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Essential roles of endogenous glucocorticoids and TNF/TNFR1 in promoting bone-marrow eosinopoiesis in ovalbumin-sensitized, airway-challenged mice

Daniela Masid-de-Brito ^{a,1}, Pedro Xavier-Elsas ^{a,*,1}, Ricardo Alves Luz ^{a,b}, Túlio Queto ^b,
Cássio Luiz Coutinho Almeida da Silva ^a, Rodrigo Soares Lopes ^a,
Bruno Marques Vieira ^b, Maria Ignez Capella Gaspar-Elsas ^b

^a Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil

^b Depto Pediatria, Instituto Fernandes Figueira, FIOCRUZ, Rio de Janeiro, Brazil

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ABSTRACT

Aims: Stress mechanisms paradoxically contribute to allergic episodes in humans and mice. Glucocorticoids (GC) and interleukin (IL)-5 synergically upregulate murine bone-marrow eosinophil production. Here we explored the role of endogenous GC in allergen-stimulated bone-marrow eosinophil production in ovalbumin-sensitized/challenged mice.

Main methods: In BALB/c or C57BL/6 mice, sensitized and intranasally challenged with ovalbumin, we monitored eosinophil numbers in freshly harvested or cultured bone-marrow, and plasma corticosterone levels. Metyrapone (MET) was used to inhibit GC synthesis, and RU486 to block GC actions. In sensitized mice challenged intraperitoneally, we examined the relationship between eosinophilia of bone-marrow and peritoneal cavity, in the absence or presence of RU486. In experiments involving in vivo neutralization of tumor necrosis factor- α (TNF) by specific antibodies, or using mice which lack functional type I TNF receptors (TNFR1), we evaluated the relationship between TNF blockade, corticosterone levels, RU486 or MET treatment and challenge-induced bone-marrow eosinophilia.

Key findings: RU486 or MET pretreatments abolished challenge-induced increases in eosinophil numbers in bone-marrow (in vivo and ex vivo), and in the peritoneal cavity. MET, but not RU486, prevented the challenge-induced increase in corticosterone levels. Challenge-induced bone-marrow eosinophilia and corticosterone surge were abolished in TNFR1-deficient mice. Anti-TNF-treatment very effectively prevented challenge-induced bone-marrow eosinophilia, in the absence of RU486 or MET, but had no independent effect in the presence of either drug.

Significance: Endogenous GC was essential for allergen challenge-induced increases in eosinophil numbers inside bone-marrow. This effect required TNF and TNFR1, which suggests an immunoendocrine mechanism.

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Introduction

Numerous reports suggest that stress mechanisms, dependent on adrenal glucocorticoid (GC) production, either predispose to, or enhance allergic inflammation, both in humans (Sandberg et al., 2000; Liu et al., 2002; Ritz et al., 2000) and experimental models (Bailey et al., 2009; Joachim et al., 2003; Chida et al., 2007; Datti et al., 2002). Furthermore, exposure to exogenous GC as part of therapeutic (Wiley et al., 2004) or immunomodulatory (Stock et al., 2005) regimens may also contribute to long-term aggravation of asthma in mice.

These paradoxical effects have been proposed to involve differential regulation of Th1 versus Th2 cytokine production (Iwakabe et al., 1998; Kang and Fox, 2001), and through the latter, of IgE antibody production, or, alternatively, from blunting of anti-inflammatory and immunoregulatory effects (Bailey et al., 2009; Stock et al., 2005). However, eosinophils, which play major roles in allergen-induced tissue damage, can also respond to GC in ways that ultimately promote, rather than inhibit, allergic inflammation.

We previously demonstrated that a single airway challenge in ovalbumin-sensitized mice rapidly upregulates bone-marrow eosinophilia, eosinophil progenitor responses to IL-3 and IL-5 in culture, and eosinophil progenitor numbers in lung tissue (Gaspar-Elsas et al., 1997; Gaspar-Elsas et al., 2003; Maximiano et al., 2005). We further reported that eosinophil production in bone-marrow culture is enhanced by dexamethasone, acting synergically with IL-5 (Gaspar-Elsas et al., 2000) and prostaglandin E2 (Gaspar-Elsas et al., 2009). Furthermore,

* Corresponding author at: Dept. Immunology, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil. Tel.: +55 21 9636 0108; fax: +55 21 2560 8344.

E-mail address: pxelsas@micro.ufrj.br (P. Xavier-Elsas).

¹ Daniela Masid-de-Brito and Pedro Xavier-Elsas are joint first authors.

surgical stress, in the absence of infection or allergic sensitization, upregulates eosinophil production in bone-marrow culture through adrenal GC production (Xavier Elsas et al., 2004). The unexpected parallelism of the effects of dexamethasone in vitro, and of stress-induced GC in vivo, to those of allergen challenge, prompted us to reexamine the role of endogenous GC in regulation of bone-marrow eosinophilia and eosinopoiesis following airway challenge.

Stress, both acute and chronic, enhances allergic airway inflammation in murine models, selectively increasing BAL eosinophilia (Joachim et al., 2003; Chida et al., 2007). Circulating corticosterone levels increase rapidly in allergen-challenged mice (Chida et al., 2007) without any identifiable external stressor. No mechanism has been demonstrated for the association of corticosterone surge and BAL eosinophilia, which apparently contradicts the well-established blocking effects of GC therapy on respiratory allergy (Bateman et al., 2010; Jonasson et al., 2010). Furthermore, no mechanism has been proposed to account for the strong corticosterone surge in allergen-challenged mice, which indeed suggests that allergen exposure itself mobilizes immunoendocrine mechanisms that ultimately lead to adrenal GC production comparable to that elicited by widely recognized stressors (Chida et al., 2007).

We hypothesized that adrenal GC release is required for bone-marrow eosinophil lineage-selective upregulation in sensitized/challenged mice, driving eosinopoiesis in bone-marrow, in the absence of any additional stressful maneuver. Accordingly, an endogenous GC surge should follow allergen challenge, and interference with either GC production or action should prevent all of the allergen-induced changes in bone-marrow eosinophils. To test this hypothesis, we directly examined whether blockade of endogenous GC production or action prevents upregulation of eosinophil production in the bone-marrow, and whether both the stimulatory effect of allergen and the inhibitory effects of the blockers correlated with their respective effects on endogenous GC.

Methods

Suppliers

The suppliers are as follows: FCS and media, Hyclone (Logan, UT); rmlL-5, R&D systems (Minneapolis, MN); ovalbumin, ICN Biomedicals (Aurora, OH); alum, Carlo Erba (Limite, Italy); RU486, metyrapone and methylcellulose, Sigma-Aldrich (St. Louis, MO); Corticosterone EIA Kit, Cayman Chemicals, Ann Arbor, MI; purified rat anti-mouse TNF- α neutralizing antibody, CAT. 554414, from BD-PharMingen, Franklin Lakes, NJ, USA; rat IgG1 CAT. 0116-0, used as an irrelevant specificity isotype control for anti-mouse TNF- α , Southern Biotechnology Associates, Birmingham, AL, USA.

