinoma of head and neck (SCCHN). *J Biol Chem* 2012; 287:3581-90.

215 **FIGURE LEGENDS**

216 **FIG 1. Functional CCR7 expression was induced in human mast cells, strongly by**
217 **IL-33 and weakly by anti-IgE Ab stimulation.**

218 **A.** qPCR analysis of CCR7 mRNA expression in human mast cells after treatment with
219 IL-33 or anti-IgE Ab following IgE sensitization. In some experiments, cells were
220 pre-incubated with dexamethasone (DEX) or FK506. **B.** Western blotting analysis of
221 Erk1/2 and p38 phosphorylation in human mast cells treated with and without IL-33.
222 Cells were stimulated with a mixture of CCL19 and CCL21 for the indicated times.
223 CESS is a CCR7⁺ B-cell line used as a positive control. **C.** For neutralization assay,
224 human mast cells were pre-incubated with anti-CCR7 Ab for 1 h before CCR7 ligand
225 stimulation. **D.** Chemotaxis assay of human mast cells treated with and without IL-33
226 for 48 h. Cells were stimulated with 200 or 1000 ng/ml of CCL21. All data show the
227 mean $\pm$ SE of three independent experiments using mast cells from three separate
228 donors. * P < 0.05, ** P < 0.01.

Table I. List of gene expression levels of chemokine receptors after IL-33 or anti-IgE Ab stimulation of human mast cells.

Gene symbol	Agilent ^a probe ID	Control		IL-33		Regulation	<i>P</i> value	IgE		IgE + anti-IgE Ab	
		Raw signal	D/N ^b	Raw signal	D/N ^b			Raw signal	D/N ^b	Raw signal	D/N ^b
CCR1	A_24_P148717	35.97	D	74.94	D	down	0.978	51.11	D	24.21	D
CCR2	A_23_P324885	6.68	D	3.20	N	down	0.640	4.26	N	4.52	N
CCR3	A_23_P250302	11.87	N	2.81	N	down	0.340	4.54	N	4.84	N
CCR4	A_23_P72982	149.36	D	179.99	N	down	0.735	200.09	D	69.07	D
CCR5	A_23_P412321	241.46	D	216.63	D	down	0.963	348.27	D	427.16	D
CCR6	A_33_P3313929	405.75	D	271.11	D	down	0.576	582.85	D	589.85	D
CCR7	A_23_P343398	6.11	N	10293.30	D	up	<0.001**	34.77	D	474.74	D
CCR8	A_23_P69012	5.37	N	2.64	N	down	0.636	4.55	N	4.49	N
CCR9	A_33_P339559	5.95	N	2.89	N	down	0.631	4.77	N	4.63	N
CCR10	A_33_P3221303	37.17	D	23.37	D	down	0.655	38.84	D	36.35	D
CXCR1	A_23_P67932	10.82	N	11.95	N	up	0.125	9.32	N	33.45	N
CXCR2	A_33_P3214550	8.38	N	6.01	N	up	0.766	18.70	N	4.11	N
CXCR3	A_23_P114299	169.67	D	105.07	D	down	0.841	179.37	D	396.48	D
CXCR4	A_23_P102000	165.23	D	705.03	D	up	0.648	112.86	D	1244.14	D
CXCR5	A_24_P252945	8.75	N	31.73	D	up	0.047*	12.50	N	12.82	N
CXCR6	A_23_P109913	8.50	N	2.81	N	down	0.054	5.45	D	15.95	N
CXCR7	A_23_P131676	5.88	N	2.81	N	down	0.605	4.48	N	4.47	N
CX3CR1	A_23_P114299	1689.67	D	105.07	D	down	0.152	57.74	D	52.33	D
XCR1	A_23_P18246	132.45	D	0.00	D	down	0.747	216.61	D	253.00	D

The IL-33 stimulation was performed in two independent experiments, and the anti-IgE Ab stimulation was performed in one experiment. The raw signal data in the control and IL-33 columns show the mean of two independent experiments.

