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A case of mistaken identity: The MAR-1 antibody to mouse

FcεRIα cross-reacts with FcγRI and FcγRIV

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Key Words: Basophil, Dendritic Cell, Fc receptor, Fc epsilon RI, Fc gamma RI, Fc gamma RIV, IgE,

Macrophage, Mast cell, Monocyte, Neutrophil

Capsule Summary:

This study demonstrates that the MAR-1 antibody to mouse FcepsilonRI unexpectedly binds to two Fcgamma receptors, raising concerns regarding numerous studies that used this antibody to assess the distribution and function of FcepsilonRI+ cells.

To the Editor:

IgE is a primary driver of allergic symptoms mediated by cells expressing the high affinity Fc receptor for IgE, FcεRI. The cell types expressing FcεRI and downstream pathways have been extensively studied in mouse models of allergic disease. The MAR-1 monoclonal antibody is thought to specifically recognize the alpha chain of mouse FcεRI. Although FcεRI was initially thought to be uniquely expressed by basophils and mast cells in mice,¹ several recent studies using the MAR-1 antibody for flow cytometric analysis reported that FcεRI is expressed on dendritic cells, particularly monocyte-derived dendritic cells (moDCs), in various inflammatory conditions, such as exposure to house dust mite, the bacterial cell wall component lipopolysaccharide (LPS) or Sendai virus.²⁻⁴ The MAR-1 antibody has also been injected into mice for numerous functional studies, including depletion of basophils⁵ and rapid desensitization.⁶ Surprisingly, several results obtained with MAR-1-mediated basophil depletion have not been recapitulated in genetic models of basophil depletion⁵. These unexpected findings based on MAR-1 prompted us to reexamine the specificity of this antibody.

We first sought to clarify whether moDCs express FcεRI under inflammatory conditions. In mice treated intranasally with house dust mite extract (HDM) and examined 3 days later by flow cytometric analysis using the MAR-1 antibody, we were able to identify FcεRIα⁺ CD11c⁺ MHC-II⁺ CD11b⁺ inflammatory moDCs in the lungs and mediastinal lymph node (mLN), but we were surprised to find that these apparent FcεRIα⁺ moDCs were also present in FcεRIα-deficient mice (*Fcer1a*^{-/-}, hereafter denoted as FcεRIα KO) (Fig 1A). Since the MAR-1 antibody labeled these cells in the absence of FcεRIα expression, we reasoned it would be more appropriate to call these MAR-1⁺ moDCs. As MAR-1⁺ moDCs have also been reported in viral infection³, we next tested MAR-1 staining of moDCs in this setting. To mimic viral infection, we administered intranasal poly I:C, a TLR3 ligand, and also detected MAR-1⁺ moDCs in the lungs. Similar to HDM exposure, we found that MAR-1⁺ moDCs could also be identified in poly I:C-treated FcεRIα KO mice similar to control mice (Fig 1B). In contrast, blood basophils only stained with

MAR-1 from control mice but not from FcεRIα KO mice (Fig 1C), confirming the genotype of the FcεRIα KO mice. We next considered whether the MAR-1 antibody may bind to other Fc receptors and stained cells from mice lacking the Fc receptor common gamma chain (*Fcγ1g*^{-/-}, hereafter denoted as FcRγc KO), which is necessary for the normal surface expression of all activating Fc receptors. In FcRγc KO mice, MAR-1 staining was greatly diminished on moDCs from the lungs and mLN after HDM-treatment (Fig 1D). Macrophages and monocytes express various Fcγ receptors but are not known to express FcεRI in mice, yet one study had noted MAR-1 staining on monocytes.⁶ We tested whether MAR-1 would stain splenic red pulp macrophages, lung alveolar macrophages, peritoneal macrophages and blood monocytes. Indeed, MAR-1 stained all of these macrophages (Fig 1E) and a subset of blood Ly6C⁻ and Ly6C⁺ monocytes in both wild-type and FcεRIα KO mice (Fig 1F). Similar to moDCs, MAR-1 staining was greatly reduced in these populations in FcRγc KO mice. These observations imply that MAR-1 may be cross-reacting with other Fc receptors, thereby resulting in the detection of MAR-1⁺ cells in FcεRIα KO mice. As our findings above suggested that MAR-1 binds one of the activating Fcγ receptors, we next attempted to identify which Fc receptor(s) MAR-1 may bind to. In mice, the activating Fc receptors that bind IgG are FcγRI (CD64), FcγRIII (CD16) and FcγRIV (CD16-2). We noticed that MAR-1 staining strongly correlated with FcγRI (CD64) staining on moDCs (Fig 2A), and that MAR-1 staining correlated with FcγRIV (CD16-2) staining on Ly6C⁻ blood monocytes (Fig 2B). To definitively test whether MAR-1 was cross-reacting with FcγRI (CD64) and FcγRIV (CD16-2), we expressed specific Fc gamma receptors in a cell line and then stained with MAR-1 versus other Fc gamma receptor specific antibodies. Specifically, the Phoenix cell line, a modified 293T human embryonic kidney cell line, was individually transfected with the alpha chains of FcγRI, FcγRIII or FcγRIV together with the Fc receptor common gamma chain, and then stained with antibodies. Untransfected cells did not stain with MAR-1 or any of the activating Fc gamma receptor antibodies, as expected, confirming that this human cell line lacks endogenous reactivity with these mouse reagents (Fig 2C). In support of our observations in monocytes and

macrophages, Phoenix cells transfected individually with Fc γ RI or Fc γ RIV stained with the MAR-1 antibody (Fig 2C). In contrast, MAR-1 did not stain Fc γ RIII-transfected cells (Fig 2C), demonstrating that MAR-1 binds to the alpha chains of Fc γ RI and Fc γ RIV, but not the shared Fc receptor common gamma chain.