In vivo procedures and experimental samples

Female mice 6–8 weeks old of the BALB/c and C57BL/6 backgrounds (both wild-type and TNFR1-deficient (B6.129 Tnfrsf1a) (Huber and Sartini, 2005), certified SPF, from CECAL-FIOCRUZ/RJ, were used following institutionally approved (CEUA#L-010/04, CEUA#L-002/09) protocols for: a) *sensitization* (2 s.c. injections of 100 μ g OVA in alum 1.6 mg/400 μ l/animal, 7 days apart); and b) *single challenge* at day 14 with 10 μ g OVA in saline, i.n. (25 μ l), or i.p. (400 μ l), in BALB/c mice; 25 μ g OVA i.n. (25 μ l) in C57BL/6 mice). RU486 (20 mg/kg) was given 2 h before challenge as a 300 μ l intragastric bolus, controls received vehicle (methylcellulose, 0.1% in water); metyrapone (30 mg/kg; De Bie et al., 1996) was injected i.p. for 8 consecutive days (Xavier Elsas et al., 2004), the last injection given 2 h before challenge (controls received vehicle). Mice were adapted for 1 week to the animal facility (housing mice only, in microisolator racks with up to 7 mice per unit) with a 12 h light/12 h darkness cycle (direct), standard mouse chow (Nuvilab CR-1, São Paulo, Brazil) and bedding, and water ad libitum, before manipulation. Experimental handling (sensitization,

challenge, drug administration, euthanasia, sample collection for blood and bone-marrow analyses) was scheduled to start between 8 and 10 am, following the same order for each group, so that 24 h intervals were kept for all groups. In Results, the order in which the various groups were manipulated is portrayed in the figures from left to right in the corresponding panels. Individual mice of the same age were weighed, and the average weight (20 g) was used to calculate the amount of drug (RU486, metyrapone) administered. No attempt was made to keep track of individual mice, since that would be impractical for experiments involving up to 8 daily drug/vehicle injections, and unnecessary with genetically identical animals of the same age and sex. Where indicated, TNF- α neutralization was achieved in vivo through administration of 10 μ g anti-TNF- α or isotype control antibody of irrelevant specificity (Montinaro et al., 2012) in 200 μ l PBS, i.p., 48 h before challenge. Euthanasia was carried out in a CO₂ chamber (Beira-Mar, Rio de Janeiro, Brazil). Blood was collected into a heparinized syringe from the abdominal vena cava immediately after death, and corticosterone immunoassays were carried out in plasma after centrifugation (Xavier Elsas et al., 2004). Peritoneal lavage fluid was collected in mice challenged with ovalbumin i.p. 24 h earlier, after repeatedly (3 $\times$) flushing the peritoneal cavity with 10 ml cold RPMI medium, and used for total and differential counts of recruited leukocytes, as well as quantitation of eosinophil peroxidase activity (EPO; also termed cyanide-resistant peroxidase). Bone-marrow cells were collected 24 h after i.n. or i.p. challenge from both femurs of individual mice, counted and cytocentrifuged before staining for EPO, or quantitation of EPO activity, or further cultured (see below) to determine the effects of challenge on eosinopoiesis in vivo (Day 0) and ex vivo (Day 7), respectively (Gaspar Elsas et al., 2000; Queto et al., 2010).

Peroxidase assays

The coarse cytoplasmic granules containing EPO are easily recognizable by their reddish-brown color after staining with diaminobenzidine in the presence of cyanide, and represent an eosinophil lineage-specific marker detectable from the earliest precursors to terminally differentiated eosinophils (Horton et al., 1996; for representative images, see also Gaspar-Elsas et al., 1997, 2009). The % EPO+ cells were scored under high magnification (1000 $\times$, under oil), by counting 300 cells in random fields in diaminobenzidine/cyanide-stained cytocentrifuge smears. The total numbers of cells expressing EPO were calculated by multiplying the % EPO+ cells in cytocentrifugates by total cell counts in either Day 0 (freshly collected bone-marrow from both femurs) or Day 7 samples (liquid cultures), obtained in hemocytometers after Turk's staining. Alternatively, EPO activity was measured on 4 $\times$ 10⁶ bone-marrow cells plated in flat-bottom 96 well plates, washed twice (500 $\times$ g, 10) and resuspended in 300 μ l PBS. Samples (50 μ l) received an equal volume of reaction mixture (10 ml PBS, 15 mg OPD, 200 ml H₂O₂ 30%), with or without 6 mM KCN and were incubated for 4 min in the dark. Reaction was stopped with 50 μ l 4 N H₂SO₄. Absorbance was measured at 490 nm.

Bone-marrow culture

Where indicated: a) *liquid bone-marrow cultures* (Gaspar-Elsas et al., 1997) were established for 7 days with 10⁶ bone-marrow cells/ml in RPMI-1640 medium, 10% FCS, 1 ng/ml rmlL-5, followed by total and EPO+ cell counts; b) *semi-solid (clonal) cultures* (Gaspar-Elsas et al., 1997; Gaspar Elsas et al., 2000) were established from 2 $\times$ 10⁵ bone-marrow cells in 1 ml in 35 mm triplicate culture dishes in IMDM with 20% FCS, GM-CSF (2 ng/ml) and agar Noble (0.3% final). Colonies (>50 cells) comprising all GM-CSF-stimulated progenitor types (GM/G/M/GMEos/Eos) were scored at Day 7. Eosinophil-containing colonies (GMEos/Eos, (Gaspar-Elsas et al., 1997)) were subsequently enumerated on dried agar layers after staining for EPO (Gaspar-Elsas et al., 1997; Gaspar Elsas et al., 2000).

Statistical analysis

The experimental unit for all data shown was the individual animal. In **Results**, the numbers of animals used in each experimental group are provided in the caption to the corresponding panels. Where indicated, multiple comparisons were done with two-way ANOVA (Tukey's or Bonferroni's corrections were used for groups of identical or unequal size, respectively); where appropriate, two-group comparisons were done by two-tailed *t* tests (separate variances). For *t* tests and Tukey's, we used Systat for Windows 4 (Systat, Inc., Evanston, IL); for Bonferroni's, Prism 5 for Windows (Graph Pad, La Jolla, CA). Analyses through both software require conformity of the data with the model underlying the aforementioned tests, and lack of conformity is indicated by the software when it occurs.