^a All probe information for the Agilent microarray is described at <https://earray.chem.agilent.com/earray/>

^b D: Detected; N: Not detected.

p* < 0.05, *p* < 0.01

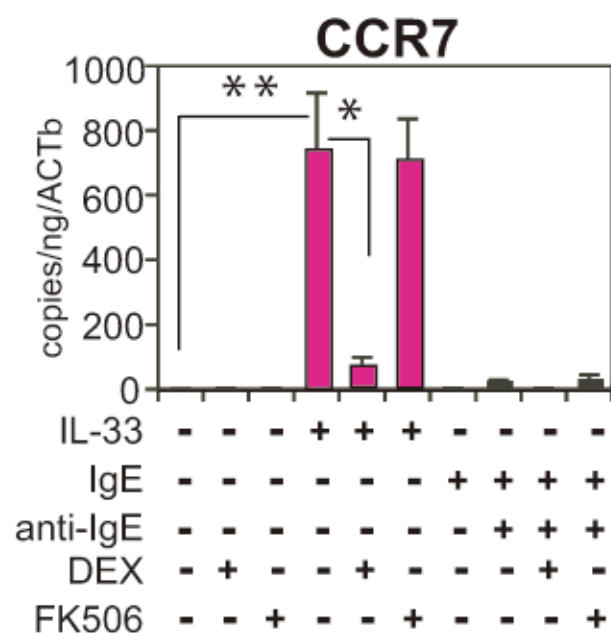
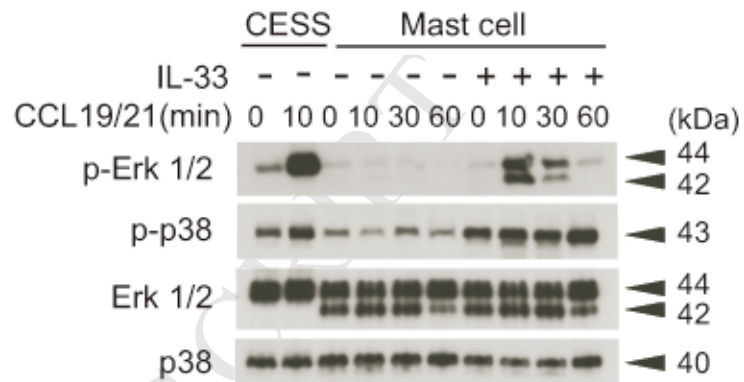
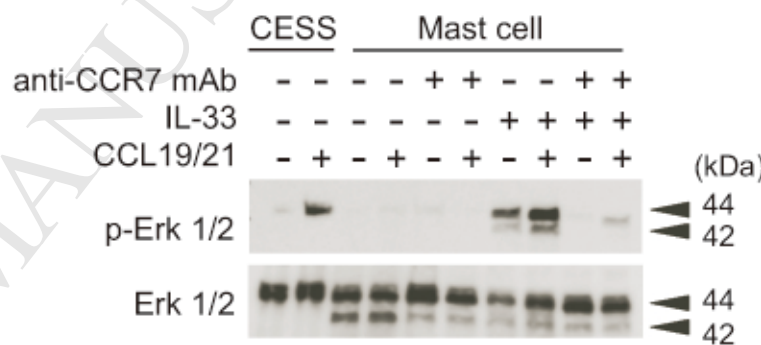
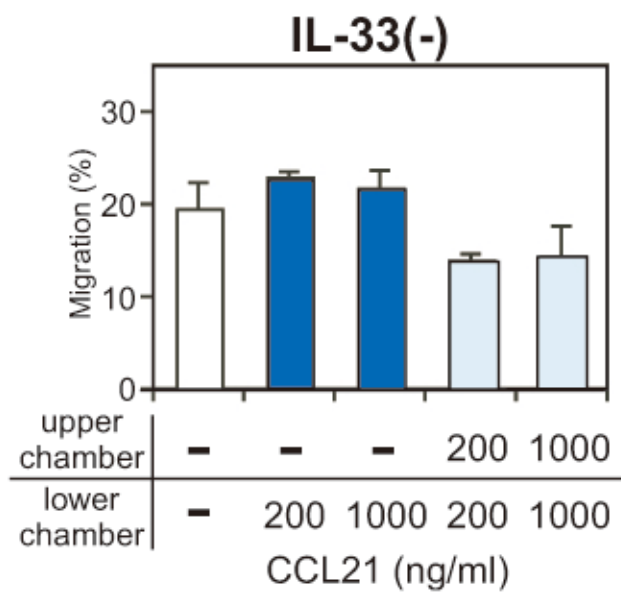
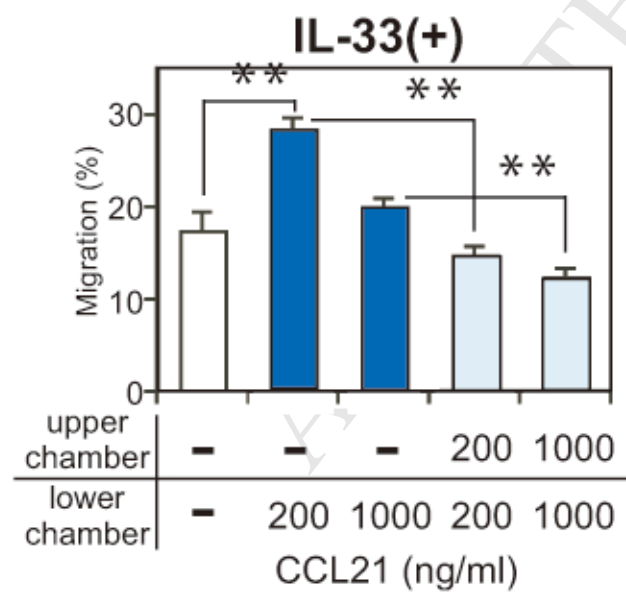
A**B****C****D**

