



5-lipoxygenase- and Glucocorticoid-dependent eosinophilia in a novel surgical model in mice

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ARTICLE INFO

Keywords:

Eosinophil
Chemokines
Animal Models
Surgical trauma
In vivo assay
Glucocorticoid

ABSTRACT

Background: Subcutaneous implants of heat-coagulated egg white (egg white implants, EWI) induce intense local eosinophilia and prime for hyperreactivity following airway ovalbumin challenge. The roles of allergen sensitization, surgical trauma-induced glucocorticoids, and the 5-lipoxygenase (5-LO) pathway were hitherto unexplored in this model, in which quantitative recovery and large-scale purification of the eosinophils from the inflammatory site for functional and immunopharmacological studies are difficult to achieve.

Methods: We overcame this limitation by shifting the implantation site to the peritoneal cavity (EWIp), thereby enabling quantitative leukocyte retrieval.

Results: By day 7 post-surgery, eosinophil counts reached ~ 30% of all leukocytes recovered. Eosinophilia was prevented by: a) induction of allergen-specific oral tolerance to ovalbumin, the main allergen in egg white; b) inactivation of the 5-lipoxygenase pathway; c) blockade of endogenous glucocorticoid signaling by pretreatment with metirapone plus mifepristone before surgery. Highly purified eosinophils (~99% pure) could be obtained from the peritoneal exudate of EWIp-carrier mice in 2 simple, antibody-free steps. Preparative-scale yields, suitable for most biochemical, pharmacological, and molecular applications, were routinely obtained, and could be further enhanced through addition of pre- or post-surgery immunization steps (active or adoptive). The recovered eosinophils were fully functional in vivo, as demonstrated by the transfer of purified eosinophils into eosinophil-deficient $\Delta\text{dbl-GATA-1-KO}$ mice, which upon subsequent challenge with eotaxin-1 present secondary accumulation of neutrophils, but not of mononuclear phagocytes.

Conclusion: These findings document glucocorticoid-, allergen- and 5-lipoxygenase-dependent eosinophilia, which makes EWIp carriers an abundant source of pure, nontransgenic eosinophils for immunopharmacological studies.

1. Introduction

The biology of eosinophils has received much attention over the last three decades [1–6]. This minority population of circulating and tissue-resident granulocytes is expanded in allergic subjects and during infection by helminth parasites, through the lineage-selective effect of interleukin(IL)-5, secreted by TH2 lymphocytes and mast cells [3–6]. A

role for eosinophils as direct effector cells against multicellular or unicellular parasites was demonstrated in some [7,8], but not all [9,10], experimental studies. Due to the variety of bioactive products contained or generated in eosinophils [6,11], and of receptors for regulatory signals expressed by these cells [6,12], eosinophils were proposed to act primarily as immunoregulatory cells in multiple contexts [9,12], including experimental allergic encephalomyelitis [13,14], pulmonary

Abbreviations: 5-LO, 5-Lipoxygenase; CysLT, Cysteinyl-leukotrienes; Eot, Eotaxin-1; EPO, Eosinophil Peroxidase; EWI, Egg white implant; EWIp, Peritoneal Egg white implant; Met, Metirapone; OVA, Ovalbumin; PEC, Peritoneal exudate cells; RU, RU486 (Mifepristone); TOL, oral tolerization to ovalbumin.

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¹ Retired.

<https://doi.org/10.1016/j.intimp.2021.107440>

Received 11 December 2020; Received in revised form 21 January 2021; Accepted 26 January 2021

Available online 12 February 2021

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fibrosis [15,16], and helminth infections [15,17]. However, biochemical, cell biological, physiological, and pharmacological studies of eosinophils can be hindered by their low frequency in human blood [18] and in most mouse tissues even during inflammation. Although several approaches to large-scale generation of eosinophils from bone-marrow of naive mice have been proposed [19,20], they may not be widely available. Moreover, mice which overexpress IL-5 and present high eosinophilia in blood and tissues [21] are not necessarily representative of physiological conditions, since their eosinophils develop in the presence of continuously elevated levels of this cytokine, which not only supports chronically increased eosinopoiesis in bone-marrow [21] but activates mature eosinophils for effector functions as well.

We have shown, in a conventional ovalbumin(OVA)-sensitization and challenge model of experimental allergy, that eosinophilia of the bone-marrow develops rapidly after allergen challenge of sensitized mice [22]. Furthermore, eosinophilia is found not only in the challenge site (the lungs, following airway challenge) but also in the spleen, where eosinophil counts surpass those from the entire bone-marrow of the same animals [23]. This suggests a role for systemically acting mediators in the eosinophilia. Importantly, the effects of challenge on eosinophilia are dependent on TNF, presumably released at the challenge site, and mediated by endogenous glucocorticoids, which points to a immunoendocrine mechanism [24] for systemic signaling. Alternatively, intense eosinophilia is observed around surgically implanted, subcutaneous pellets of heat-coagulated egg white (egg white implants, or EWI) [25]. Using this protocol, we documented a separate effect of surgery, in the absence of allergen sensitization, in mice operated as above which received no pellet (SHAM-implanted controls): these developed bone-marrow eosinophilia, that was abolished by blockade of endogenous glucocorticoid production and action. Observations in both the conventional and the surgical murine models are consistent with the effects of exogenous glucocorticoids on murine [26,27] bone-marrow eosinophils, as well as with the observations of other groups with human cord blood-derived eosinophils [28]. Furthermore, the effects of endogenous glucocorticoids on eosinophilia in conventional sensitization/challenge models are dependent on the integrity of the 5-lipoxygenase (5-LO) pathway. This is shown by the absence of challenge-induced eosinophilia in mutant mice lacking functional 5-LO, as well as in wild-type mice treated with blockers of the 5-LO pathway, despite their unimpaired ability to produce endogenous glucocorticoids [29,30]. This raises the possibility that, in the EWI model, eosinophilia at the implant site requires both endogenous glucocorticoids and 5-LO, which jointly determine the properties of the recruited eosinophils. The EWI model, in which intense eosinophilia develops in response to an association of surgical trauma and allergen sensitization, enables us to further examine this issue, provided the recovery of eosinophils from the implant site is quantitative. While this was hitherto hard to achieve with subcutaneous implants, it is now made possible by shifting the implant site to the peritoneal cavity. Using this modified, peritoneal egg white implant protocol (EWIp), we could quantitatively characterize the roles of endogenous glucocorticoids and of the 5-LO pathway, and further demonstrate the unique practical potential of this approach to provide preparative-scale numbers of pure eosinophils for in vivo, in vitro and ex vivo studies.

2. Methods

2.1. Animals and ethical aspects

Mice, 6–8 wk. old, of the BALB/c and 129S2/SvPAS (PAS) strains, as well as the respective Δ dbl-GATA-1-KO (eosinophil-deficient; BALB/c [31] background) and ALOX (5-lipoxygenase-deficient; PAS [26] background) mutants were bred at CECAL-FIOCRUZ, RJ, Brazil. The study followed institutionally approved guidelines (CEUA-CCS-UFRJ, #A12/18–181–13). All applicable ARRIVE guidelines were adhered to.

3. In vivo procedures

EWIp and SHAMp. The heat-coagulated egg white pellet was made as previously described [25]. Briefly, 50 μ L of 10% w/v solution of chicken egg white (Ito Avicultura, SP, Brazil) were transferred to a round-bottom 96-well plate and heat-coagulated in a microwave oven, set at maximum potency for 30 s, 4 times, stirring at each round. The pellet (5 mg hen egg white/pellet/mouse) was fixed in absolute ethanol for 24 h and rehydrated with PBS solution before in vivo procedures. In the EWIp surgical model, mice were anesthetized with ketamine (100 mg/kg) and xylazine (12 mg/kg), i.p. After local asepsis inside a laminar-flow hood, a $\sim$ 1 cm access to the peritoneal cavity was gained, and a sterile pellet of heat-coagulated egg white was inserted with tweezers, before suturing together tissue layers from skin to peritoneum. Sham-implanted controls (SHAMp) underwent surgery as above but received no pellet. All mice recovered from anesthesia under a 40 W lamp with their eyes protected from direct light and desiccation.