Results

Effect of pretreatment with RU486 on allergen-induced bone-marrow eosinophilia, in vivo and ex vivo

To test the hypothesis that an endogenous GC surge should follow allergen challenge, and interference with either GC production or action should prevent all of the allergen-induced changes in bone-marrow eosinophils, we initially examined the effects of pretreatment with GC/progesterone receptor antagonist RU486 on the well-established responses of the bone-marrow, both in vivo and ex vivo (Gaspar-Elsas et al., 1997; Queto et al., 2010), to allergen challenge of the airways.

As shown in Fig. 1A, allergen challenge significantly increased the total numbers of EPO+ (eosinophil-lineage, both mature and immature) cells in bone-marrow freshly collected from both femurs of each animal, relative to saline-challenged controls. Importantly, while RU486 pretreatment had no significant effect in unchallenged controls, it completely prevented the effect of allergen challenge on bone-marrow eosinophilia in vivo. These differences were not accounted for by the corresponding differences in the total numbers of nucleated bone-marrow cells (all lineages considered) in the same groups, as shown in Fig. 1B, and were therefore considered as lineage-selective.

It was important to confirm that these differences in EPO+ cell numbers were accompanied by similar differences in the activity of EPO in freshly harvested bone-marrow, which is independent of microscopic scoring and associated factors, such as the number of fields counted, the average numbers of cells per field, and the homogeneity of distribution of cells among fields. The allergen-induced increase, as well as the ability of RU486 to prevent this increase, was accurately reflected in the EPO activity of freshly harvested bone-marrow (1C), thereby providing an objective biochemical correlate of the microscopic scoring method, which can be adapted for handling larger numbers of samples.

On the other hand, allergen challenge significantly enhanced subsequent responses to IL-5 in bone-marrow culture (1D), which reflect differentiation from immature precursors (Gaspar-Elsas et al., 1997, 2009). This ex vivo effect of in vivo airway challenge was also abolished by the RU486 pretreatment of challenged mice. By contrast, RU486 had no significant effect on unchallenged controls.

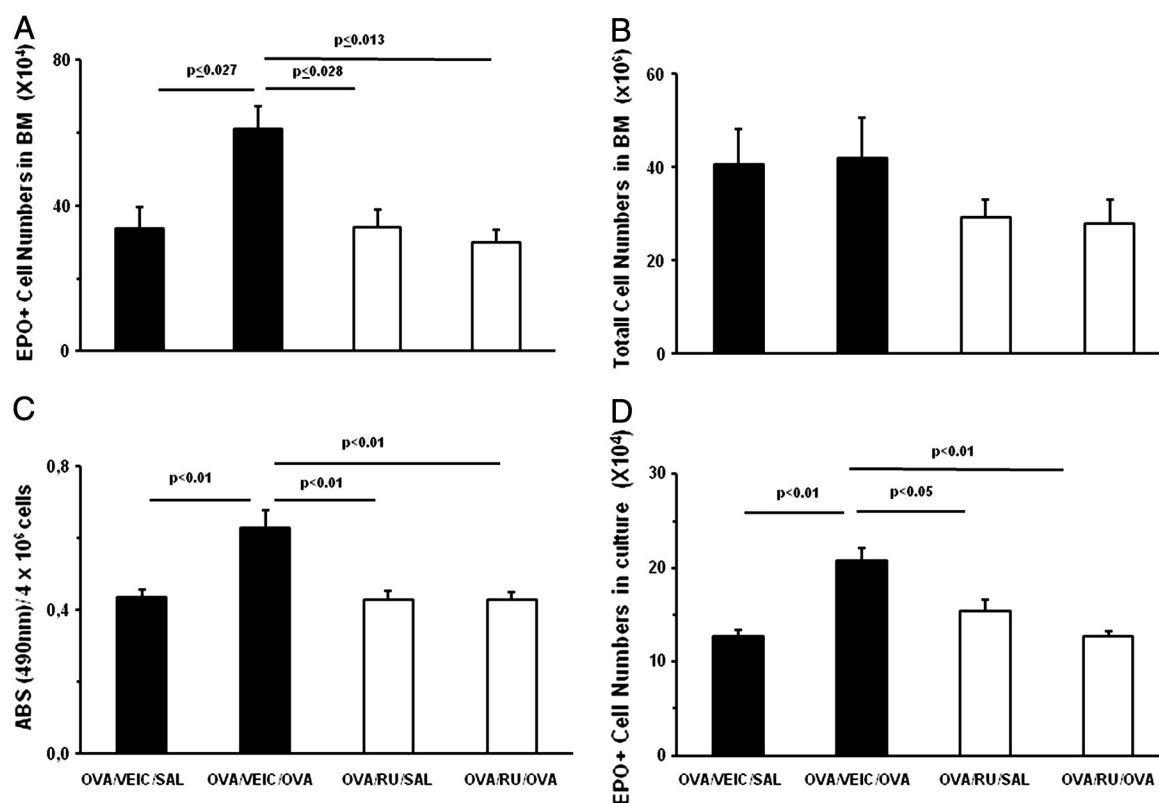


Fig. 1. Effect of pretreatment with RU486 on allergen-induced bone-marrow eosinophilia, in vivo and ex vivo. BALB/c mice, sensitized to ovalbumin (OVA, 2 s.c. 100 µg injections in alum, 7 days apart), were pretreated with vehicle (VEIC, black bars) or RU486 (RU, open bars), 300 µl as an intragastric bolus, and challenged i.n. 2 h later with saline (SAL) or OVA (10 µg/25 µl). Bone-marrow (BM) was collected 24 h after challenge from both femurs of each mouse, counted for total and EPO+ cells (Day 0 counts), assayed for EPO activity of 4×10^6 cells or further cultured for 7 days with IL-5 (Day 7 counts). Data (mean $\pm$ SEM) are as follows: A, total EPO+ cell numbers ($n = 3$ all groups); B, total femoral BM cell numbers in freshly collected BM ($n = 3$ for all groups); C, absorbance at 490 nm in the presence of 6 mM KCN, as a measurement of EPO (cyanide-resistant peroxidase) activity in freshly collected BM ($n = 4/3/5/5$, from left to right for the groups shown); D, EPO+ cell numbers in bone-marrow cultivated 7 days with IL-5 ($n = 3/4/4/4$). All significant differences between experimental groups are indicated (in B, OVA/VEIC/SAL $\times$ OVA/RU/SAL, $P = 0.631$; OVA/VEIC/SAL $\times$ OVA/RU/OVA, $P = 0.560$; OVA/VEIC/OVA $\times$ OVA/RU/SAL, $P = 0.544$; OVA/VEIC/OVA $\times$ OVA/RU/OVA, $P = 0.476$).