We next further confirmed that the MAR-1 staining on myeloid cells *in vivo*, in the absence of Fc ϵ RI, was due to binding to Fc γ RI and Fc γ RIV. We analyzed splenocytes from mice deficient in the α chains of all four Fc γ R (Fc γ R α 4 KO) which showed an absence of MAR-1 staining on splenic red pulp macrophages and monocytes (Fig E2). Neutrophils, which are known to express Fc γ RIV⁷, also stained weakly with MAR-1 in control but not in Fc γ R α 4 KO mice (Fig E2). Some residual surface Fc γ RIV expression was also noted in the FcR γ c KO mice compared with the Fc γ R α 4 KO mice (Fig E2), providing a potential explanation for the weak MAR-1 staining observed on some cell populations in the FcR γ c KO mice. While our findings do not formally exclude the possibility that MAR-1 may bind additional receptors, taken together, our data indicate that MAR-1 binding to Fc γ RI and Fc γ RIV accounts for most of the MAR-1 staining on macrophages, monocytes, and neutrophils. Similar MAR-1 staining was observed on splenocytes from wild-type B6 and BALB/c mice, indicating our findings are broadly applicable (Fig E3). We conclude that MAR-1 antibody, which was thought to specifically recognize Fc ϵ RI α , in fact cross-reacts with Fc γ RI and Fc γ RIV. As a result, MAR-1 staining is specific for Fc ϵ RI α only on mast cells and basophils, whereas MAR-1 staining on dendritic cells, macrophages, monocytes, and neutrophils is due to Fc γ R expression. Contrary to prior reports,²⁻⁴ our results indicate that the MAR-1⁺ moDCs observed in inflammatory conditions do not express Fc ϵ RI. Therefore, normal mouse moDCs do not model the Fc ϵ RI expression by human DCs in allergic disease. However, some monocytes and macrophages that stain with MAR-1 express Fc γ RIV instead of Fc ϵ RI. Interestingly, Fc γ RIV is a low affinity receptor for IgE that binds to IgE-immune complexes.⁷ Engagement of Fc γ RIV by IgE-immune complexes on monocytes/macrophages may contribute to local inflammation.⁷

Early structural studies of rodent FcεRI demonstrated that surface expression could only be achieved when the alpha, beta and gamma chains were all transfected.¹ Unlike rodents, in humans the beta chain is not necessary for FcεRI surface expression, and a trimeric form of FcεRI, consisting of one alpha chain and two gamma chains, is expressed on human dendritic cells and monocytes.¹ Some recent studies have suggested that a trimeric form of mouse FcεRI lacking the beta chain may be naturally induced during inflammation, as a functional equivalent to human trimeric FcεRI.^{3,8} However, in these studies, MAR-1 was used for co-immunoprecipitation of FcεRIα, which, based on our findings above, would likely have instead captured FcγRIα or FcγRIVα complexed with the shared Fcγ chain. Two mouse lines have been engineered to express human FcεRIα in DCs, which can form a trimeric receptor with mouse Fcγ chains, representing a potentially better model system for studies of IgE-mediated activation and antigen uptake.^{1,9}

Our findings also raise the possibility that the injection of the MAR-1 antibody into mice, as in the aforementioned basophil depletion studies, could have significant off-target effects. Hammad et al. reported that MAR-1 depleted certain populations of dendritic cells.⁴ A technical caveat, however, is that the authors used the same MAR-1 antibody to both deplete and detect cells, and thus the lack of MAR-1 staining may have been due to receptor occupancy. Another study noted that MAR-1 depleted a subpopulation of splenic dendritic cells that were CD11c^{lo} CD11b⁺ IgE⁺ and MAR-1⁺⁶; however, our flow cytometric analysis suggested that these cells were in fact splenic basophils, which express this set of markers (data not shown). In our own experiments, MAR-1 antibody administration did not significantly deplete known macrophage, monocyte, or moDC subsets (Fig E4). Nevertheless, it seems likely that injection of the MAR-1 antibody would cross-link activating Fcγ receptors on these and other myeloid cells, thereby potentially modifying immune responses. We therefore caution investigators in the use of the MAR-1 antibody, and we suggest that in future investigations, FcεRIα KO mice should be used as a critical control.