Glucocorticoid blockade. Mice received the glucocorticoid biosynthesis inhibitor metyrapone (30 mg/kg; 300 μ L, i.p. Sigma-Aldrich, St. Louis, MO) for 8 consecutive days (last dose 2 h before EWIp surgery). In addition, the glucocorticoid/progesterone receptor antagonist mifepristone (RU486, 20 mg/Kg, 300 μ L in methylcellulose, both from Sigma-Aldrich) was administered by gavage, 16 h before EWIp surgery. Controls for both treatments received only the corresponding vehicle at each step.

Induction of OVA-specific tolerance. Mice were given rehydrated egg white ($\sim$ 50% OVA) as 1% w/v solution in the drinking water, for 5 consecutive days [32], with daily changes. This procedure was previously shown [32] to induce OVA-specific tolerance. Non-tolerized controls received only drinking water. After a 2-day resting period, mice were submitted to surgery (EWIp, or SHAMp as a control). Throughout this period, both groups received standard mouse chow *ad libitum*.

Eosinophil transfer and in vivo neutrophil recruitment assay. Eosinophil-deficient Δ dbl-GATA-1 (which produce no eosinophils and do not respond to eotaxin with recruitment of neutrophils [31]) mice received purified BALB/c eosinophils (10^6 cells/0.2 mL, i.p.) from EWIp donors followed by 0.2 mL of RPMI 1640 medium (as controls), or by 0.2 mL eotaxin-1 (50 ng/cavity; PeproTech; Rocky Hill, NJ). The recruitment of neutrophils to the peritoneal cavity [31] of Δ dbl-GATA-1 recipients of BALB/c eosinophils was evaluated in the absence and in the presence of eotaxin after 4 h.

Boosting EWIp through protocols of sensitization and challenge. To maximize the yield of pure eosinophils, various manouvers were added before the basic EWIp design; in all cases, mice were euthanized 7 days after surgery and samples were collected. For conventional (active) sensitization, mice were given 2 s.c. (days 0 and 7) injections of 400 μ L saline, containing 100 μ g OVA adsorbed on 1,6 mg alum [22]. For challenge, mice were given 10 μ g OVA grade V i.n. in a total 50 μ L volume at day 14 [22]. Pre-surgery challenge was done in sensitized mice 24 h before surgery; alternatively, post-surgery challenge was done in sensitized mice bearing EWIp 24 h before euthanasia and sample collection. Comparability of these experiments with the standard EWIp protocol is ensured by using mice from the same source, animal facility, strain and age, to collect cells with identical procedures and materials at the same time point following the same surgical procedure, over the same period of experimentation in the lab.

4. Ex vivo procedures

Harvest and quantification of leukocyte populations. Peritoneal exudate cells (PEC) were collected by flushing the peritoneal cavity with 10 mL of RPMI1640 medium (RPMI) containing 25 U/mL heparin, washing 1x, and recovering 9 mL for leukocyte counts and purification. PEC were centrifuged (500xg, 15 min, 4°C), and resuspended in 2 mL medium for counts in a haemocytometer after dilution in Turk's solution (total) and in cytocentrifuge smears (differential). Cytocentrifugates were stained

for cyanide-resistant peroxidase [33] (eosinophil peroxidase; EPO), an eosinophil-lineage marker expressed from the earliest precursors to the mature leukocytes in tissues, and counterstained with Hematoxylin. Eosinophils are recognizable by their donut-shaped nucleus and coarse cytoplasmic granules stained positively for EPO (EPO +). Neutrophils and mononuclear phagocytes have no EPO + coarse granules and present typical nuclear morphologies distinct from that of eosinophils. The numbers of eosinophils, neutrophils and mononuclear phagocytes in PEC were calculated from total and differential counts.

Eosinophil purification. Purification of eosinophils from PEC for in vitro and in vivo assays included an initial depletion of plastic-adherent PEC (mostly mononuclear phagocytes), followed by collection of the nonadherent cells, including eosinophils; further depletion of contaminants (which are predominantly neutrophils) was achieved by cell sorting, based both on positive selection by eosinophil autofluorescence [34] and on FSC and SSC profiles. To carry out this two-step purification, the PEC were seeded at 10^6 cells/mL, 5 mL per tissue culture flask, for a 2 h-period at 37 °C, 95% air/5% CO₂. Next, the nonadherent cells in the supernatant (including eosinophils, neutrophils and lymphocytes) were recovered by centrifugation (500xg for 10 min) and resuspended at 5×10^6 cells/mL before separation in a fluorescent-activated cell sorter (MoFlo XDP, Beckman Coulter). Initially, the parameters FSC-H $\times$ FSC-A were used to remove cell doublets. Subsequently, the parameters FSC-H $\times$ SSC-H were used to isolate the cells of interest by size and granularity, since eosinophils are medium-sized (9–15 nm diameter), with high complexity scores. Cells from a Δ dbl-GATA-1-KO mouse, devoid of eosinophils, were run in parallel as negative controls. Next, eosinophils were gated in the positive region for 530 ± 20 nm emission in the cytometer. At the end of the sorting, the cells recovered were 95–99% pure eosinophils.

In vitro migration assay. Eosinophil migration in response to eotaxin-1 (250 ng/mL) was directly assayed using Transwell™ inserts (pore size 5 μ m; Thermo-Fischer, Waltham, MA) and 24-well tissue culture plates. Cells in RPMI/1% FCS were added to the upper compartment of the transwell insert. Eotaxin-1 in this medium was added to the lower compartment. RPMI/1% FCS without eotaxin-1 was used as a control. This assay allows the detection of both chemotactic and chemokinetic responses to eotaxin-1 (see Results). After 4 h incubation at 37°C in an atmosphere with 95% air/5% CO₂, cells in the lower compartment were recovered and counted.

4.1. Data analysis

Data (presented as Mean + SEM throughout) were analyzed with SigmaPlot for Windows version 11.0 software. Multiple comparisons were carried out by factorial ANOVA with Bonferroni's correction. Where only two groups were compared, we used the two-tailed t-test with separate variances. $P < 0.05$ was taken to show significant differences between group means.

5. Results

5.1. Eosinophilia at the implant site (peripheral) and bone-marrow (central)

In pilot experiments with the EWIp protocol, eosinophilia was nonsignificant at the implant site 24 h after surgery; it increased subsequently and became fully established by day 7, decreasing thereafter (not shown). Based on these preliminary results, we next examined whether the well-established eosinophilia at day 7 required the presence of an allergen pellet at the operated site, or could be induced by surgical trauma alone. BALB/c mice submitted to surgical trauma alone (SHAMp) or associated with pellet-induced allergic sensitization (EWIp), had peritoneal lavage fluid collected at day 7, when leukocyte accumulation had peaked. Naive controls were also included. In the peritoneal cavity, the EWIp group presented a significant increase in

total cell counts (Fig. 1, Panel A) relative to both the SHAMp (3.3-fold increase) and the naive (6-fold increase) control groups at Day 7. This was accompanied by significant increases in the counts of eosinophils (14.7-fold and 29.2-fold increase relative to SHAMp and the naive control groups respectively - Panel B); neutrophils (11.1-fold increase relative to SHAMp control group - Panel C; naive mice had no neutrophils in the peritoneal cavity) and mononuclear cells (2.5-fold and 4.5-fold increase relative to SHAMp and the naive control groups respectively - Panel D). The surplus total cell counts (Panel A) in EWIp carriers were accounted for, in decreasing order, by increases in mononuclear cells, eosinophils and neutrophils (Fig. 1, Panel E), as determined by subtracting, for each leukocyte class, the naive cell counts from the respective EWIp and SHAMp cell counts, and expressing the results as % of each class.