Table E1. The top 4 up-regulated genes following IL-33 stimulation of human mast cells.

Gene symbol	Agilent probe ID	Control		IL-33		Fold change	IgE		Anti-IgE Ab		Fold change
		Raw signal	D/N ^a	Raw signal	D/N ^a		Raw signal	D/N ^a	Raw signal	D/N ^a	
CCR7	A_23_P343398	6.11	N	10293.30	D	2316.5	34.77	D	474.74	D	13.65
IL-13	A_23_P251031	5.74	N	8001.20	D	1918.0	4.62	N	1871.63	D	405.11
IL-5	A_24_P209047	5.66	N	5052.88	D	1227.9	7.20	N	6121.31	D	5.00
AREG	A_23_P259071	5.80	N	4238.60	D	1005.2	176.60	D	11846.64	D	67.08

The IL-33 or anti-IgE Ab stimulation was performed in two or one independent experiments, respectively. The fold-change data were filtered using the gene expression in IL-33 stimulation versus the control. The raw signal data in the control and IL-33 columns show the mean of two independent experiments.

^a D: Detected; N: Not detected

9 **Table E2. Expression profile list of 5-fold-changed genes in IL-33-stimulated human mast cells with**
 10 **and without DEX or FK506 treatment.**

Agilent probe ID	Fold change (IL-33 / control)	Raw signal						Gene symbol
		Control	FK506	DEX	IL-33	FK506+ IL-33	DEX+ IL-33	
A_23_P343398	2316.5	6.1	11.4	3.1	10293.3	14254.7	922.9	CCR7
A_23_P251031	1918.0	5.7	6.0	3.4	8001.2	9109.4	410.4	IL13
A_24_P209047	1227.9	5.7	5.9	2.8	5052.9	5604.3	155.2	IL5
A_23_P259071	1005.2	5.8	494.0	2.9	4238.6	6407.2	7586.6	AREG
A_32_P108156	944.5	8.6	6.9	6.1	5911.9	9384.6	3658.9	MIR155HG
A_33_P3268304	874.7	5.2	5.6	4.0	3298.0	4506.5	3670.0	LIMS2
A_23_P121064	762.8	64.0	238.7	44.2	35469.2	46963.7	41175.5	PTX3
A_23_P121533	577.1	6.6	39.7	3.1	2754.3	2871.4	6681.1	SPON2
A_23_P49759	552.4	46.4	32.0	19.6	18631.3	20234.0	15322.0	CCL1
A_33_P3301709	416.9	5.2	5.4	2.7	1585.1	2600.1	549.4	GNG4
A_33_P3419190	413.8	5.7	218.3	3.4	1710.5	2562.6	3077.8	AREG
A_33_P3329013	408.3	8.0	185.1	11.3	2387.3	3236.7	464.6	SSTR2
A_23_P356616	370.2	14.3	32.1	16.7	3858.7	5302.1	1481.1	ABTB2
A_23_P68031	343.8	9.3	13.4	11.7	2319.0	3693.2	785.7	STAT4
A_33_P3354607	341.4	55.6	15.2	26.6	13789.1	19330.8	3113.9	CCL4
A_19_P00812504	331.1	16.5	29.3	17.5	3978.0	4759.5	667.4	
A_23_P119478	252.2	133.6	598.7	79.4	24482.2	38259.5	46311.7	EBI3
A_23_P207564	241.0	88.1	49.3	41.2	15424.1	21382.1	3712.9	CCL4
A_23_P51936	195.3	26.6	6.0	22.9	3778.6	6113.6	1658.5	TNFRSF9
A_23_P251075	188.8	11.5	8.3	14.7	1580.6	2446.7	656.8	MAMLD1