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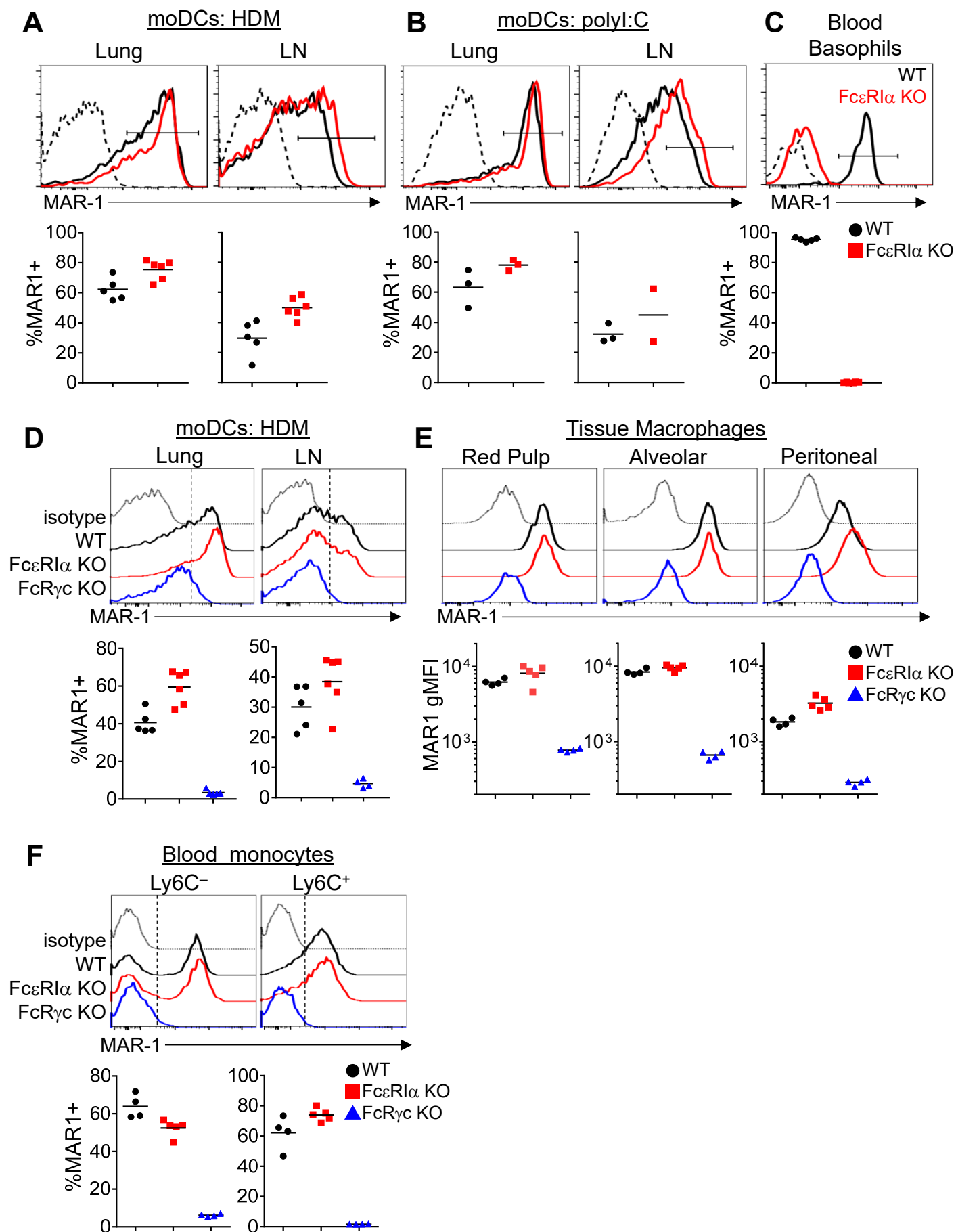
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Figure Legends

Figure 1: The MAR-1 antibody stains cells from FcεRIα KO mice but not from FcRγc KO mice. Flow cytometric analysis of MAR-1 staining; representative flow cytometric plots and summary graphs are shown. (A, B) MAR-1 staining on moDCs from the lung and mediastinal lymph node (LN) 3 d after intranasal HDM (A) or polyI:C (B) exposure in wild-type (WT, black) and FcεRIα KO (red) mice. (C) MAR-1 staining on blood basophils to confirm FcεRIα KO genotype. (D) MAR-1 staining on moDCs in the lung and mediastinal LN 3 d after HDM exposure in FcεRIα KO (red) and FcRγc KO mice (blue) compared to WT (black). (E) MAR-1 staining on splenic red pulp macrophages, alveolar macrophages and peritoneal macrophages from untreated mice of the indicated genotypes. (F) MAR-1 staining on blood monocytes from untreated mice. In histograms, dashed lines in A-C and gray lines in D-F denote isotype control staining (“isotype”). Data shown are representative of at least two independent experiments. In summary graphs, each data point represents one mouse, bars indicate the mean. The gating strategies to identify the represented populations of moDCs, basophils, macrophages and monocytes are shown in Fig E1.

Figure 2: MAR-1 cross reacts with FcγRI and FcγRIV. (A) Representative plots showing flow cytometric analysis of MAR-1 and FcγRI (CD64) co-staining on moDCs in lung and mediastinal LN 3 d after HDM exposure. (B) Representative plots showing flow cytometric analysis of MAR-1 co-staining with FcγRI or FcγRIV on blood monocytes. For (A) and (B), black dots denote isotype control (“Isotype”) staining whereas red dots denote MAR-1 staining. The gating strategy to identify moDCs and monocytes is shown in Fig E1. (C) Phoenix cells were transfected individually with the indicated Fc receptors along with a YFP reporter gene. Transfected cells were gated by YFP expression and stained with MAR-1 or antibodies to FcγRI, FcγRII/III (CD16/32 clone 2.4G2) or FcγRIV (CD16-2), as indicated. Data shown are representative of at least 3 independent experiments.