Because previous investigations with the original EWI model [25] examined changes in bone-marrow, we also examined the bone-marrow from these three experimental groups (naive, SHAMp and EWIp) at day 7 (Table 1). In EWIp carriers, there was a significant increase in the numbers of eosinophils relative to both SHAMp and naive controls, indicating that eosinophilia induced by EWIp is both peripheral (peritoneal cavity) and central (bone-marrow). Besides, there was a significant increase in the numbers of mononuclear cells and total nucleated cells in the bone-marrow of EWIp carriers relative to naive controls (but not relative to SHAMp controls); mononuclear cells were the major component in the total increase in cell numbers. By contrast, the three groups did not significantly differ in numbers of neutrophils. Also, differences between SHAMp and naive controls were nonsignificant for all the parameters examined.

Together, the data in Fig. 1 and Table 1 show that eosinophils, unlike other leukocyte populations, are selectively increased by EWIp in both the inflammatory site and the bone-marrow at day 7 post-surgery. The EWIp model therefore induces both central (bone-marrow) and peripheral (implant site) eosinophilia.

6. Role of specific immune responses in the EWIp model

Impact of oral tolerance induction on the peripheral accumulation of leukocyte populations. We have previously shown [32] that oral tolerization followed by conventional sensitization and challenge with ovalbumin profoundly reduces the bone-marrow eosinophilia relative to nontolerized controls which underwent both sensitization and challenge. Despite the lack of separation of the sensitization and challenge events in the EWIp model, we next examined the possibility that oral tolerization would also reduce the peripheral eosinophilia induced by EWIp at the implant site, thus providing evidence of an important role for specific immunity in this response. BALB/c mice underwent a 5-day course of oral tolerization to ovalbumin (TOL) [32], and were submitted to EWIp 48 h later. At day 7 after surgery, PEC were collected. TOL/EWIp mice had significantly decreased counts of eosinophils, neutrophils, mononuclear cells and total nucleated cells, relative to the EWIp positive control group (Fig. 2). By contrast, there was no significant difference between TOL/SHAMp mice relative to the respective positive control SHAMp in the eosinophil and neutrophil populations (Panels B and C). On the other hand, a significant effect of oral tolerization was observed in both SHAMp and EWIp mice for the counts of mononuclear cells, which were reduced by this treatment (Panel D). The effect on mononuclear cells was sufficient to significantly reduce total PEC numbers (Panel A). These observations demonstrate an important effect of oral tolerization followed by exposure to the implant on all infiltrating leukocyte populations in EWIp carriers. Because SHAMp control mice suffered surgical trauma without further exposure to allergen in the form of a heat-coagulated pellet, the absence of an effect on the eosinophilic and neutrophilic populations was expected [32]; for the same reason, however, the clear effect on mononuclear cells was unexpected. Even though the underlying mechanism of this effect remains to be determined, the observation in itself shows that TOL/SHAMp differs

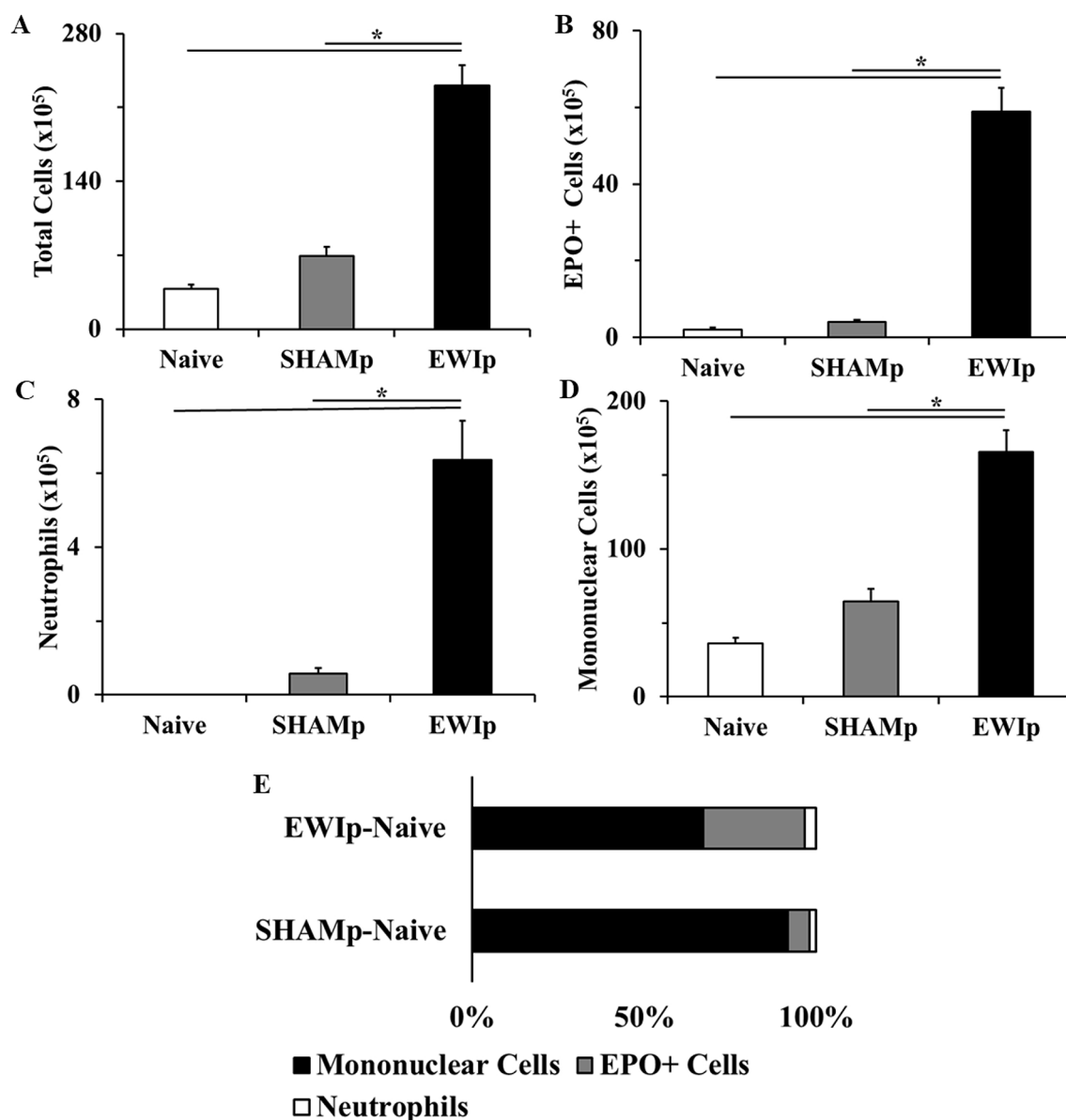


Fig. 1. Effect of EWIp surgery on total and differential counts of leukocytes in the peritoneal lavage fluid at day 7. Data are numbers of: (A) Total cells, (B) Eosinophils, (C) Neutrophils and (D) Mononuclear cells collected from the peritoneal cavity from naive (white bars), SHAMp (grey bars) or EWIp (black bars) BALB/c mice. In (E), the composition of the surplus cells in the comparison between EWIp and naive mice (top) or between SHAMp and naive mice (bottom) is expressed as % Mononuclear cells (black), Eosinophils (grey) and Neutrophils (white). *, significant differences relative to the indicated controls ($p < 0.05$). $n = 6, 20$, and 24 for the Naive, SHAMp and EWIp groups, respectively in all panels.

Table 1

Effect of trauma, alone or associated with sensitization, on leukocyte populations of the bone-marrow, 7 days after surgery in BALB/c mice.