A_23_P26024	175.0	325.6	171.2	217.9	41419.7	58677.3	19939.0	C15orf48
A_33_P3353791	170.9	8.8	5.7	10.3	1088.7	2252.4	251.7	ITGA1
A_23_P133408	151.2	206.5	11.5	82.8	22697.7	32346.1	3189.1	CSF2
A_23_P79518	130.1	985.4	948.5	639.1	93184.1	117615.9	11073.6	IL1B
A_32_P87013	127.2	25.7	6.4	12.3	2371.8	2780.1	108.6	IL8
A_33_P3242543	119.0	99.0	162.0	70.7	8566.6	14231.1	7278.1	MAOA
A_23_P19226	107.2	248.5	553.1	160.5	19360.1	29960.7	23838.0	DSE
A_33_P3718269	101.6	18.5	6.6	11.0	1367.6	1905.0	470.8	
A_24_P351283	89.0	18.9	11.3	15.1	1223.9	1707.7	774.7	MREG
A_23_P81898	87.1	40.1	32.4	25.0	2538.6	4616.4	2078.9	UBD
A_23_P122924	72.5	101.2	70.2	72.0	5332.1	8378.4	336.1	INHBA
A_23_P108948	71.4	123.4	92.1	73.7	6400.3	8498.1	3797.6	MREG
A_23_P7402	55.4	45.1	27.6	35.9	1814.6	3225.6	1817.5	PDZD2
A_23_P109034	54.1	110.2	123.4	68.1	4327.8	5664.6	2510.4	SDC4
A_24_P257416	50.3	63.7	17.0	42.1	2328.6	3294.4	328.7	CXCL2

A_23_P57760	49.3	90.2	2360.1	51.4	3233.4	4289.5	10325.5	ACPL2
A_24_P89891	46.4	167.3	122.3	104.5	5639.8	9919.6	2207.5	TRAF1
A_23_P404494	44.9	155.6	571.3	85.4	5072.0	8270.3	8194.6	IL7R
A_24_P353638	40.8	146.4	201.8	84.3	4344.5	5347.1	671.8	SLAMF7
A_23_P5903	40.4	38.6	108.7	2.7	1133.1	1605.2	2144.7	SLCO4A1
A_24_P411121	40.3	81.4	46.2	65.8	2384.8	2894.2	1664.2	TNFRSF18
A_23_P315364	39.9	138.1	40.7	81.0	4007.5	4961.9	465.3	CXCL2
A_23_P134176	34.7	3246.6	4646.4	2529.2	81942.2	113198.6	65497.8	SOD2
A_23_P98350	33.4	3249.7	5144.1	1739.6	78938.9	97223.9	61046.0	BIRC3
A_23_P152002	32.1	267.0	305.6	158.9	6222.1	9346.0	2522.9	BCL2A1
A_33_P3393766	30.3	126.5	49.2	36.6	2786.5	3206.8	2327.2	C17orf96
A_33_P3342628	29.5	76.1	45.1	28.2	1631.9	2594.6	768.0	HES4
A_24_P157926	28.0	149.3	927.5	101.2	3041.1	4590.3	9829.8	TNFAIP3
A_23_P210482	26.2	1257.2	718.8	840.0	23978.1	37932.5	11620.0	ADA
A_33_P3316273	26.1	242.6	70.5	109.6	4608.3	4792.8	377.6	CCL3
A_23_P421423	22.4	2167.0	1251.2	1410.4	35317.9	49931.1	27528.9	TNFAIP2
A_33_P3272231	20.2	106.9	41.7	67.1	1571.7	1931.2	527.0	MFSD2A
A_33_P3375934	20.0	442.5	628.4	315.6	6433.4	8926.9	7555.6	NAMPT

IL-33 stimulation of human mast cells was performed in two independent experiments using cells from three separate donors. Data were filtered using IL-33 stimulation with fold change > 20 (compared with control) and raw signal > 1000. The raw signal data in the control and IL-33 columns show the mean of the two independent experiments.