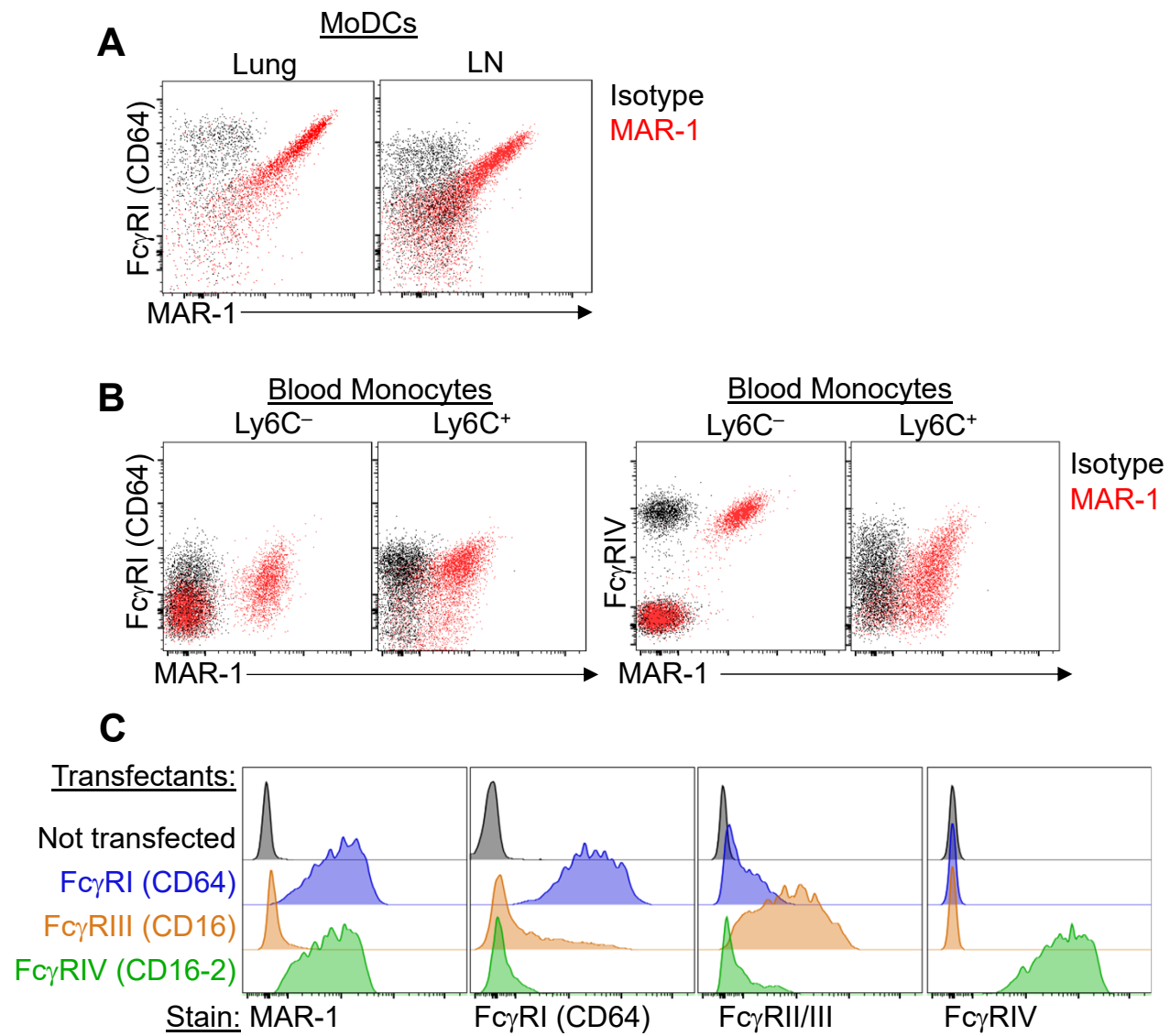