Effect of pretreatment with RU486 on allergen-induced expansion of bone-marrow progenitors (colony-forming cells) ex vivo

We further examined whether the recovery of increased total femoral bone-marrow EPO+ cell numbers at Day 0 (1A), which reflects the condition of bone-marrow in vivo, and the increased responses to IL-5 ex vivo (1D), which reflect an increased responsiveness of precursors (Gaspar-Elsas et al., 1997), would be accompanied by a selective expansion of the bone-marrow pool of lineage-committed progenitors (colony-forming cells) detectable in semisolid culture (Gaspar-Elsas et al., 1997; Gaspar-Elsas et al., 2000). As shown in Fig. 2A, the numbers of eosinophil-containing colonies derived from plating 2×10^5 bone-marrow cells in the presence of the multilineage hemopoietic growth factor, GM-CSF, were significantly increased by airway allergen challenge of sensitized mice. This increase was abolished by the RU486 pretreatment of the challenged mice. The differences between groups in numbers of eosinophil progenitors were not accounted for by the corresponding differences in the total number of GM-CSF-responsive progenitors (all lineages considered), which did not significantly differ between the same groups of animals (2B), and therefore are considered eosinophil lineage-selective.

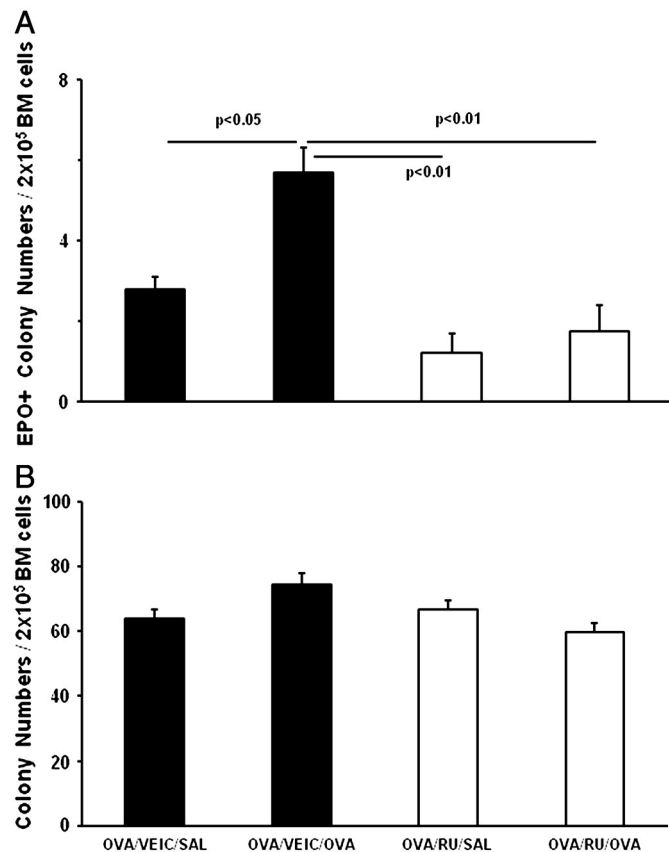


Fig. 2. Effect of pretreatment with RU486 on allergen-induced expansion of bone-marrow progenitors (colony-forming cells) ex vivo. Groups of BALB/c mice, pretreatment (black and open bars), airway challenge and BM harvest were as described in caption to Fig. 1. Semisolid cultures were established with 2×10^5 BM cells per 35 mm culture dish in 1 ml IMDM/20% FCS/2 ng/ml GM-CSF/0.3% agar Noble. Colonies (>50 cells) arising in all GM-CSF-responsive progenitor types were scored at Day 7; eosinophil-containing colonies were subsequently enumerated on dried agar layers stained for EPO. Data (mean $\pm$ SEM) are as follows: A, numbers of eosinophil-containing (EPO+) colonies ($n = 3/4/4/4$), B, total numbers of colonies, irrespective of lineage, formed by 2×10^5 bone-marrow cells plated, after 7 days in GM-CSF-stimulated semisolid cultures ($n = 3/4/4/4$). All significant differences between experimental groups are indicated. Calculations of total EPO+ colony forming cells in femoral BM of mice in the OVA/VEIC/OVA and OVA/RU/OVA groups (823 ± 102 and 403 ± 153 , mean $\pm$ SEM) showed a reduction of ~50%, averaging 419 progenitors less in the RU-treated group.

Effect of pretreatment with metyrapone on allergen-induced bone-marrow eosinophilia, in vivo and ex vivo

We further examined the effects of pretreatment with metyrapone (MET), an inhibitor of adrenal GC biosynthesis, on allergen challenge-induced bone-marrow eosinophilia. The challenge-induced increase in total femoral bone-marrow EPO+ cell numbers was totally prevented by MET pretreatment, while MET had no significant effect in unchallenged controls (Fig. 3A). Moreover, the enhanced response to IL-5 in bone-marrow culture, in challenged mice relative to unchallenged controls, was also abolished by MET pretreatment (3B). Again, MET had no significant effect in unchallenged mice.

Effect of pretreatments with RU486 and metyrapone on allergen-induced increases in plasma corticosterone levels

Our hypothesis required the demonstration of a link between allergen challenge and a surge of GC hormones. We directly examined this link, by measuring plasma corticosterone levels at the time point in which allergen-induced increases bone-marrow EPO+ cells and

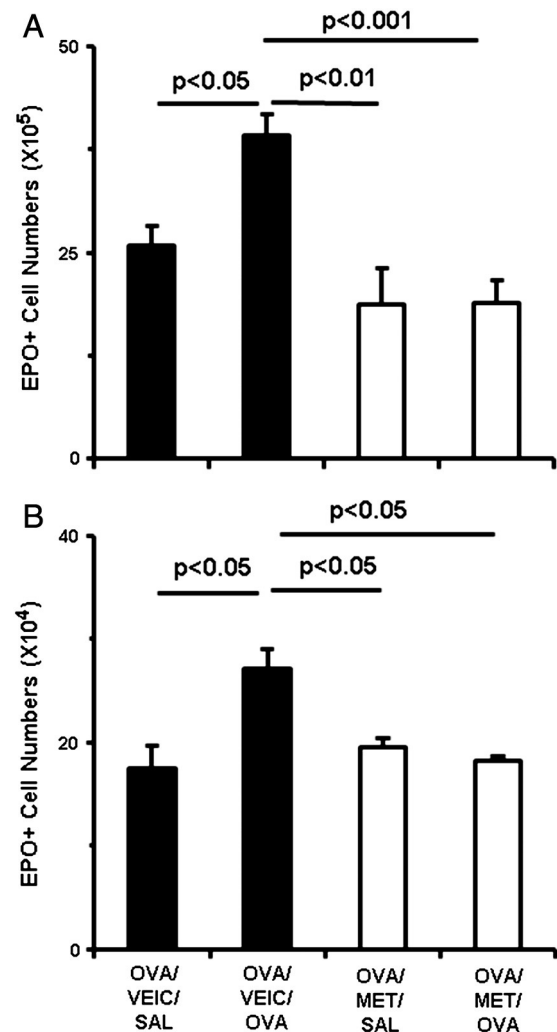


Fig. 3. Effect of pretreatment with metyrapone on allergen-induced bone-marrow eosinophilia, in vivo and ex vivo. Separate groups of BALB/c sensitized to OVA as described in caption to Fig. 1 were pretreated with vehicle (VEIC, black bars) or metyrapone (MET, open bars), 8 daily i.p. injections. Airway challenge, BM harvest and sample handling for generation of Day 0 and Day 7 counts were as in caption to Fig. 1. Data (mean $\pm$ SEM) are as follows: A, total EPO+ cell numbers in freshly collected BM ($n = 7/7/3/7$); total bone-marrow cell counts, like those in Fig. 1B, did not significantly differ between groups (not shown); B, EPO+ cell numbers in bone-marrow cultivated 7 days with IL-5 ($n = 3$ for all groups). All significant differences between experimental groups are indicated.