17 **Table E3. Expression profile list of 5-fold-changed genes in anti-IgE Ab-stimulated human mast cells**
 18 **with and without DEX or FK506 treatment.**

Agilent probe ID	Fold change (IgE+anti-IgE Ab / IgE)	Raw signal				Gene symbol
		IgE	IgE+anti-IgE Ab	IgE+anti-IgE Ab + DEX	IgE+anti-IgE Ab + FK506	
A_33_P3354607	3845.4	45.1	180639.2	136662.1	40422.3	CCL4
A_33_P3419264	3312.6	4.8	16667.5	830.4	3.8	IL3
A_23_P207564	1830.7	94.5	180121.0	140621.3	48026.4	CCL4
A_24_P209047	817.1	7.2	6121.3	228.3	65.9	IL5
A_33_P3304065	670.1	14.1	9853.9	3291.3	15.0	AQP2
A_32_P87013	647.3	41.2	27743.3	4445.8	19563.2	IL8
A_23_P49759	571.2	263.7	156781.5	102388.1	8415.5	CCL1
A_24_P228130	519.2	31.2	16868.8	7334.6	2145.8	CCL3L3
A_33_P3316273	427.9	188.4	83935.8	42217.7	16155.2	CCL3
A_19_P00320202	407.7	4.2	1774.3	597.1	586.9	
A_23_P251031	389.5	4.6	1871.6	61.4	573.2	IL13
A_24_P183150	373.9	23.2	9012.1	1434.1	18536.3	CXCL3
A_23_P125233	253.4	8.9	2356.5	1330.0	9.8	CNN1
A_23_P80570	238.9	7.2	1800.0	820.9	411.5	AADAC
A_32_P345659	235.0	13.3	3254.6	2702.7	21.9	XIRP1
A_23_P305092	227.0	4.5	1067.3	123.1	64.8	CRTAM
A_24_P38290	209.1	4.9	1073.7	246.9	6.4	TAC1
A_33_P3296181	195.8	35.3	7196.9	3188.0	1065.7	CCL3L3
A_33_P3386547	163.9	12.3	2097.3	1242.8	657.0	SGPP2
A_32_P108156	145.6	68.3	10350.1	4180.1	3841.7	MIR155HG
A_23_P122924	128.6	180.6	24176.0	6240.3	23577.1	INHBA
A_24_P252497	108.4	56.1	6328.6	4398.7	5887.1	TRIB1
A_33_P3301709	107.1	46.7	5205.9	2612.9	6247.8	GNG4
A_24_P257416	106.7	87.5	9719.1	2607.4	13400.4	CXCL2
A_33_P3332018	103.7	71.7	7738.1	4737.9	820.2	FGL2
A_23_P62647	100.5	65.2	6819.8	14583.8	439.3	SLAMF1
A_24_P158089	94.3	15.9	1561.0	2727.5	631.0	SERPINE1
A_23_P121533	90.0	17.5	1637.4	1126.3	36.8	SPON2
A_23_P315364	86.0	115.4	10327.3	2380.4	7340.6	CXCL2
A_33_P3237150	73.1	35.8	2724.2	682.7	50.9	BMP2
A_23_P133408	70.7	563.8	41472.5	4959.0	4858.3	CSF2
A_19_P00812504	65.3	139.0	9444.2	1601.1	2197.6	
A_23_P259071	64.4	176.6	11846.6	21191.7	26367.8	AREG
A_33_P3419190	62.2	74.3	4807.4	8402.1	13677.0	AREG
A_33_P3330264	62.1	39.8	2571.5	793.6	4316.3	CXCL1