Protocol	Total Cells			EPO + Cells			Neutrophils			Mononuclear Cells			n=
	Mean	SEM	p-Value	Mean	SEM	p-Value	Mean	SEM	p-Value	Mean	SEM	p-Value	
Naive	27.3	3.0	–	1.5	0.4	–	8.4	0.8	–	17.4	2.5	–	6
SHAMp	37.4	3.0	0.201	2.2	0.3	0.601	10.6	1.4	0.377	24.4	2.0	0.205	20
EWIp	42.6	2.2	0.016	3.5	0.3	0.001	11.8	1.3	0.231	27.2	1.4	0.02	24

BALB/c mice were either naive or subjected to surgery following either protocol, SHAMp (surgery without implant) or EWIp (surgery with egg white implant). After 7 days, mice were euthanized and bone-marrow was collected. The data are mean + SEM of the number of total cells, EPO + cells (eosinophils), neutrophils, or mononuclear cells (all expressed in millions [10^6]) in bone-marrow from the two femurs. p-Value is from ANOVA comparison between the indicated groups and the Naive group. n (number of animals used for calculations of means and SEM) is indicated at the extreme right column.

from conventional TOL/sensitization/challenge, which requires 3 sequential allergen exposures to have a detectable effect [32]. Taken together, the results point to a major role for specific immune responses

in the development of an inflammatory exudate rich in eosinophils in the EWIp mice.

Effect of additional immunization/challenge manouvers on final pure

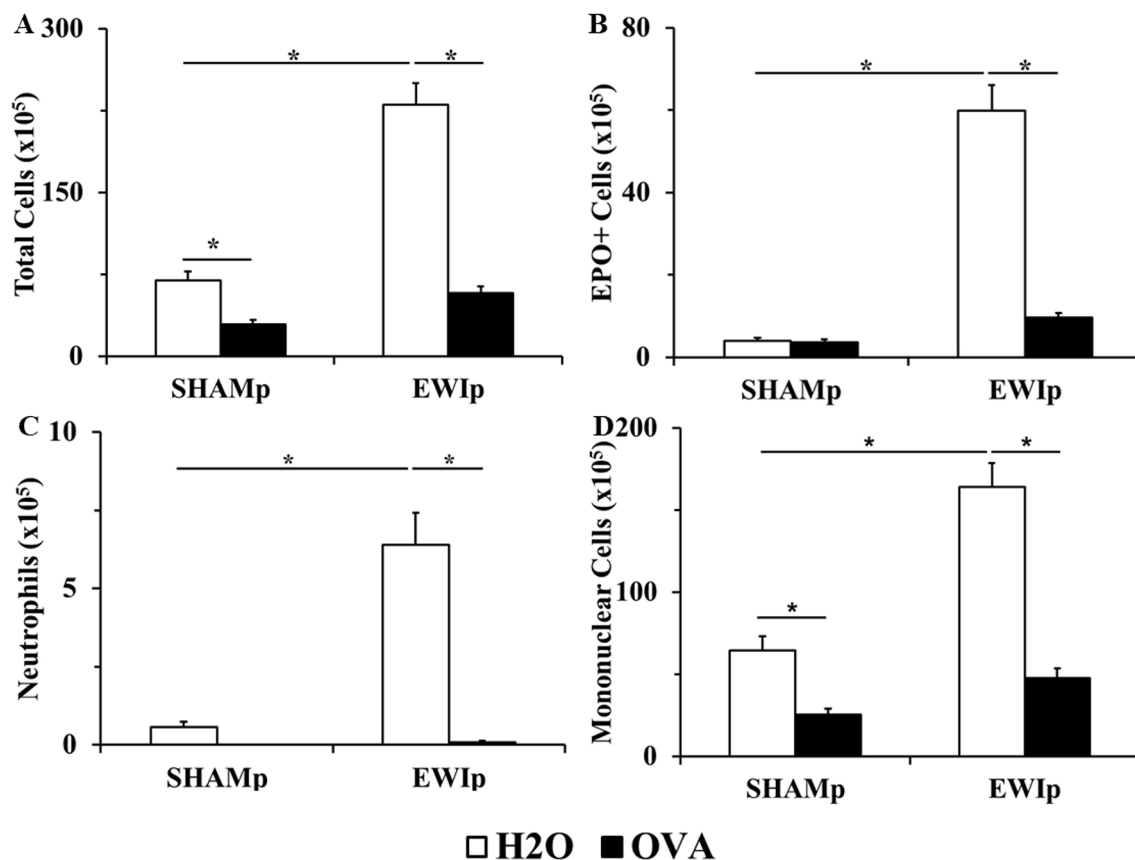


Fig. 2. Effect of orally induced tolerance to ovalbumin on total and differential counts of leukocytes in the peritoneal lavage fluid of EWIp mice at day 7. Data are numbers of: (A) Total cells, (B) Eosinophils, (C) Neutrophils, (D) Mononuclear cells collected from the peritoneal cavity of the H₂O (non-tolerized) control group (white bars) or OVA (orally tolerized) experimental group (black bars) of BALB/c mice. *, significant differences relative to the indicated control group ($p < 0.05$). $n = 20$ for H₂O control group and $n = 11$ for OVA experimental group in all panels.

eosinophil yield. We further approached the role of specific immunity in the EWIp model by testing whether the peripheral eosinophilia could be boosted by additional steps of sensitization and challenge. A strong boosting effect was observed in a series of experiments (Table 2), comparing the effects of active sensitization (AS2X) alone or followed by intranasal challenge (i.n. Ch; either pre- or post-surgery) on subsequent responses to EWIp. In all protocols, cells were collected at day 7 after EWIp. Since we planned to pursue purification of eosinophils and to perform functional characterization (see below), the most interesting combination should combine high eosinophil numbers with low neutrophil contamination; this outcome was provided by a combination of active sensitization before surgery, followed by EWIp and by i.n. Ch. with ovalbumin 24 h before collection of peritoneal exudate (protocol 4 in Table 2; $p = 0.001$ for comparison with both protocols 1 and 2). There

was no difference between i.n. Ch. prior versus following EWIp (i.e., between protocols 3 and 4; p -Value = 1.00). Protocol 4 yielded over 35×10^6 eosinophils per donor mouse with minimal neutrophil contamination; because the main contaminant (mononuclear phagocytes) is effectively depleted by a simple step of plastic adherence, this modified protocol made further purification of eosinophils in the cell sorter (see below) much easier.

Together, the data in Fig. 2 and Table 2 support an important role for ovalbumin-specific immune responses in promoting the intense inflammatory response found in EWIp carriers. Importantly, while all three leukocyte populations are decreased by the oral tolerization protocol, boosting protocols (especially protocol 4) selectively promote eosinophilia as opposed to neutrophilia in the implant site.

Table 2
Enhancement of I.P. eosinophilia by immunization/challenge procedures.

Procedures	Protocols	Total Cells		EPO + Cells		Neutrophils		Mononuclear Cells		n=
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
EWIp	1	23.1	2.0	6.0	0.6	0.6	0.1	16.4	1.5	24
AS2X/EWIp	2	52.6	3.0	17.6	1.0	0.0	0.0	34.9	2.4	5
AS2X/i.n. Ch/EWIp	3	70.5	8.4	31.1	4.0	0.1	0.1	39.9	4.5	5
AS2X/EWIp/i.n. Ch	4	77.1	9.9	36.4	4.2	0.0	0.0	40.7	6.2	5

Data are Mean or SEM of the numbers of cells (in multiples of 10^6) collected from BALB/c recipients of EWIp, consisting of: total cells, EPO + cells (eosinophils), neutrophils, or mononuclear cells. Abbreviations: i.p., intraperitoneal; AS2X, active sensitization by 2X injection of OVA; i.n. Ch, intranasal challenge with OVA. In all cases, BALB/c mice were recipients of EWIp, and PEC were collected at Day 7 after surgery. Protocols: 1: EWIp with no additional procedures. 2: BALB/c mice underwent AS2X and received EWIp 7 days after the second injection. 3: BALB/c mice underwent AS2X, followed by i.n. Ch with OVA 7 days after the second injection, and 24 h before EWIp surgery. 4: BALB/c mice underwent AS2X, received EWIp 7 days after the second injection, and were i.n. Ch 24 h before euthanasia (6 days after surgery). n is indicated at the extreme right column. For p values of the relevant comparisons, see Results.