eosinophil progenitor numbers, as well as precursor responsiveness to IL-5, were all significant, as shown by comparison between OVA/OVA mice and OVA/SAL controls. Corticosterone levels were significantly increased in challenged mice relative to unchallenged, sensitized controls (Fig. 4A). RU486 pretreatment, which blocks GC signaling rather than production, had no significant effect on corticosterone levels in challenged or control mice (4A). Importantly, MET pretreatment, which prevents GC production rather than signaling, completely abolished the challenge-induced surge in corticosterone levels (compare the vehicle-treated group, challenged in 4B with the vehicle-treated, challenged group in 4A) bringing them back to the unchallenged control level (compare the MET-treated, challenged group 4B with the vehicle-treated, unchallenged group in 4A). Together, these data show that allergen challenge induces a strong endogenous GC response, which closely parallels the eosinophilia of bone-marrow.

Effect of RU486 on eosinophil numbers in BM and peritoneal cavity following i.p. OVA challenge of sensitized mice

One important aspect of this model is whether it depends on mechanisms which can be mobilized by allergen challenge in the airways, but not in other sites. We therefore examined the ability of i.p. OVA challenge to induce an RU486-preventable increase in bone-marrow EPO+ cell numbers, and took further advantage of the favorable conditions of this alternative challenge site to carry out accurate quantification of recruited eosinophils, which is difficult to perform in the lungs by conventional histopathological approaches, and rather laborious to do in BAL samples.

OVA challenge i.p. significantly increased total EPO+ cell numbers in freshly collected bone-marrow from both femurs of each animal, thereby documenting that this effect does not require any specific property (anatomical or functional) of the airways (Fig. 5A). Importantly, this effect was abolished by the RU486 pretreatment as effectively as that of airway challenge (5A). In addition, OVA challenge i.p. induced a significant accumulation of eosinophils in the peritoneal cavity (5B), which was significantly reduced (by ~50%) by the RU486 pretreatment, thereby confirming that the positive association between endogenous GC and eosinophil inflammation is not restricted to the airways.

Evidence for a TNF/TNFR1-dependent mechanism for challenge-induced increases in bone-marrow eosinophils and plasma corticosterone levels

It was important to establish a plausible sequence of events that accounts for the ability of a local immune challenge with specific allergen to induce a strong endocrine response from the adrenal glands, thereby supporting a complex hematological and inflammatory response with apparent selectivity for eosinophils. Previous studies have shown that proinflammatory cytokines such as TNF- α and IL-1 possess important systemic effects, including the ability to activate the hypothalamus–pituitary–adrenal (HPA) axis (Berthold-Losleben and Himmerich, 2008; Joachim et al., 2006).

We took initially advantage of the availability of mutant C57BL/6 mice lacking TNF- α type I receptors (TNFR1) to examine the effects of allergen challenge on the hematological (Fig. 6A) and immunoendocrine (Fig. 6B) parameters previously examined in the BALB/c strain. Unlike wild-type C57BL/6 (positive) controls, OVA-challenged TNFR1-deficient mutants did not show a significantly increased number of EPO+ cells in bone-marrow, relative to their unchallenged control group (Fig. 6A). Most importantly, there was a clear relationship to the lack of an endogenous GC surge: TNFR1 mutant mice, unlike C57BL/6 controls, showed no increase in corticosterone levels following allergen challenge, consistently with a mechanism in which TNF- α receptors provide an indispensable link between OVA challenge and the immunoendocrine response (6B). Furthermore, these findings could not be accounted for an inability of TNFR1 mutant mice to mount an inflammatory response or to produce eosinophils, because they presented larger numbers of eosinophils recruited by allergen challenge into the peritoneal cavity than the C57BL/6 controls (6C), even though they failed to present challenge-associated bone-marrow eosinophilia (6D).

We next examined whether the association between TNFR1 function and bone-marrow eosinophilia was coincidental, or, alternatively, whether TNF- α would be relevant to bone-marrow eosinophilia through a mechanism unrelated to GC production. In the first case, the use of a different approach to disrupting TNF- α signaling would not support our hypothesis; in the second case, if TNF- α had a role of its own, separate and independent of GC production, this role should be demonstrable both in the absence and in the presence of RU486/MET.

To test these hypotheses within the same experimental framework that was used in the preceding experiments, we chose to neutralize

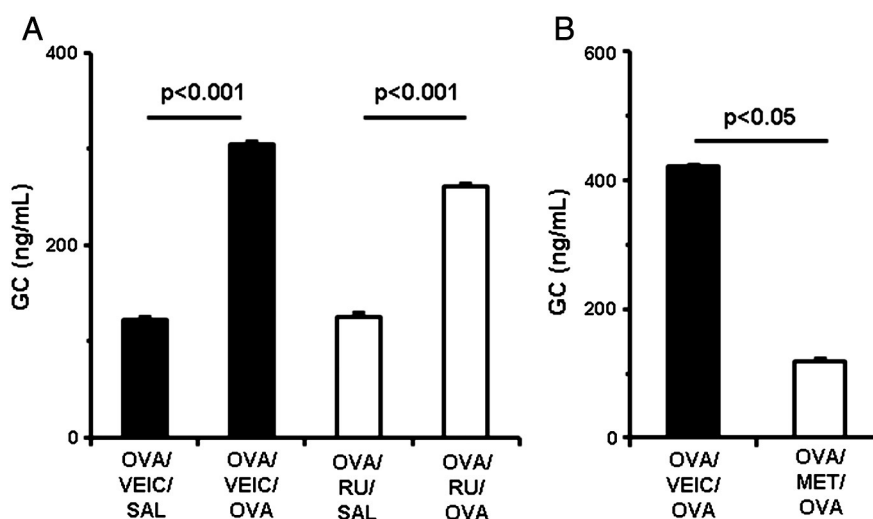


Fig. 4. Effect of pretreatments with RU486 and metyrapone on allergen-induced increases in plasma corticosterone levels. Separate groups of BALB/c sensitized to OVA as described in caption to Fig. 1 were pretreated with vehicle (VEIC, black bars), RU486 (RU, open bars in Panel A, as detailed in caption to Fig. 1) or metyrapone (MET, open bars in Panel B, as detailed in caption to Fig. 3). Airway challenge was as in caption to Fig. 1. Heparinized blood was collected 24 h after challenge and used for quantitation of corticosterone (Gc) levels by immunoassay (17). Data (mean $\pm$ SEM) are plasma corticosterone (Gc) levels ($n = 3$). A, $n = 3/4/3/4$; B, $n = 3$ for both groups. All significant differences between experimental groups are indicated.