A_23_P356616	60.0	60.0	3746.1	2432.4	1996.5	ABTB2
A_23_P46936	58.3	75.1	4557.8	1539.9	3756.7	EGR2
A_23_P257043	54.1	128.1	7213.5	3409.6	4239.2	GEM
A_32_P74409	41.5	102.5	4422.3	2946.5	463.1	C11orf96
A_23_P68031	39.3	40.2	1642.4	904.0	3297.1	STAT4
A_23_P398566	39.1	73.8	3006.3	926.4	1164.6	NR4A3
A_23_P10385	37.5	104.5	4075.8	3980.1	924.8	DTL
A_24_P10137	37.2	360.4	13959.2	8834.9	21429.4	C13orf15
A_24_P237036	37.2	89.1	3450.5	1805.7	1911.6	TNFSF14
A_23_P51936	37.1	392.2	15148.5	6307.7	4074.0	TNFRSF9
A_24_P122921	36.1	59.4	2229.3	2060.3	587.9	BCL2L11
A_23_P102731	35.3	863.7	31706.7	12896.8	22232.4	SMOX
A_23_P78037	32.3	72.1	2420.4	444.9	3949.3	CCL7
A_33_P3251841	32.0	47.3	1577.3	431.6	130.6	DSEL
A_23_P5903	29.4	55.1	1689.7	1589.7	583.5	SLCO4A1
A_33_P3342628	25.9	505.1	13600.8	6906.9	9784.5	HES4
A_23_P251075	24.9	99.4	2572.7	1035.8	2910.2	MAMLD1
A_23_P167096	24.6	40.1	1027.7	1107.1	925.6	VEGFC
A_23_P152002	24.0	358.8	8969.2	3070.2	11104.4	BCL2A1
A_23_P117602	23.0	8864.1	212610.8	166895.0	151410.0	GZMB
A_23_P108751	22.7	135.3	3191.6	1605.0	1910.1	FHL2
A_23_P57760	22.2	135.8	3142.8	10553.7	4569.2	ACPL2
A_23_P134176	22.1	6457.5	148849.5	129934.3	51236.6	SOD2
A_23_P150018	21.5	146.8	3284.2	1740.9	2300.9	DUSP5
A_33_P3354935	21.2	475.3	10470.6	5417.9	1737.7	CSF1
A_19_P00317560	21.1	47.6	1046.4	705.7	1079.7	
A_33_P3474319	20.3	100.2	2121.6	4109.7	30.0	SLC37A2
A_23_P109034	20.3	286.9	6054.3	3248.2	1740.5	SDC4
A_23_P369815	20.3	53.1	1120.6	437.1	668.7	FASLG
A_23_P203475	20.0	201.2	4186.7	2779.5	338.5	PRKCDBP

Anti-IgE Ab stimulation was performed in one experiment using cells from three separate donors. Data were filtered using anti-IgE Ab stimulation with fold change > 20 (compared with control) and raw signal > 1000.

Table E4. Adhesion-related molecules upregulated by IL-33 or anti-IgE Ab stimulation of human mast cells.

Gene symbol	Agilent Probe ID	Control		IL-33		Fold change (IL-33/control)	Regulation	IgE		IgE+anti-IgE		Fold change (IgE+anti-IgE/IgE)	Gene name
		Raw signal	D/N ^a	Raw signal	D/N ^a			Raw signal	D/N ^a	Raw signal	D/N ^a		
CDH13	A_32_P85999	3.35	N	2081.83	D	621.44	up	4.68	N	4.69	N	1.00	cadherin 13, H-cadherin
ITGB4	A_33_P3377364	75.38	D	1128.10	D	14.97	up	34.43	D	7.95	N	0.23	integrin, beta 4
SELL	A_33_P3400273	533.73	D	4800.37	D	8.99	up	461.27	D	223.34	D	0.48	selectin L
MMP9	A_23_P40174	20850.41	D	53224.91	D	2.64	up	1508.61	D	3455.35	D	2.20	matrix metalloproteinase 9, mRNA
ICAM3	A_23_P164691	3013.32	D	5793.83	D	1.92	up	5427.81	D	4478.01	D	0.83	intercellular adhesion molecule 3

Human peripheral CD34⁺-derived mast cells from three separate donors were stimulated for 6 h with 100 ng/ml IL-33 in two independent experiments. The raw signal data in the control and IL-33 columns show the mean of two independent experiments.