7. Role of the 5-lipoxygenase pathway

Eosinophilia induced by allergen challenge in conventionally sensitized mice requires both an initial glucocorticoid surge [24] and a later contribution of the 5-LO pathway [20,23,26,30]. The EWIp model differs from conventional sensitization/challenge because it does not distinguish between sensitization and challenge, as insoluble antigen pellets provide continuous stimulation from day 0. We examined if a functional 5-LO is required for the development of peripheral eosinophilia in the EWIp model, using mice lacking 5-LO (ALOX) as well as wild-type controls of the same background (PAS). In wild-type control mice, the counts of total cells in the peritoneal cavity did not significantly differ between the EWIp and SHAMp groups (Fig. 3, Panel A, left); both operated groups showed higher counts than the naive controls. By contrast, the EWIp group presented eosinophilia, with significantly higher counts than those in both SHAMp and naive controls (Fig. 3, Panel B, left). On the other hand, neither neutrophil (Panel C, left) nor mononuclear cell (Panel D, left) counts showed significant differences in EWIp mice relative to the respective SHAMp controls. For neutrophils and mononuclear cells, only the EWIp group was significantly different from naive controls. Hence, in the PAS background (unlike the BALB/c background; see Fig. 1) the EWIp protocol induces a selective increase in eosinophils at the implant site relative to SHAMp controls. On the other hand, in ALOX mutants, the total cell numbers in the exudate were not significantly different between the EWIp, SHAMp and naive experimental groups; all three ALOX groups were comparable to the naive control group of wild-type PAS mice (Panel A, right). This absence of an

inflammatory response to either surgical procedure is paralleled by a lack of eosinophilia in all of the ALOX experimental groups (Panel B, right). A significant difference between SHAMp and EWIp groups was observed for neutrophils in ALOX mice, although the absolute numbers were low in both (Panel C, right). Concerning mononuclear cells, no significant difference between groups was observed in ALOX mice, since counts were comparable to those in the naive group of PAS controls (Panel D, right). Together, these observations suggest that 5-LO is required in EWIp mice for the selective induction of eosinophilia. In both EWIp and SHAMp groups, functional 5-LO is also required for the accumulation of other leukocyte populations (neutrophils and mononuclear cells) in the implant site.

8. Role of endogenous glucocorticoids in eosinophilia of the implant site

In the conventional immunization model, endogenous glucocorticoids are essential for the eosinophilia (both central and peripheral) that follows allergen challenge in sensitized mice [24], as shown by complete blockade by both metyrapone (a glucocorticoid synthesis inhibitor) and mifepristone (glucocorticoid receptor antagonist). In the surgical EWIp model, endogenous glucocorticoids might be increased in response to both allergen exposure (as in the conventional model [24]) and surgical trauma (as in SHAMp animals [25]). For this reason, we designed a double blocking procedure associating metyrapone and mifepristone before surgery (see *Methods*). The double treatment, with metyrapone given over an 8-day period and mifepristone given 16 h before surgery,

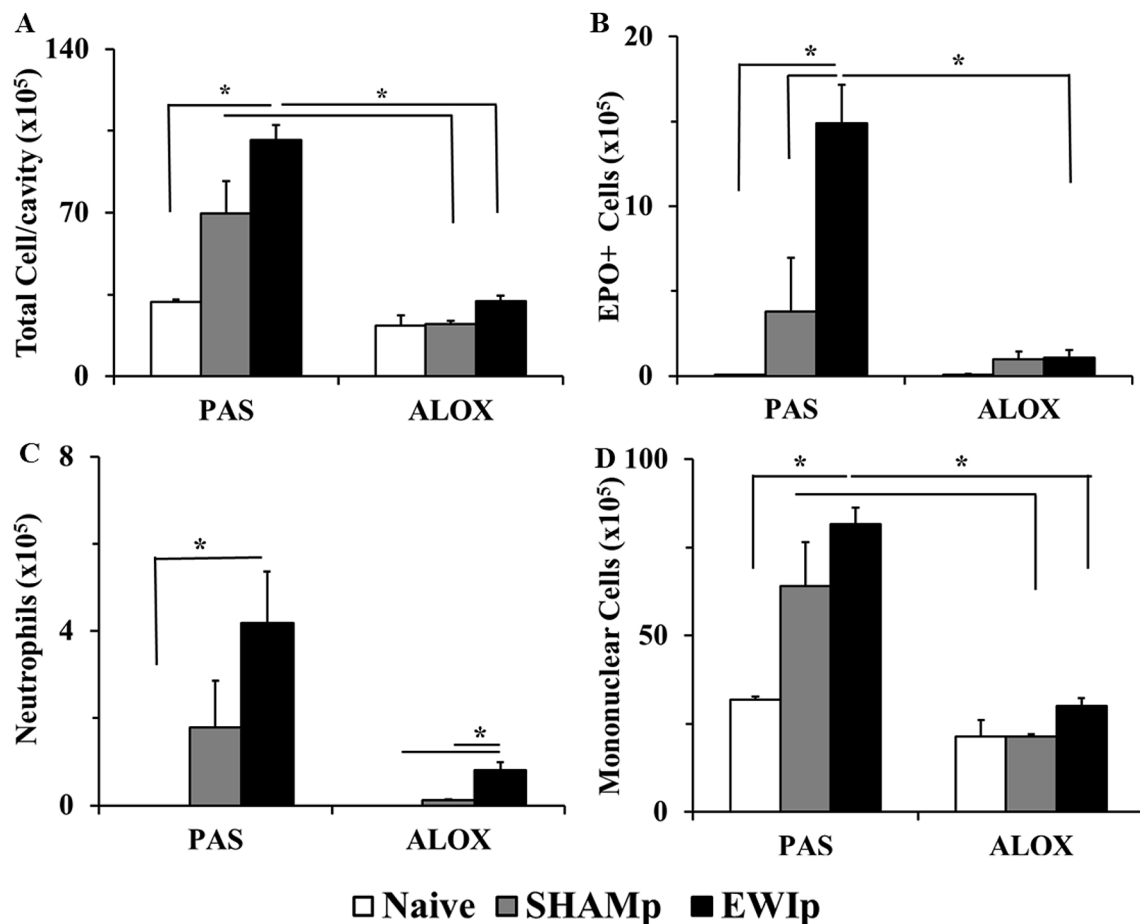


Fig. 3. Effect of 5-LO-inactivation on total and differential counts of leukocytes in the peritoneal lavage fluid at day 7. Data are numbers of: (A) Total cells, (B) Eosinophils, (C) Neutrophils and (D) Mononuclear cells collected from the peritoneal cavity from naive (white bars), SHAMp (grey bars) or EWIp (black bars) PAS (wild-type control) or ALOX (5-LO-deficient mutants of the PAS background) mice at day 7 post-surgery. *, significant differences relative to the indicated controls ($p < 0.05$). $n = 6, 11-12$, and 6 for the naive, PAS and ALOX groups respectively, in all panels.

significantly reduced total cell numbers in the peritoneal cavity at day 7 post-surgery, in Met/RU/EWIp mice relative to VEIC/VEIC/EWIp positive controls (Fig. 4, Panel A). This reduction was accounted for by a significant decrease in eosinophils (Panel B). By contrast, neutrophils (Panel C) and mononuclear cells (Panel D) were not decreased by this treatment. These data document an important role for endogenous glucocorticoids in the eosinophilia of the EWIp model, but not in the accumulation of other leukocyte classes.

9. Purification and features of EWIp-induced eosinophils

Eosinophils from EWIp donor mice were collected from peritoneal exudate, separated from mononuclear cells by a single step of plastic adherence and further purified by cell sorting, based on their cytometry profile (Fig. 5A, left) and on the eosinophil autofluorescence [34] (Fig. 5A, right). Gating (as outlined in blue in Fig. 5A, left) corresponds to the eosinophil region, as confirmed by its paucicellularity in eosinophil-deficient GATA-1 control mice (data not shown). The morphology of eosinophils obtained after purification was consistent with that of mature murine eosinophils: cells were medium-sized, with abundant peroxidase-containing granules, and a donut-shaped nucleus (Fig. 5B). Characteristic features were visible in transmission electron microscopy, including a polymorphic nucleus, lipid bodies, and the unique crystalloid-containing granules (Fig. 5C). Neither cytocentrifugates nor electron micrographs showed evidence of degranulation.