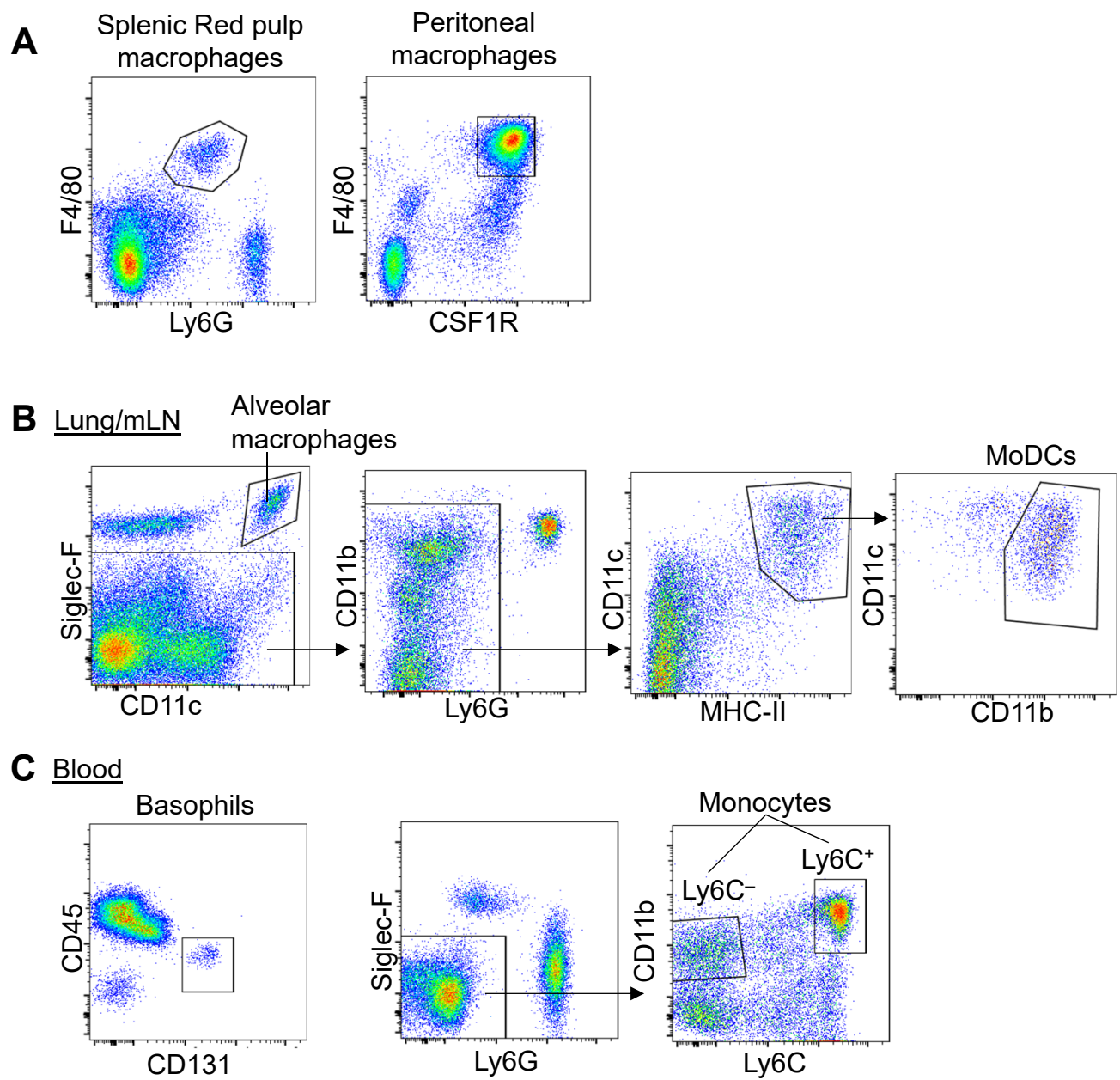
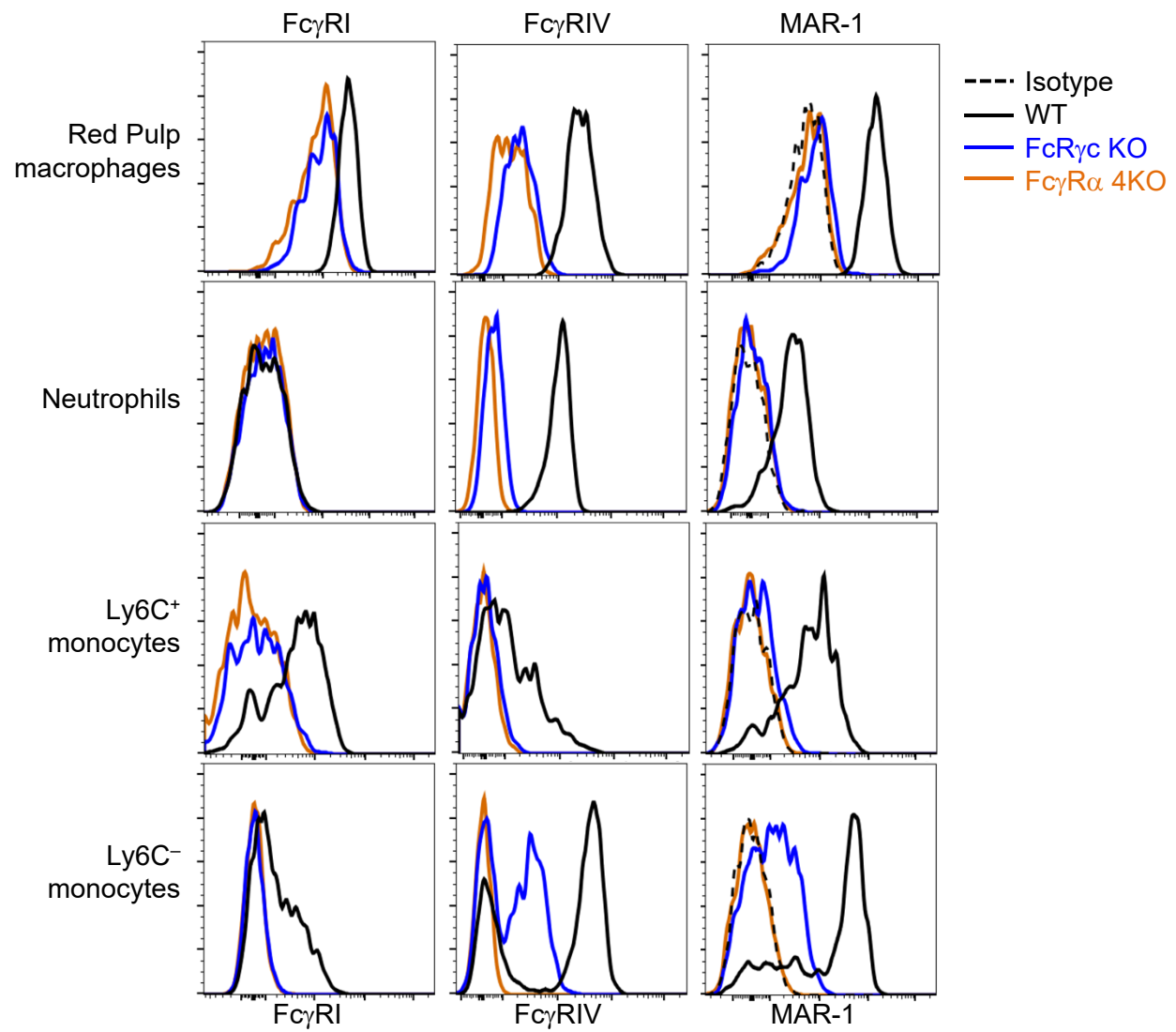