^a D: Detected; N: Not detected.

Table E5. Genes upregulated by stimulation of IL-33-primed human mast cells with CCR7 ligands (CCL19 and CCL21).

Gene symbol	Agilent Probe ID	Control		CCL19/21		IL-33		IL-33+ CCL19/21		Fold Change (IL33+CCL19/21 vs IL33)	Regulation	Gene name
		Raw signal	D/N ^a	Raw signal	D/N ^a	Raw signal	D/N ^a	Raw signal	D/N ^a			
MYRIP	A_23_P326760	622.60	D	569.81	D	457.61	D	1353.14	D	2.96	up	myosin VIIA and Rab interacting protein
IL10	A_23_P126735	394.76	D	318.35	D	1015.31	D	2184.00	D	2.15	up	interleukin 10
IL13	A_23_P251031	7.96	N	3.90	N	7285.45	D	15183.22	D	2.08	up	interleukin 13
SYTL4	A_24_P122337	508.78	D	407.73	D	970.87	D	1972.24	D	2.03	up	synaptotagmin-like 4

Human peripheral CD34⁺-derived mast cells from three separate donors were stimulated for 48 h with 100 ng/ml IL-33 and then stimulated for 6 h with 1000 ng/ml CCL19 and 200 ng/ml CCL21. The raw signal data in the control and IL-33 columns show the mean of two independent experiments.

^a D: Detected; N: Not detected.

Letter to the Editor, JACI

Online Repository Materials

IL-33 induces functional CCR7 expression in human mast cells

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Online repository Methods

Mast cell culture, stimulation and mRNA expression analysis

Human mast cells were derived from hematopoietic progenitor cells according to a protocol established previously in our laboratory.¹ We generated human mast cells from peripheral blood CD34⁺ cells from three separate donors (Lonza, Basel, Switzerland), and the final purity of the mast cells always exceeded 98% by Giemsa staining. Cells were sensitized with 1 µg/ml human myeloma IgE (Calibiochem, La Jolla, CA) at 37°C for 24 h, and then washed, preincubated with 0.1 µM dexamethasone (DEX) (Sigma-Aldrich, St. Louis, MO), 100 nM FK506 (Calibiochem) or 0.01% DMSO (Sigma-Aldrich) for 1 h and finally stimulated with 1.5 µg/ml anti-IgE Ab (Dako, Carpinteria, CA) for 6 h (mRNA) or 48 h (functional assay). For interleukin-33 (IL-33: Pepro Tech, Rocky Hill, NJ) stimulation, cells were preincubated with 0.1 µM DEX, 0.1 µM FK506 or 0.01 % DMSO for 1 h and then stimulated with 100 ng/ml IL-33.

For CCR7 ligands stimulation, IL-33-stimulated mast cells from three separate donors were further stimulated for 6 h with 1000 ng/ml recombinant human chemokine (C-C motif) ligand 19 (CCL19) and 200 ng/ml chemokine (C-C motif) ligand 21 (CCL21) (R & D Systems, Minneapolis MN).

Microarray analysis and qPCR analysis were performed using microarray technology (Agilent Technologies, Santa Clara, CA) as described elsewhere.²

CCR7 upstream signaling pathway analysis

In silico analysis was performed by the following steps. (1) We hypothesized that a gene set containing CCR7 that was upregulated by IL-33 and downregulated by dexamethasone treatment but not by FK506 treatment has a shared signal transduction pathway. Therefore, we first extracted, from the microarray data, 1124 genes that were upregulated (>2 fold) and meaningfully upregulated (raw signal >1000) by IL-33 stimulation of human mast cells (**Fig. E1C1**). (2) From among those upregulated genes, we further extracted 285 genes that were downregulated (<2 fold) by dexamethasone treatment but not by FK506 treatment (**Fig. E1C2**). And (3), using Ingenuity Pathway Analysis (IPA: Ingenuity Systems, Inc., Redwood City, CA) we searched for a common upstream signaling pathway of those extracted 285 genes that include CCR7 (**Fig. E1C3**). The identified upstream regulators were filtered using a cut-off of 3.6-fold (top 6 upregulated gene) in the z-score algorithm, which was calculated using the gene expression (fold change) in the microarray data and the expected effects for on their known target genes in the Ingenuity Knowledge Base. The statistical significance between our uploaded genes and the upstream regulators (predicted target molecules) was calculated by Fisher's exact test. A z-score of >2 and $p < 0.01$ were considered to indicate a significantly "activated" upstream regulator.