10. In vivo and in vitro functionality of purified eosinophils from EWIp donor mice

Because the EWIp protocol enables us to obtain preparative-scale

yields of highly purified eosinophils, which could be used for a variety of biochemical, physiological or pharmacological studies, it was important to establish the ability of the purified eosinophils from EWIp donors to function both in vivo and in vitro. To establish in vivo functionality, we examined first (Fig. 6, Panels A and B) the ability of BALB/c eosinophils transferred i.p. into eosinophil-deficient Δ dbl-GATA-1 recipients to reconstitute the fast (4 h) neutrophil accumulation in response to subsequent i.p. administration of eotaxin-1, which was previously shown to be strictly dependent on eosinophils [31]. As expected, Δ dbl-GATA-1 mice had no eosinophils in the absence of eosinophil transfer; 4.5 h after transfer, ~30% of all injected eosinophils were recovered in the lavage fluid (data not shown). Controls included recipients injected with medium alone, or with eosinophils followed by RPMI medium (vehicle). In the experimental (eosinophil and eotaxin-1-injected) group (dark gray bar), the peritoneal lavage fluid presented a significant increase in neutrophils (Panel A) relative to the two control groups (without eotaxin-1, light gray bar; without eosinophils and eotaxin-1, open bars). Eosinophil transfer without further administration of eotaxin-1 did significantly increase neutrophil counts relative to the negative control, but the magnitude of this effect was not sufficient to significantly increase total cell numbers (data not shown). Eosinophil transfer, with or without further eotaxin-1 exposure, did not significantly affect mononuclear cell counts (Panel B), showing the selectivity of the assay for the eosinophil and neutrophil lineages. In the absence of eosinophil transfer, the administration of eotaxin-1 does not induce migration of neutrophils or mononuclear cells to the peritoneal cavity [31].

To establish in vitro functionality, we next examined the chemotactic and chemokinetic responses of eosinophils from EWIp donor mice to eotaxin-1 in a transwell system, comparing purified eosinophils to

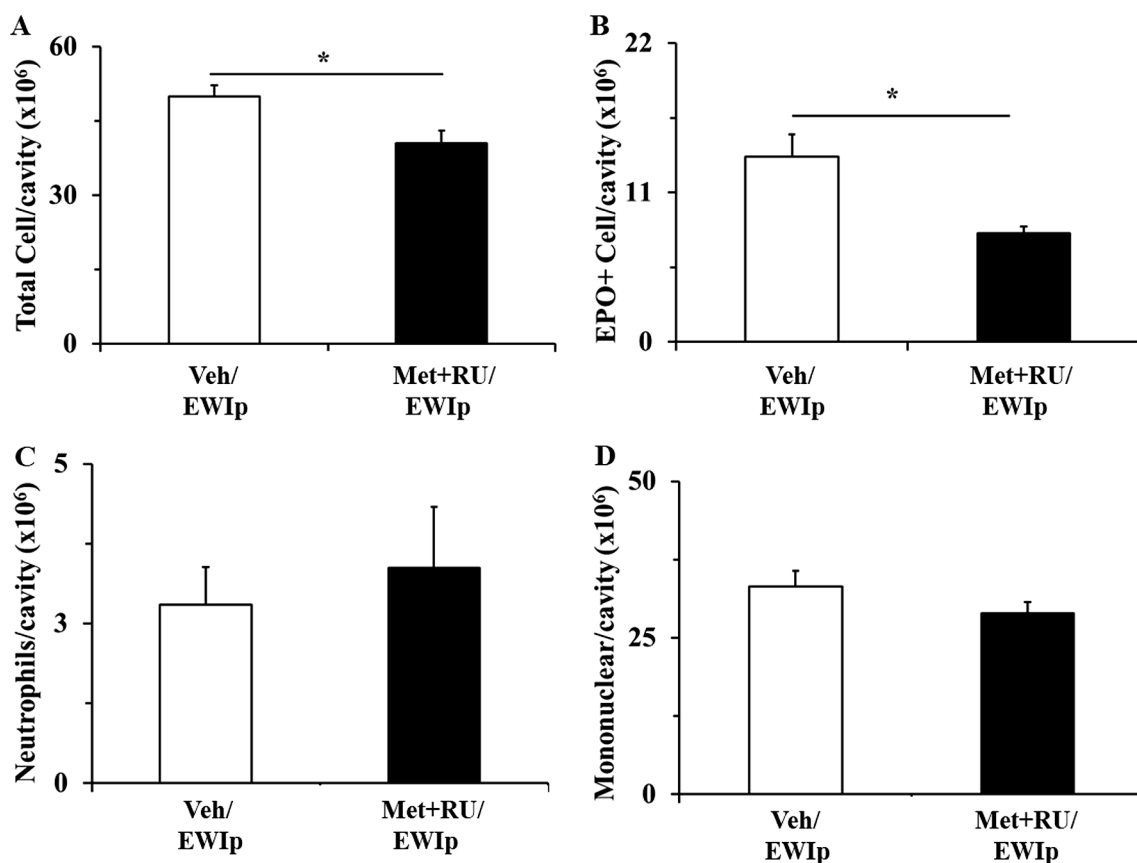


Fig. 4. Effect of EndoGC blockade on total and differential counts of leukocytes in the peritoneal lavage fluid at day 7. Data are numbers of: (A) Total cells, (B) Eosinophils, (C) Neutrophils and (D) Mononuclear cells collected from the peritoneal cavity of metyrapone- and RU486-treated (black bars) or vehicle-treated (white bars) BALB/c mice subsequently submitted to EWIp and euthanized at day 7. *, significant differences relative to the indicated controls ($p < 0.05$). $n = 7$ in all panels.