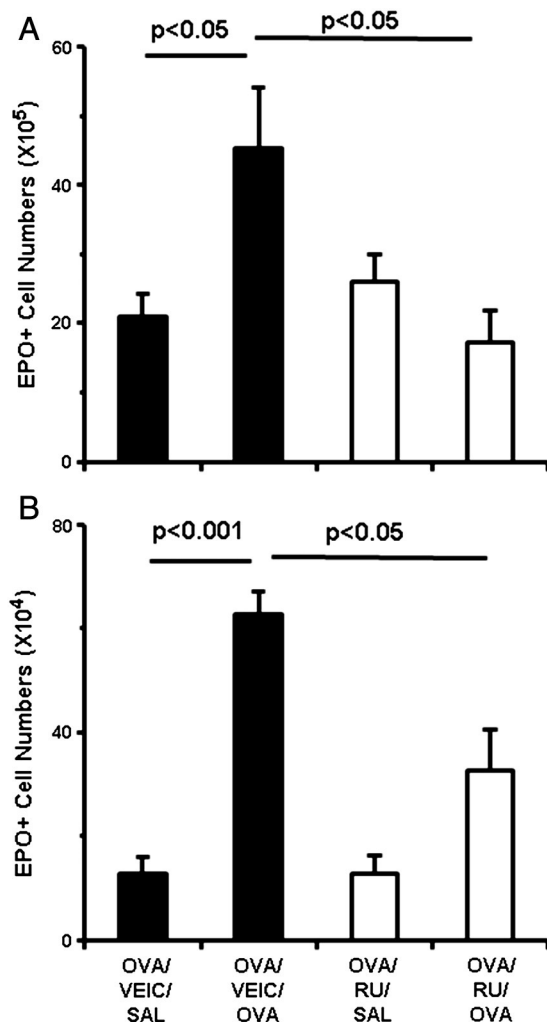


Fig. 5. Effect of RU486 on eosinophil numbers in BM and peritoneal cavity following i.p. OVA challenge of sensitized mice. Separate groups of BALB/c mice were sensitized, pretreated with vehicle (VEIC, black bars) or RU486 (RU, open bars), and challenged with SAL or OVA (10 mg/400 μ l) as detailed in caption to Fig. 1. BM (A) and peritoneal lavage fluid (B) were harvested 24 h after challenge. Sample handling to obtain total EPO+ cell counts was as detailed in caption to Fig. 1. Data (mean $\pm$ SEM) are total EPO+ cell numbers in freshly collected BM from both femurs of each animal (A) or peritoneal lavage fluid (B) in OVA/SAL or OVA/OVA BALB/c mice, pretreated with vehicle (VEIC) or RU486 before challenge. A, $n = 4$ all groups. B, $n = 4/4/4/5$. All significant differences between experimental groups are indicated.

TNF- α in vivo in sensitized/challenged BALB/c (Fig. 7A) and C57BL/6 (7B) mice with specific antibody. These experiments included the appropriate control antibody of the same isotype, as well as a route of administration (i.p.) and a period of antibody equilibration among compartments (48 h), prior to challenge, which were adequate to our purpose. The outcomes of interest were, in both cases, the effects of anti-TNF- α on bone-marrow eosinophilia in the *absence* and in the *presence* of either RU486 or MET. As shown in 7A and 7B, anti-TNF- α had a clear effect in the *absence* of endocrine blockers, preventing the up-regulation of eosinophilia by allergen challenge, in both BALB/c (7A) and C57BL/6 mice (7B). By contrast, anti-TNF- α had no effect in the *presence* of RU486 (7A) or MET (7B). Together, these observations support the view that TNF and its type I receptors play an important role in up-regulating bone-marrow eosinophilia through induction of endogenous GC.

Discussion

We provide direct evidence that endogenous GC are required for the eosinophilia of the bone-marrow, as well as enhanced eosinophil

differentiation from bone-marrow cells in response to both IL-5 and GM-CSF, which rapidly develop following airway allergen exposure in sensitized mice (Gaspar-Elsas et al., 1997). These effects, which reflect both an increase in numbers of eosinophil progenitors and in responsiveness of eosinophil precursors to IL-5 (as analyzed in detail by Gaspar-Elsas et al., 1997) resemble those previously demonstrated following in vitro and in vivo exposure to dexamethasone (Gaspar-Elsas et al., 2000) and to stress-induced GC (Xavier-Elsas et al., 2004), which are often referred to as priming. In a previous study, priming by stress-induced GC was shown to last as long as 2 weeks (Xavier-Elsas et al., 2004). In more recent, as yet unpublished, observations, dexamethasone priming for increased myeloid colony formation (as defined in Gaspar-Elsas et al., 2000) lasted over 1 month. These findings are compatible with a target cell that is relatively long-lived, and with a mechanism of priming that involves long-term changes in gene expression, a well-characterized effect of GC. By contrast, they would be difficult to account for mechanisms such as phosphorylation or dephosphorylation, which are known to rapidly and transiently affect intracellular signaling.

Our data confirm that *specific allergen challenge in the airways induces a strong surge of endogenous GC production, which drives the stimulatory effects of challenge on the eosinophil lineage*. We have used complementary, but independent, approaches to investigate GC roles: while RU486 interferes with GC receptor-mediated signaling, MET suppresses GC synthesis by the adrenal glands. As expected, MET was highly effective in suppressing challenge-induced corticosterone production, while RU486 had no effect on the corticosterone surge. Both approaches prevented the selective effects of allergen challenge on bone-marrow eosinophils to a comparable extent, reducing by ~50 % the total numbers of EPO+ cells in bone-marrow, the numbers of EPO+ cells differentiated in culture, and the numbers of progenitors forming EPO+ colonies. This identity of effects between agents which disrupt GC-dependent signaling at distinct steps (production and receptor activation, respectively) shows an essential requirement for endogenous GC in the effects of airway challenge.

According to our hypothesis, blockade of GC production or action, which effectively opposes the consequences of allergen challenge, should have no effect in unchallenged controls. RU486 or MET treatment had no effect in unchallenged controls, but had very consistent effects in challenged mice. This argues for corticosterone playing a significant role in this model only in the context of challenge. This conclusion is reinforced by the results from challenged TNF- α $-/-$ mice, which failed to develop both corticosterone surge and the bone-marrow eosinophilia, only in the context of challenge.