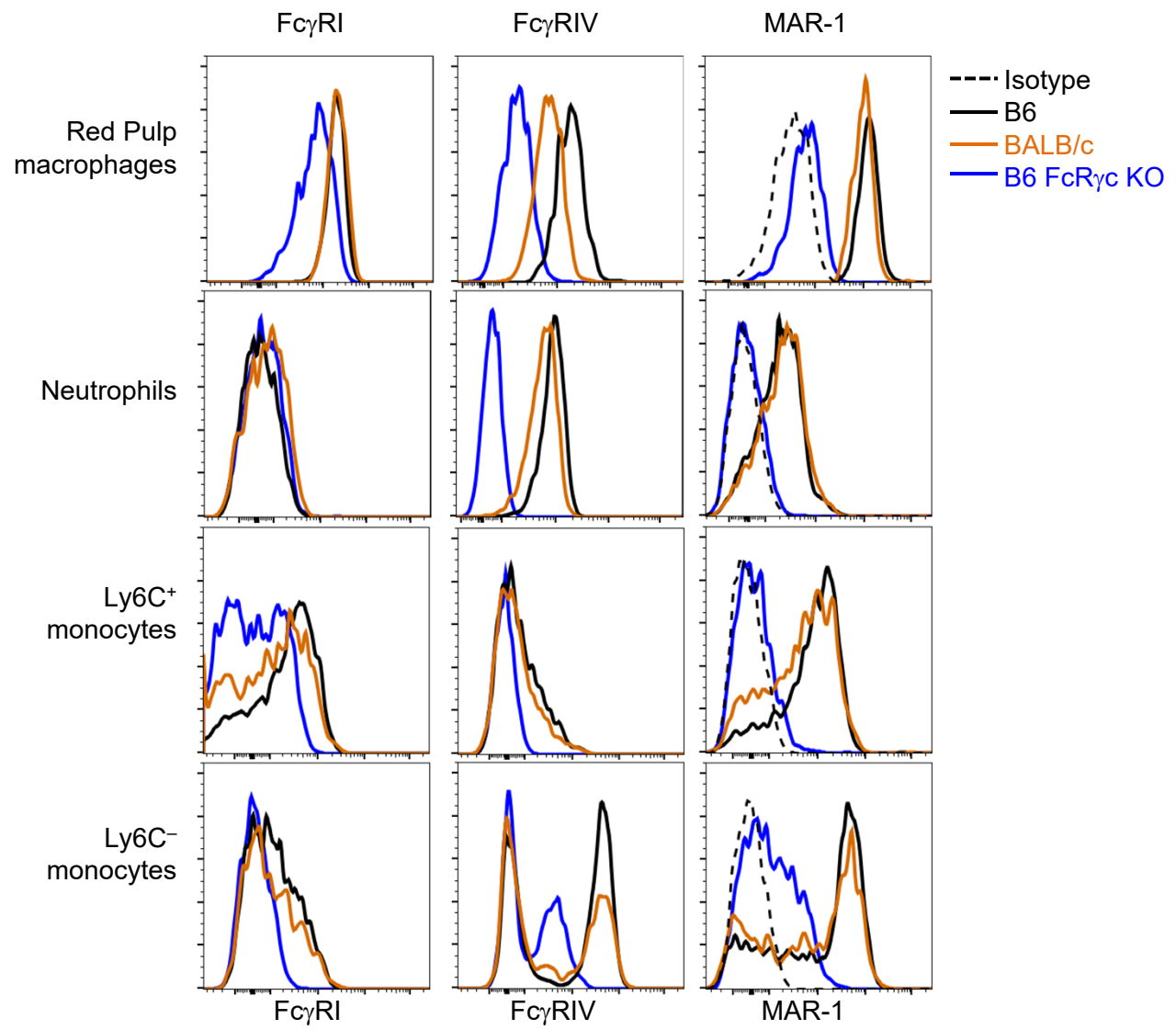