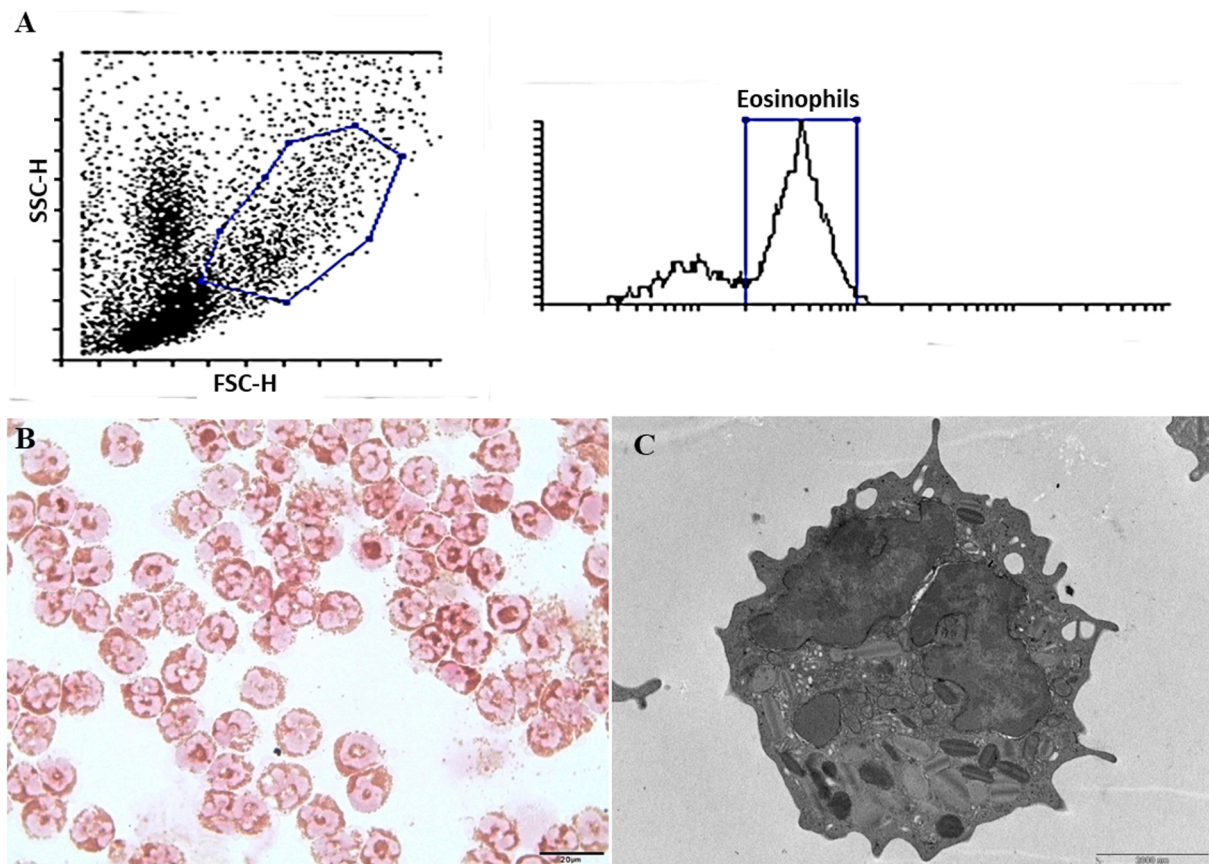


Fig. 5. Isolation and features of high purity eosinophils from EWIp carriers. Cells were obtained from peritoneal lavage fluid of BALB/c mice 7 days after EWIp surgery. Data are: (A, left) FSC $\times$ SSC plot from cells after the first step of eosinophil purification (gating in the eosinophil region outlined in blue); (A, right), histogram from gated cells presenting autofluorescence (fluorescence intensity at 488 nm), which is the parameter used for cell sorting. (B) Representative image of the eosinophils recovered from cell sorting in optical microscopy of cytocentrifugates, after staining for eosinophil peroxidase and counterstaining with Harris' hematoxylin (1000x magnification). (C) Representative image of eosinophil recovered from cell sorting in transmission electron microscopy (15000x magnification).

unseparated PEC containing 30% eosinophils and to reconstituted populations consisting of one-third purified eosinophils and two-thirds PEC collected from EWIp-carrying eosinophil-deficient Δ dbl-GATA-1 mice. Fig. 6, Panel C shows migration in a transwell of the following: a) 10^6 purified eosinophils from EWIp BALB/c donor mice (left); b) unseparated PEC from EWIp BALB/c donor mice (containing 10^6 eosinophils and 2×10^6 non-eosinophil cells; center); c) 10^6 purified eosinophils from EWIp BALB/c donor mice reconstituted with 2×10^6 total PEC from EWIp eosinophil-deficient Δ dbl-GATA-1 donor mice (right). All samples were assayed without (open bars) or with eotaxin-1 (black bars). Neither purification nor reconstitution diminished the chemotactic response of eosinophils in the presence of eotaxin. By contrast, in the absence of eotaxin-1, purified eosinophils showed significantly lesser migration to the lower chamber in comparison compared with the eosinophils in unseparated PEC or in reconstituted populations. This suggests eosinophil random chemokinesis (which, unlike chemotaxis, does not require an eotaxin concentration gradient) is increased in the presence of non-eosinophil cell types present in peritoneal exudate of EWIp carriers.

11. Discussion

Central (bone-marrow) and peripheral (implant site) eosinophilia was selectively induced by EWIp. We showed that peripheral eosinophilia was dependent on: a) a specific immune response to egg white antigens; b) the integrity of the 5-LO pathway; and c) signaling by endogenous glucocorticoids. We could, for the first time, demonstrate the requirement for antigen-specific immunity, products of the 5-LO

pathway, and endogenous glucocorticoids in sustaining the eosinophil-rich inflammation caused by the unique combination of surgical trauma and allergic sensitization. We achieved quantitative recovery of infiltrating leukocyte populations from the implant site, with preparative-scale yields of pure eosinophils from nontransgenic donors following two simple, antibody-free steps. This approach consisted of an initial *negative* selection (depletion of plastic-adherent cells) followed by *positive* selection (enrichment in eosinophils by cell sorting based on autofluorescence), aiming to prevent unintentional activation/deactivation of the selected cells by antibody-based methods, which target surface structures. Purity and functional preservation of the eosinophils were therefore directly demonstrated.

Multiple leukocyte types accumulated in the peritoneal cavity of EWIp carriers over a week. Eosinophils were in this model very abundant, amounting to over one-third of the PEC. Two complementary sets of observations point to an important role of specific immune responses in promoting and sustaining the eosinophil-rich mixed inflammatory infiltrate: a) the heterogeneous infiltrate was abolished by oral tolerization prior to EWIp surgery; b) the inflammation, and especially eosinophilia, were strongly enhanced by manoeuvres aimed at inducing (or boosting) OVA-specific sensitization. The boosting effect of supplementary immunization suggests that the adoptive transfer of immune T lymphocytes into naive recipient mice before EWIp surgery will allow us to identify a T cell subset endowed with the ability to initiate and/or accelerate the inflammatory response to EWIp. In this respect, DO11.10 mutants, which express a transgenic α/β receptor with specificity for one ovalbumin peptide in association with autologous MHC molecules [35], are likely to be very useful in the characterization of the relevant subset,

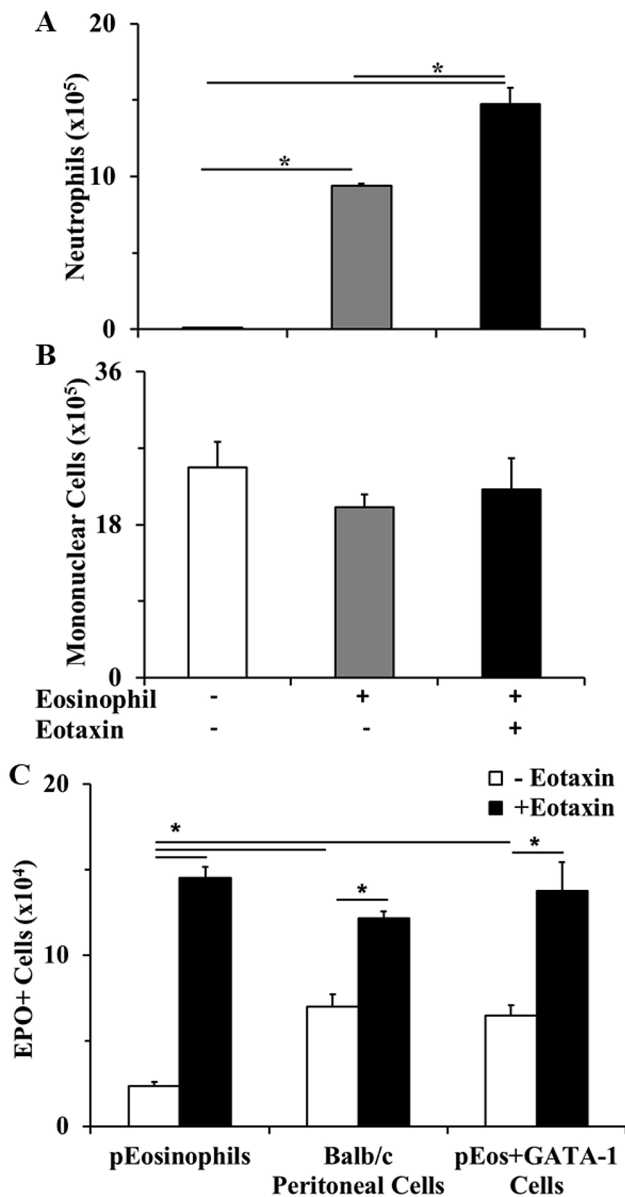


Fig. 6. Purified eosinophils are functional both *in vivo* and *in vitro*. A–B, *In vivo* actions—secondary recruitment of neutrophils. Eosinophil-deficient GATA-1 mice were injected with medium (open bars) or with 10^6 purified eosinophils, alone (grey bars) or in association with eotaxin-1, 50 ng/cavity (black bars). Data are numbers of: Neutrophils (A) and Mononuclear cells (B) obtained from peritoneal lavage fluid 4.5 h after eosinophil transfer. $n = 6$ in all panels. C, *In vitro* actions—chemotaxis and chemokinesis of eosinophils. In a transwell assay, the upper chamber received 10^6 purified eosinophils (p-Eosinophils; left), unseparated PEC from EWip-carrying BALB/c donors containing 10^6 eosinophils (center), or 10^6 purified eosinophils mixed with peritoneal cells from EWip-GATA-1 donor to match the proportion in the BALB/c peritoneal lavage fluid (right). Data are eosinophil numbers accumulating in the transwell lower chambers in response to 250 ng/mL eotaxin-1 (black bars) or vehicle (white bars). *, significant differences relative to the indicated controls ($p < 0.05$). $n = 6$ in all panels.