Importantly, the data establish a requirement for endogenous GC in the effects of airway challenge on the bone-marrow, which is independent of any added stressor. This is a substantive advance relative to previous reports on the effects of stress and stress hormones as modifiers of allergic airway inflammation, since they did not explore the possibility that both the basic phenomenon (respiratory allergy) and the modifier (stress) involved production of endogenous GC, and had outcomes similarly dependent on these hormones. Notably, the study by Chida et al. (2007) documented an intense and early corticosterone surge following allergen challenge in their nonstressed control group (which actually produced more GC than both their stressed groups), but did not examine how corticosterone could become irrelevant in this group if it contributed to the proallergic effects of stress in the other groups.

In a peritoneal challenge model, we further provided evidence that allergen-induced eosinophilia of the bone-marrow is paralleled by significantly increased eosinophil infiltration of the peritoneal cavity. Both effects were blocked by RU486. The model is convenient for future studies of the differences between the effects of endogenous GC and those of therapeutic doses of GC in allergic responses.

We were able to identify one possible mechanism to account for the upregulation of GC production following allergen challenge, which ultimately increases bone-marrow eosinophil numbers, since both

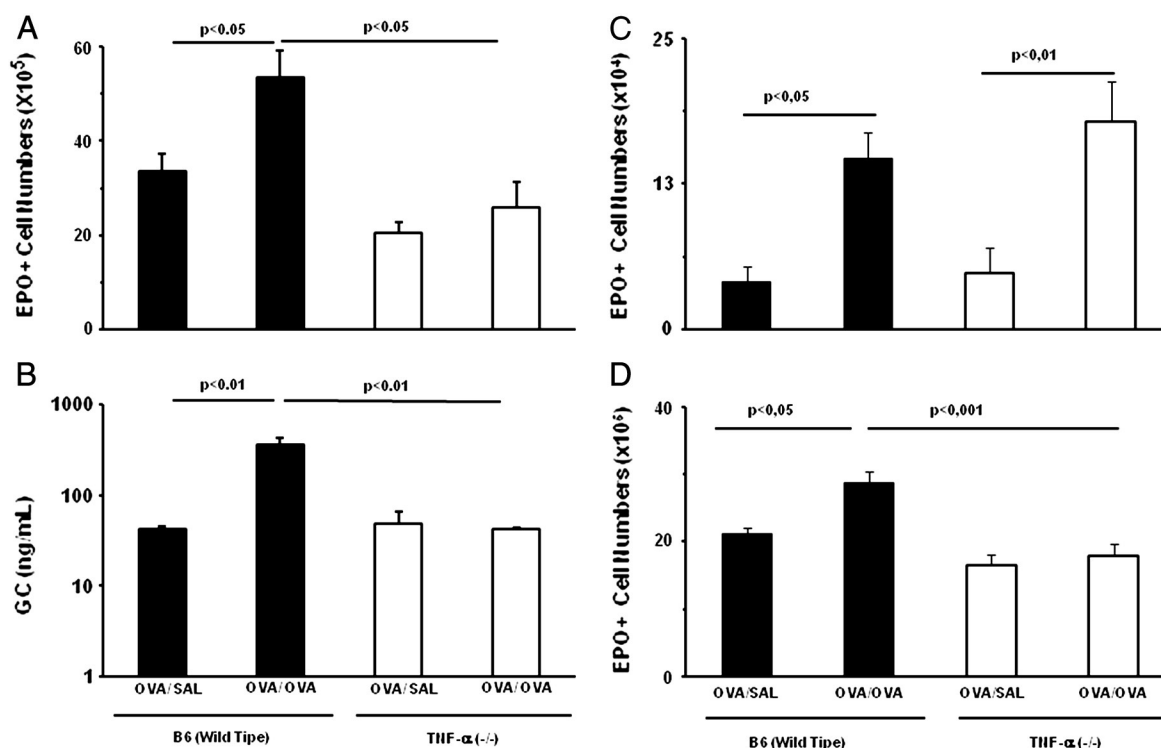


Fig. 6. Absence of allergen-induced increase in bone-marrow eosinophils and plasma corticosterone levels in mice lacking the type I TNF receptor. C57BL/6 mice, either wild-type (B6, black bars), or mutants lacking the type I TNF- α receptor (TNF- α $-/-$, open bars), were sensitized to OVA as detailed in the caption of Fig. 1. Separate groups were challenged at Day 14 with saline (SAL) or OVA (25 μ g/25 μ l), by the i.n. (A, B) or i.p. (C, D) routes. Bone-marrow (A, D) was collected and total and EPO+ cells counted as detailed in caption to Fig. 1. Heparinized blood (B) was used for corticosterone (Gc) quantitation as detailed in caption to Fig. 4. Peritoneal lavage fluid (C) was collected for total and EPO+ cell counts as detailed in caption to Fig. 5. A, Data (mean $\pm$ SEM) are total EPO+ cell numbers in freshly collected BM from B6, or from TNF- α $-/-$ mice, sensitized and i.n. challenged ($n = 5/5/3/3$). B, Data (mean $\pm$ SEM) are plasma GC levels in the experimental groups shown in A ($n = 5/5/3/3$). C, D, Data (mean $\pm$ SEM) are total EPO+ cell numbers in peritoneal lavage fluid (C) or freshly collected BM (D) from B6, or from TNF- α $-/-$ mice, sensitized and i.p. challenged (C, $n = 6/10/7/8$; D, $n = 7/9/8/8$). All significant differences between experimental groups are indicated.

outcomes were present in wild-type controls but absent in TNFRI-KO mice. It might be argued that, since TNF- α is essential to normal inflammatory/immune homeostasis, an animal lacking TNFRI cannot mount an appropriate immune/inflammatory response, and exposure of the bone-marrow to substances that are necessary to upregulate eosinophilia, such as IL-5, would not take place. This hypothesis is, however, contradicted by direct evidence that sensitized, TNFRI-deficient mice challenged with allergen present more intense leukocyte infiltration than the wild-type controls, and have eosinophils that can be recruited, presumably from the peripheral blood. It is noteworthy that TNFRI deficiency in the C57BL/6 mutant mice did not prevent leukocyte migration into the peritoneal cavity. By contrast, in sensitized wild-type BALB/c mice, RU486 reduced infiltration by one-half (compare Fig. 6C with Fig. 5B). Future studies should examine whether these apparent discrepancies between the pharmacological short-term model (BALB/c) and the genetical long-term model (C57BL/6 mutants lacking TNFRI) can be accounted for by background strain differences, by hitherto unidentified effects of TNFRI deficiency, or by adaptive changes over a lifetime of immune deficiency on the part of the mutant mice.

