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

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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 (TNFRI), 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 TNFRI-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 TNFRI, which suggests an immunoendocrine mechanism.

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,

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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); rmIL-5, R&D systems (Minneapolis, MN); ovalbumin, ICN Biomedicals (Aurora, OH); alum, Carlo Erba (Limito, 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 TNFRI-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 μg OVA in alum 1.6 mg/400 μl/animal, 7 days apart); and b) *single challenge* at day 14 with 10 μg OVA in saline, i.n. (25 μl), or i.p. (400 μl), in BALB/c mice; 25 μg OVA i.n. (25 μl) in C57BL/6 mice). RU486 (20 mg/kg) was given 2 h before challenge as a 300 μl 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 μg anti-TNF-α or isotype control antibody of irrelevant specificity (Montinaro et al., 2012) in 200 μl PBS, i.p., 48 h before challenge. Euthanasia was carried out in a CO_2 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×) 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×, 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 4×10^6 bone-marrow cells plated in flat-bottom 96 well plates, washed twice (500×*g*, 10) and resuspended in 300 μl PBS. Samples (50 μl) received an equal volume of reaction mixture (10 ml PBS, 15 mg OPD, 200 ml H_2O_2 30%), with or without 6 mM KCN and were incubated for 4 min in the dark. Reaction was stopped with 50 μl 4 N H_2SO_4. 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 10^6 bone-marrow cells/ml in RPMI-1640 medium, 10% FCS, 1 ng/ml rmIL-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 2×10^5 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).