since their antigen-specific T cells are abundant and easy to isolate and tag in pure form for transfer and tracking experiments. Such cell transfer experiments would also reinforce the role of cell-mediated immunity in this model.

Accumulation of different leukocyte classes in the peritoneal exudate of EWip carriers requires functional 5-LO. One possibility is that the specific immune response to the implant antigens is expressed through

one or more products of the 5-LO pathway which induce the secondary recruitment of leukocytes. This hypothesis would predict that the 5-LO-dependent step is located *downstream* from the generation of an immune response to the implant, and therefore the reconstitution of the inflammatory response in EWip carriers of the ALOX strain would be achieved by local transfer of wild-type cells expressing 5-LO, obtained from nonsensitized donors, as a source of the relevant 5-LO product(s). This hypothesis is consistent with observations of the roles of various leukotrienes in attracting leukocyte subpopulations. Leukotriene B₄ (LTB₄), for instance, is a potent neutrophil chemoattractant, which also recruits mononuclear phagocytes and eosinophils [36,37]. The cysteinyl-leukotrienes (CysLT: LTC₄, LTD₄, and LTE₄) are eosinophil chemoattractants that promote mobilization of eosinophils from bone-marrow; CysLT also increase survival and cytokine production by eosinophils [38,39]. However, there is an alternative possibility, namely that 5-LO function is only required to *generate* the antigen-specific immune response that drives inflammation in this model. This alternative hypothesis would predict that the defect in ALOX EWip carriers is located *upstream* from specific sensitization, and the defect in EWip carriers of the ALOX strain would be corrected by local transfer of wild-type antigen-specific immune cells, such as T lymphocytes purified from the 7-day exudate of PAS EWip carriers.

Previous studies showed that endogenous glucocorticoids are essential for bone-marrow eosinophilia induced by subcutaneous EWip [24]. We demonstrated here that endogenous glucocorticoids also sustain eosinophilic inflammation at the implant site. The blockade performed before surgery was effective even though eosinophilia was examined at day 7, i. e., its effects outlast the glucocorticoid surge associated with surgery as well as with the pre-surgery procedures (8 injections of vehicle or metyrapone, followed by gavage with vehicle/mifepristone). A comparison of data in Figs. 1 and 4 suggests that with the addition of the pre-surgery procedures, the baseline inflammation (Panel A in both) and eosinophilia (Panel B in both) is significantly increased in the vehicle controls (Fig. 4) over the original EWip response (Fig. 1), as if these pre-surgery procedures, in the absence of endocrine blockers, were sufficiently stressful to strongly boost the glucocorticoid-dependent eosinophilia.

The experiment in Fig. 4 shows both a significant reduction of eosinophil numbers (which alone accounts for a significant reduction in total cells), and the persistence of a subpopulation of infiltrating eosinophils despite the double blockade by metyrapone and mifepristone. This suggests that two populations of infiltrating eosinophils exist, only one of which is sensitive to glucocorticoid blockade. We cannot, at this point, rule out the alternative possibility that the persistence of an eosinophil population at the site after the double blockade is due to residual glucocorticoid effects. However, we think this is unlikely, because in a previous study [24] both mifepristone and metyrapone were separately able to abolish the eosinophilic response to allergen-challenge by bringing eosinophil counts back to the baseline level; neither mifepristone nor metyrapone decreased eosinophil counts below the baseline of unchallenged controls [24]. In the present study, the association of mifepristone and metyrapone was synergistic, when compared to either separate treatment (data not shown). The comparison of flow cytometry profiles between eosinophils obtained with and without endocrine blockade might be useful in helping us to identify subpopulation markers to develop subset separation strategies. The concept of eosinophil subsets is consistent with previous studies: glucocorticoids have powerful and long-lasting stimulatory effects on eosinophil progenitors and precursors [26–29]. Dexamethasone augments eosinophil yield in bone-marrow cultures established in the presence of IL-5 by increasing the number of cell cycles that eosinophil precursors undergo before terminal differentiation; while dexamethasone-exposed cultures show eosinophil precursors with immature cytological features [26], dexamethasone-primed cultures respond to maturation-promoting agents, such as prostaglandin E₂, by producing larger numbers of mature eosinophils [27]. Similar effects were

shown in humans by other investigators [28]. Similarly, endogenous glucocorticoids, acting *in vivo*, are required for the eosinophil differentiation-promoting effects of CysLT [29] in murine bone-marrow cultures established *ex vivo* in the presence of IL-5 [30]. In the EWIp model the target of endogenous glucocorticoids might be a subset of eosinophil progenitors/precursors, which is kept proliferating over a larger number of cell cycles and finally matures in the presence of CysLT or other 5-LO end products.

Potential applications. The eosinophils were morphologically and functionally intact, and in sufficient numbers to support many novel applications. Considering that in physiological conditions eosinophils are scarce in blood and tissues, and that the use of EWIp donors from different wild-type and mutant strains of mice has the potential to generate a variety of purified eosinophil populations with different properties, amenable to both *in vivo* and *in vitro* experimentation, we think this modified EWI model is a useful addition to the existing tools in eosinophil biology. Isolation of eosinophil-specific organelles, granules, or crystals, in preparative scale for biochemical and ultrastructural studies would be thereby facilitated. For instance, the possibility of transferring pure eosinophils into eosinophil-deficient GATA-1 recipients would allow us to evaluate the role of 5-LO products in eosinophil-mediated recruitment of neutrophils and mononuclear phagocytes by eotaxin [31]. Alternatively, our approach may provide reliable *in vivo* assays, with potential applications to the study of eosinophil roles in the immune response to cancer [40], bleomycin-induced fibrosis or experimental allergic encephalomyelitis [13–16].

CCRediT authorship contribution statement

Bruno Marques Vieira: Conceptualization, Writing - original draft, Methodology, Investigation. **Carina Corrêa de Souza:** Investigation. **Daniela Masid-de-Brito:** Investigation. **Renato Nunes Ferreira:** Investigation. **Rebeca S. Brum:** Investigation. **Maria Ignez C. Gaspar-Elsas:** Resources, Supervision, Data curation. **Pedro Xavier-Elsas:** Conceptualization, Writing - review & editing, Methodology, Investigation.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES)-Finance Code 001. Supported by: a) fellowships from CNPq to BMV (PhD student), PXE and MIGE (Research Productivity Fellowships); b) research grants to PXE from CNPq (470377/2011-9, Edital Universal) and FAPERJ (E-26/103.138/2011 Cientista do Nosso Estado; E-26-112.694-2012 Collaborative Network on Immunomodulators; E-26/010.000918/2016 APQ1; E-26/010.001631/2019 APQ1.)

PXE conceived and supervised the study, reviewed the data and wrote the paper. MIGE provided essential materials, supervised the morphological aspects of the study and reviewed all the primary data. BMV developed the purification procedure and carried out most of the experiments, in collaboration with the following (who are listed in decreasing order of contribution to the final contents of Results): CCS, DMB, RNF, RSB.

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