Further evidence for a role of TNF- α in the upregulation of bone-marrow eosinophilia was obtained through *in vivo* neutralization, in which antibody is given in excess, so that the concentration of cytokine found *in vivo* no longer influences the outcome. *Anti-TNF had a clear effect in the absence of endocrine blockers*: it prevented the upregulation of bone-marrow eosinophilia by allergen challenge, in both BALB/c and C57BL/6 mice. By contrast, *anti-TNF- α had no effect in the presence of either endocrine blocker*: hence, its effectiveness was abolished by interference with GC signaling, in agreement with the view that this effectiveness depends on the ability of TNF- α to induce GC. These observations reinforce the idea that TNF-TNFR1 link allergen challenge, through a GC surge, to bone-marrow eosinophilia.

Based on the immunoendocrine interactions involving proinflammatory cytokines and activation of the HPA axis (Berthold-Losleben and Himmerich, 2008; Joachim et al., 2006), we propose that local TNF- α production initiated at the challenge site evokes systemic effects, driving endogenous GC production. In sensitized mice, GC administration before allergen exposure effectively prevents respiratory allergy (Wiley et al., 2004; Jonasson et al., 2010), presumably by interfering at a very early step with generation of cytokines, including TNF- α , ultimately preventing the endogenous GC surge that depends on TNFRI. In future studies, it is important that such a TNF- α -mediated process be experimentally dissociated from any maneuver intended to evoke stress, because stress induces TNF- α in mice (Berthold-Losleben and Himmerich, 2008; Joachim et al., 2006).

At present, it is clear that TNF and TNFRI are *necessary* for the GC surge following challenge; it remains to be established, however, whether TNF- α alone is *sufficient* to evoke the GC surge. A similar, and related, issue is *whether GC are necessary, but not sufficient*, to induce bone-marrow eosinophilia *in vivo*. These questions are prompted by a comparison of GC effects in different experimental settings. In naive BALB/c mice, *in vivo* injection of dexamethasone primes bone-marrow for increased responses to IL-5 *ex vivo*, with no increase in bone-marrow EPO+ cell numbers (Gaspar Elsas et al., 2000). By contrast, in naive C57BL/6, the same treatment increases both *ex vivo* IL-5 responses and *in vivo* EPO+ cell numbers (manuscript in preparation). On the other hand, GC-dependent increases in bone-marrow eosinophils were seen in sensitized/challenged BALB/c mice, but not naive mice of the same strain. This highlights the importance of context, including both genetic background and allergen exposure, for the GC effect.

One hypothesis that reconciles these apparently incompatible observations of GC effects can be advanced as follows: in sensitized/

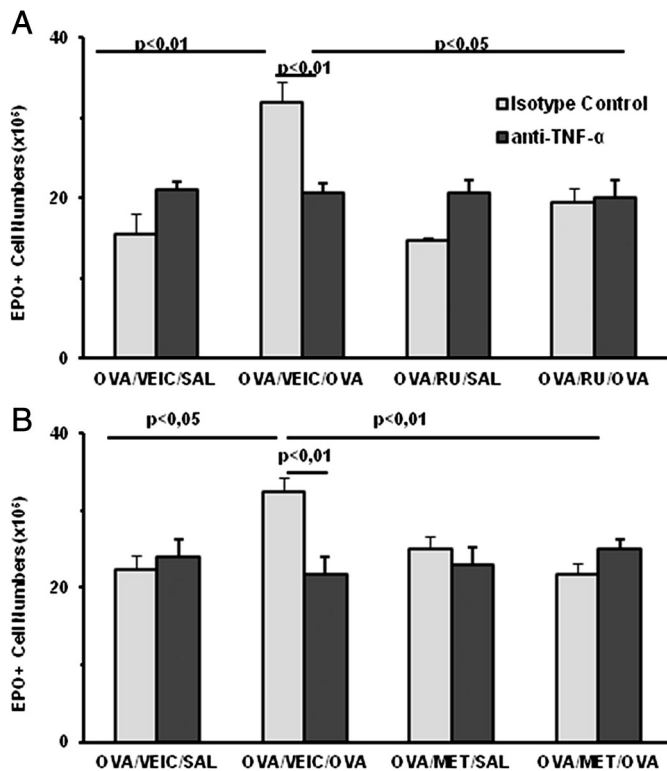


Fig. 7. The effectiveness of anti-TNF neutralizing antibodies in allergen-challenged, sensitized mice depends on undisrupted GC signaling. **A**, BALB/c mice were sensitized, pretreated with vehicle (VEIC) or RU486 (RU), and challenged i.n. 2 h later with SAL or OVA as detailed in caption to Fig. 1; **B**, C57BL/6 mice were sensitized as detailed in caption to Fig. 1, pretreated with vehicle (VEIC) or metyrapone (MET), as detailed in caption to Fig. 3, and challenged i.n. with SAL or OVA as detailed in caption to Fig. 6. The indicated groups of mice in both A and B were given anti-TNF neutralizing (dark gray bars) or irrelevant isotype control (light gray bars) antibodies (10 µg/200 µl/mouse), i.p., 48 h before challenge. Bone-marrow was collected, and total and EPO+ cells counted, as detailed in caption to Fig. 1. Data (mean ± SEM) are EPO+ cell numbers in freshly collected bone-marrow. **A**, isotype control-treated, $n = 3/5/3/3$; anti-TNF-treated, $n = 4/5/4/5$; **B**, isotype-control treated, $n = 3/5/3/4$; anti-TNF-treated, $n = 4/5/4/5$. All significant differences between experimental groups are indicated.

challenged mice, TNF- α would be transiently present in the circulation, drive adrenal GC production, and also prime targets for responding to this GC and to IL-5 with increased proliferation and differentiation in vivo. Such a synergic ("priming") effect of TNF- α might account for the differences between sensitized/challenged and naive mice, because the latter, unlike the former, would not have been exposed to systemically elevated TNF- α . These TNF- α -primed effects on bone-marrow responses to GC would be, by definition, dependent on GC, and therefore compatible with the evidence presented here, that anti-TNF treatment reveals no effect of TNF that is independent of GC signaling. Future studies should directly address the possibility that, in sensitized/challenged mice, TNF- α prepares bone-marrow to support increased in vivo eosinophil production in response to a combination of GC and IL-5.

Conclusion

To our knowledge, this is the first demonstration that bone-marrow eosinophilia and eosinopoietic activity are upregulated in allergen-challenged mice through an immunoendocrine mechanism requiring both TNF/TNFR1 signaling and adrenal GC production. We further provided evidence that adrenal GC is important for eosinophil accumulation in an inflammatory site (peritoneal cavity) in BALB/c mice. This should prompt further reexamination of proallergic GC effects, which were repeatedly reported but remain little understood.

Conflict of interest statement

None to declare.

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