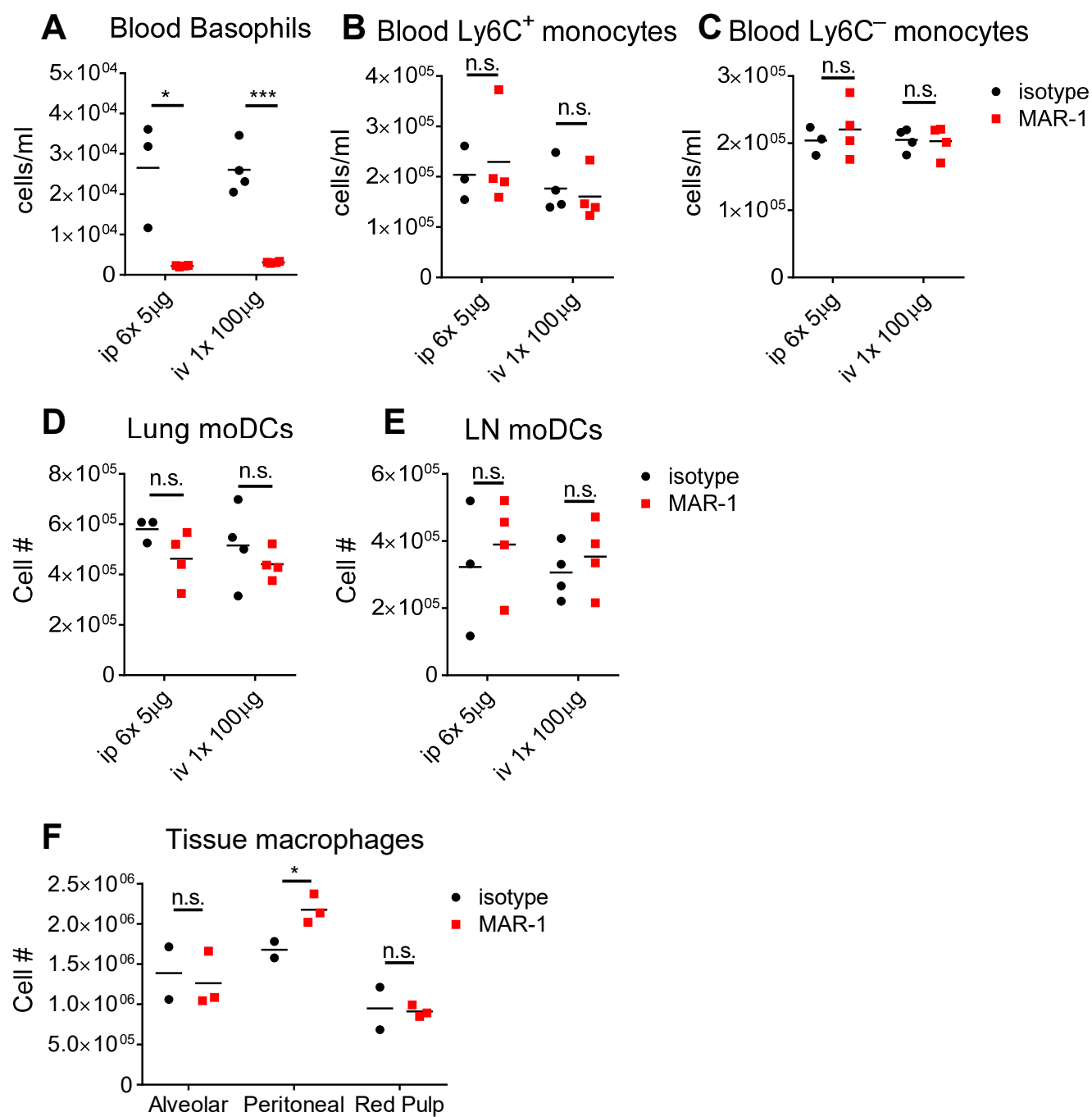
Table E1. Antibodies for flow cytometry

Target	Conjugate	Clone	Vendor	Dilution Factor
CD11b	BV785	M1/70	Biolegend	1:400
CD11c	BV650	N418	Biolegend	1:100
CD16/32 (Fc-block TruStain FcX)	purified	93	Biolegend	1:100
CD16/32	purified	2.4G2	BD Biosciences	1:100
CD16/32	A647	2.4G2	UCSF MAb core	1:100
CD16.2	APC	9E9	Biolegend	1:100
CD131	PE	JOR050	BD	1:100
CD19	PE/Dazzle594	6D5	Biolegend	1:200
CD45	A700	30-F11	Biolegend	1:100
CD64	PE-Cy7	X54-5/7.1	Biolegend	1:75
CSF-1R	Biotin/APC	AFS98	Biolegend	1:100
F4/80	BV421	BM8	Biolegend	1:100
FcεRIα	Biotin	MAR-1	Biolegend	1:100
I-A ^b	FITC	KH74	Biolegend	1:1000
Isotype Control (Armenian Hamster IgG)	Biotin	HTK888	Biolegend	1:100
Ly-6C	BV711	HK1.4	Biolegend	1:500
Ly-6G	BV510, FITC	1A8	Biolegend	1:100
Siglec-F	BV421, PE	E50-2440	BD	1:100
Streptavidin	Qdot 605		Life Technologies	1:400
Viability dye	eFluor780		eBioscience	1:600









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Methods

Mice and Tissues

C57BL/6J (B6, 000664) and BALB/cJ (000651) mice were originally from The Jackson Laboratories. B6-CD45.1 congenic mice, which were used as wild type mice in some experiments, were from the National Cancer Institute / Charles River Frederick National Laboratory (01B96; B6-Ly5.2/Cr, later renamed to B6-Ly5.1/Cr). FcεRIα KO mice (*Fcer1a*^{tm1Knt})¹ and FcRγc KO mice (*Fcer1g*^{tm1Rav})² were maintained on B6 background. All mice were maintained in specific pathogen free facilities and protocols were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Spleens from FcγRα 4KO mice were kindly provided by the laboratory of J. Ravetch. Intact spleens were isolated into tubes with media containing 10% FBS and then shipped overnight on ice. For comparison, we similarly isolated intact spleens from B6 WT and FcRγc KO into tubes with media containing 10% FBS and kept these on ice overnight in a 4°C cold room. We then prepared single cell suspensions from the spleens as indicated below.

Immunizations

House dust mite extract (HDM) (Greer Laboratories) was resuspended in sterile PBS at 100 µg/30 µl and stored at -20°C, and then thawed before intranasal droplet administration to mice that were lightly anesthetized with isoflurane at 100 µg/mouse. Poly I:C (pl:C, Invivogen) was resuspended at 10 mg/ml in sterile saline (Invivogen), stored at -20°C, and administered to mice intranasally at 15 µg/30 µl/mouse. Lungs and the right posterior mediastinal lymph nodes were examined 3 d later.