Western Blot analysis

IL-33-stimulated mast cells were pre-treated with 200 $\mu\text{g/ml}$ monoclonal mouse anti-human CCR7 mAb (clone #150503: R & D Systems) for 1 h at 4°C, and then stimulated with 1000 ng/ml CCL19 and 200 ng/ml CCL21 for the indicated times. Immunoblotting was performed using rabbit anti-human phospho-p44/42 mitogen-activated protein kinase (MAPK) (p-Erk 1/2) monoclonal Ab (mAb; clone E10) rabbit anti-human phospho-p38 MAPK (p-p38) mAb (clone D3F9), rabbit anti-human Erk 1/2 mAb (clone #9102) and rabbit anti-human p38 MAPK (p38) mAb (clone #9212: Cell Signaling Technology, Danvers, MA) and horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) as the 2nd Ab.

Mast cell chemotaxis assays

Mast cell migration was measured by the Boyden chamber technique (Neuro Probe Inc., Bethesda, MD) and calcein-AM as described previously³ with slight modification, and conducted 5 or 3 independent experiments using IL-33-treated or untreated human mast cells. According to our microarray data, IL-33 stimulation for 48 h induced phenotypic changes in human mast cells with regard to the expression of adhesion molecules (CDH13, ITGB4, SELL and ICAM3) and protease (MMP9) (**Table E4**). Therefore, we thought that direct comparison between IL-33-stimulated and unstimulated mast cells might be inappropriate, and we compared the CCL21-induced chemotaxis in IL-33-stimulated and unstimulated cells separately (**Fig. 1D** and **Fig. E1**).

Statistical analysis

Data are presented as the mean $\pm$ SE and analyzed using Student's t-test (two-tailed) for microarray analysis, and multiple comparisons between treatment and control conditions were performed by ANOVA with Bonferroni's *post hoc* test. * $P < 0.05$, ** $P < 0.01$

REFERENCES

- E1. Saito H, Kato A, Matsumoto K, Okayama Y. Culture of human mast cells from peripheral blood progenitors. *Nat Protoc* 2006; 1:2178-83.
- E2 Shoda T, Matsuda A, Arai K, Shimizu H, Morita H, Orihara K, et al. Sera of patients with infantile eosinophilic gastroenteritis showed a specific increase in both thymic stromal lymphopoietin and IL-33 levels. *J Allergy Clin Immunol* 2016;138:299-303.
- E3. Smrz D, Bandara G, Beaven MA, Metcalfe DD, Gilfillan AM. Prevention of F-actin assembly switches the response to SCF from chemotaxis to degranulation in human mast cells. *Eur J Immunol* 2013;43:1873-82

82 **Supplemental Fig. E1.**

83 **A. CCL19 induced modest migration of IL-33-stimulated human mast cells. B. The numbers of**
84 **upregulated genes (IL-33 and/or anti-IgE Ab treatments;**

85 **B1) and downregulated genes (dexamethasone and/or FK506 treatments; B2 and B3).**

86 **C. NF- κ B is located in the upstream pathway of CCR7. D. IL-33-activated mast cells into TLS and may**
87 **play critical roles in the pathogenesis of type 2 inflammation-related allergic diseases.**

88 **E1D. Diagram of the proposed mechanism by which mast cells migrate into tertiary lymphoid**
89 **structures (TLS) and play critical roles in the pathogenesis of type 2 inflammation-related allergic**
90 **diseases.**

91 IL-33 is released by epithelial/endothelial cells as a result of activation or tissue damage by viral
92 infection or protease allergen exposure (innate immune responses).

93 Functional CCR7, a receptor for key cytokines (CCL19 and CCL21) for TLS formation, is induced
94 in human mast cells by IL-33, but not by anti-IgE mAb (an acquired immune response). Migration
95 of mast cells toward TLS is thought to contribute to exacerbation of type 2 inflammation.
96
97