Tissue preparation

Whole blood was collected from the retro-orbital plexus of anesthetized mice or from the inferior vena cava of euthanized mice. Red blood cells were lysed using ACK Lysing buffer (Quality Biological). 30 µl of

blood was added to 200 μ l of ACK Lysing buffer and incubated for 15 mins at room temperature, then the cells were centrifuged and the supernatant was discarded. The blood cells were resuspended in 200 μ l ACK Lysing buffer for another 7-10 mins at room temperature, centrifuged and resuspended in FACS buffer (See Flow Cytometry) for antibody staining. To collect peritoneal macrophages, 5 mL of PBS was injected into the peritoneal cavity, then the animal was moved side-to-side while holding the injection site closed with forceps, and then a glass Pasteur pipette was introduced through the injection site to collect the peritoneal lavage, which was then kept on ice before antibody staining. Spleens were mechanically dissociated through 70 μ m nylon cell strainers (Falcon) in DMEM (Cellgro) supplemented with antibiotics (100 IU/ml of penicillin and 100 μ g/ml of streptomycin; Gibco), 1X L-glutamine (Gibco), 10 mM HEPES (Gibco) (supplemented DMEM) and 2% FBS (Gibco), and then cells were washed and kept on ice. Lungs and mediastinal lymph nodes were collected in supplemented DMEM without FBS, snipped into smaller pieces, and enzymatically digested with 25 μ g/ml of DNase I (Roche) and 0.28 Wunitz Units/ml of Liberase TM (Roche) at 37°C shaking at 700 rpm for 45 min on a ThermoMixer F1.5 (Eppendorf). The digestion reaction was quenched with one-tenth volume of DMEM supplemented with 50% FBS and 0.1 M EDTA before mechanical dissociation through 70 μ m nylon cell strainers (Falcon). Cells were then washed and resuspended in supplemented DMEM with 2% FBS and kept on ice before antibody staining.

Flow Cytometry

2-3 x 10⁶ cells were plated per well in 96-well U-bottomed plates (Falcon) for flow cytometry staining. Plated lung cells were washed with 150 μ l of ACK Lysing buffer (Quality Biological) and resuspended in PBS with 2% FBS (Gibco), 1mM EDTA (Gibco) and 0.1% sodium azide (VWR) (FACS buffer). Cells were stained with antibodies for analysis by flow cytometry as described.^{3, 4} Antibodies used are listed in Table E1. Data were collected on an LSRFortessa (BD) using FACS DiVa software (BD) and analyzed with FlowJo software (TreeStar).

Transfections

Plasmids encoding Fc receptors were kind gifts from Falk Nimmerjahn. Phoenix-Eco cells were cultured as described⁵ and plated to 70-80% confluence in 24-well plates. 200ng each of the indicated Fc receptor alpha chain and the common gamma chain, together with 100ng of a plasmid encoding a YFP reporter gene (pQEF-Venus),⁶ were transfected using the TransIT-LT1 transfection reagent (Mirus Bio) according to manufacturer's protocol. 24 h later, cells were resuspended by repeated pipetting into single cell suspensions, washed and then stained with antibodies and analyzed by flow cytometry as described above.

MAR-1 antibody treatment

Mice were injected with two different protocols for MAR-1 antibody-mediated cell depletion. In one protocol, mice were injected intraperitoneally with 5 µg MAR-1 antibody (eBioscience) or isotype control antibody (clone PIP, Bio X-Cell) twice a day over 3 d, before intranasal immunization 3 d after the final injection, as reported.⁷ In the other protocol, modified slightly from previous reports,^{8,9} mice were injected intravenously with 100 µg MAR-1 antibody or isotype control antibody 3 d before intranasal immunization.

Statistical analysis

GraphPad Prism v6 or v7 was used for statistical analysis and graphical representations. The appropriate statistical tests were chosen based on experimental design after consulting the GraphPad Statistics Guide. All tests were two-tailed.

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Figure Legends

Fig E1: Gating Strategy. (A) Gating strategy for splenic red pulp macrophages and peritoneal macrophages. (B) Gating strategy for alveolar macrophages and moDCs from the lung. A similar gating scheme was used for moDCs in mediastinal lymph nodes. (C) Gating strategy for blood basophils and monocytes.

Fig E2: The majority of MAR-1 staining is accounted for by Fc γ R expression. Flow cytometric analysis of Fc receptor expression and MAR-1 staining in spleen cells from WT (black lines), FcR γ KO mice (blue lines) and mice lacking α chains from all 4 Fc γ R (Fc γ R α 4KO) mice (brown lines). Histograms are representative of 3 mice of each genotype.

Fig E3: Myeloid cells in BALB/c and B6 mice show similar staining with MAR-1. Flow cytometric analysis of Fc receptor expression and MAR-1 staining in spleen cells from B6 (black lines), BALB/c (brown lines) and B6 FcR γ KO mice (blue lines). Histograms are representative of 3 mice of each genotype.

Fig E4: MAR-1 does not deplete known macrophages, monocytes or moDCs. Cell counts of blood basophils (A), Ly6C⁺ (B) and Ly6C⁻ (C) monocytes, moDCs in the lung (D) and mediastinal lymph node (LN) (E), and tissue macrophages (F) after MAR-1 or isotype control ("isotype") antibody treatment. In panels (A-E), mice underwent two protocols for MAR-1 treatment. One set of mice were injected intraperitoneally with 5 μ g of MAR-1 or isotype control antibody every 12h on d-2, d-3, and d-4 for a total of 6 times, while another set of mice were injected intravenously with 100 μ g of MAR-1 or isotype control antibody on once on d-3. All mice were immunized with HDM intranasally on d0. Myeloid cell populations were assessed on d3. In panel (F), mice received 100 μ g of MAR-1 or isotype control antibody 1 day before macrophage populations were assessed. Each data point represents one mouse, horizontal bars indicate mean. * $p < 0.05$, *** $p < 0.001$, n.s. not significant.