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Potent stimulation of eosinopoiesis in murine bone-marrow by myriadenolide is mediated by cysteinyl-leukotriene signaling

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ABSTRACT

We describe the potent effect of myriadenolide (Myr), a naturally occurring labdane diterpene, in promoting the production of eosinophils in cultured bone-marrow from several inbred mouse strains. This enhancing effect is lineage-selective and requires the eosinophil growth factors, Interleukin(IL)-5 or GM-CSF. Myr acts over a very low concentration range (10^{-10}–10^{-14} M), if added at the beginning of the cell cultivation. Its enhancing effect increases between 24 h and 10 days of culture. We used both pharmacological and genetical tools to analyze its mechanism of action. Several lines of evidence show that the enhancing effect of Myr requires functional integrity of the 5-lipoxygenase (5-LO) pathway, and of $CysLT_1$ receptors, which transduce the effects of cysteinyl-leukotrienes generated through this pathway. Myr also protects developing eosinophils from apoptosis induced by exogenous prostaglandin E2 (PGE_2), but not by NO, indicating that it acts upstream of NO in the PGE_2-initiated proapoptotic pathway which requires iNOS and CD95. Exposure to NO concentrations insufficient to induce apoptosis abolished the ability of eosinophils to respond to Myr, suggesting the involvement of a NO-sensitive cellular target. Myr has potential as a chemically defined research tool, which can be used to generate large numbers of eosinophils, thereby overcoming current limitations in the biochemical and molecular biological study of murine eosinophils, which has so far depended on complex, labor-intensive and long-term culture protocols for in vitro expansion.

Summary: Potent enhancing effects of Myr on eosinophil production in bone marrow stimulated by GM-CSF and IL-5 are mediated by the 5-LO pathway.

1. Introduction

Eosinophil granulocytes, a subpopulation of blood and tissue leukocytes, prominent in allergic disease infiltrates and associated with helminth infections, have been the focus of intensive investigation over the last two decades [1–6]. Recent studies have highlighted cytokine production [1,7–8], antimicrobial peptide secretion [9–10], antigen presentation [11–12], extracellular trap formation [13–14], among other important, novel functions of eosinophils; they also evidenced several novel processes and organelles whose significance is currently being established. Gradually, the interest of eosinophil biology has expanded from its original framework to include conditions other than allergic processes and helminth-caused disease.

Eosinophils, in both humans and mice, are a minority of the circulating leukocytes and undergo homing to different mucosal sites (especially the gut [15–16]), from which they can be isolated only by a laborious digestion/separation procedure. As a result, their purification from either blood or tissues is a limiting factor in the study of their

Abbreviations: FLAP, 5-lipoxygenase activating protein; 5-LO, 5-lipoxygenase; CysLT, Cysteinyl-leukotriene; $CysLT_1R$, type 1 cysteinyl-leukotriene receptors; EPO, eosinophil peroxidase; IL, interleukin; iNOS, inducible NO synthase; MKT, montelukast; Myr, myriadenolide; PGE_2, prostaglandin E2; SNAP, S-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside

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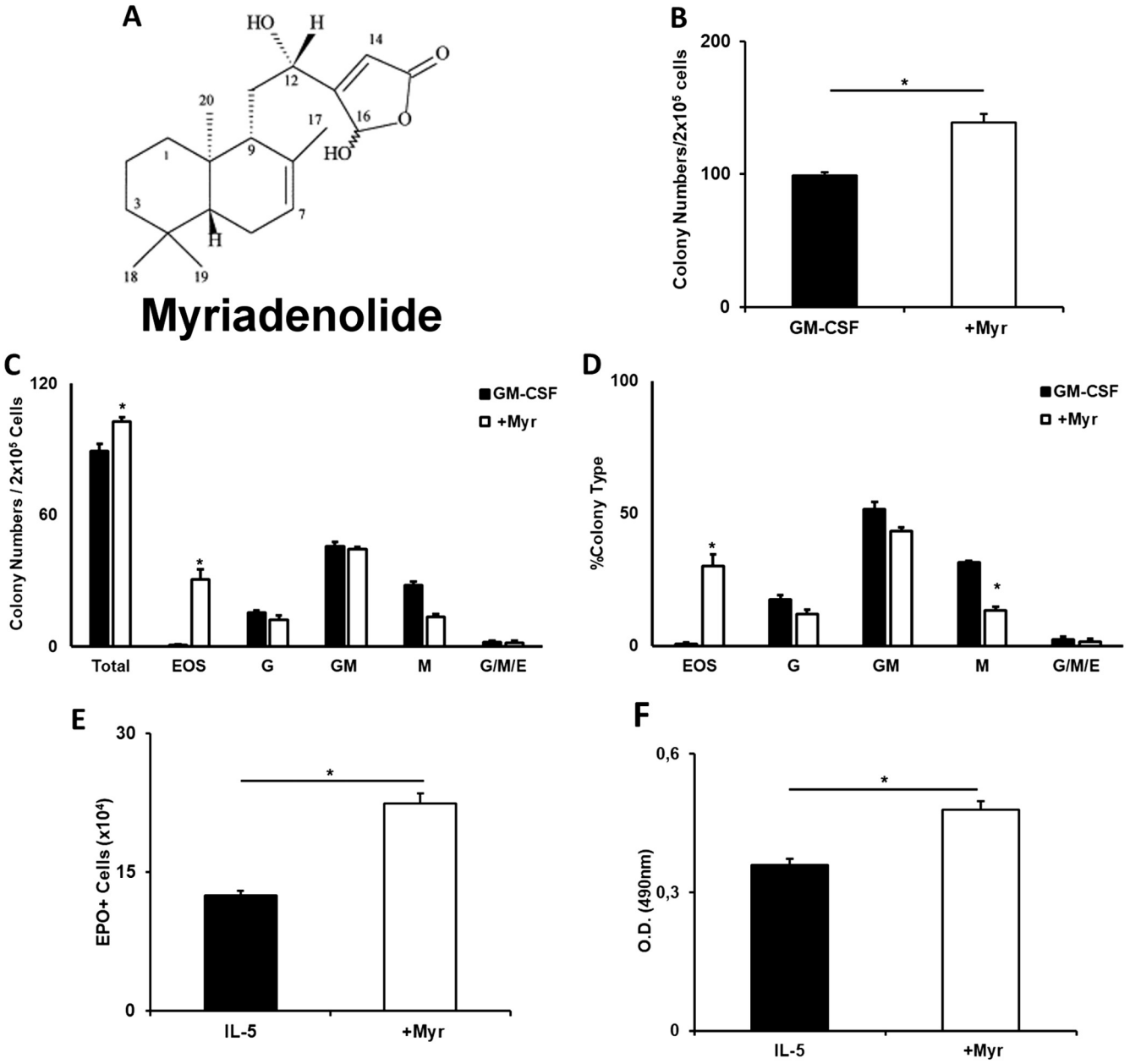

Fig. 1. Myr selectively stimulates eosinopoiesis in murine bone marrow cultures. In (A) structure from Myr, obtained from [38]. In (B–F), cultures were established in presence of GM-CSF (B-D) or IL-5 (E, F) with (open bars) or without (closed bars) Myr [10^{-10} M], from bone-marrow of naïve BALB/c donor mice. Data from semi-solid (B–D) and liquid cultures (E–F) established from freshly collected bone marrow are, respectively, Mean (+SEM) of: (B) total colony counts; (C) numbers of the different myeloid colony types; (D) percent of the different myeloid colony types after colony staining; (E) total EPO+ cell numbers; (F) absorbance at 490 nm as a measurement of EPO activity. In C and D, the following colony types can be unambiguously identified: pure eosinophil colonies (Eos), pure neutrophil colonies (G), neutrophil/macrophage containing, mixed colonies (GM), pure macrophage colonies (M) and eosinophil/neutrophil/macrophage-containing (mixed) colonies (G/M/E). *, significant differences relative to the indicated controls ($p < 0.05$). n = 7 in B–D; n = 8 in E, n = 4 in F.

properties in a physiological setting. Therefore, there is interest in experimental approaches that provide numbers of eosinophils in high purity and adequate for biochemical, immunological or pharmacological characterization.

Human blood eosinophils can be isolated in high purity by several methods [17–18]; however, ethical and practical considerations preclude in vivo and ex vivo experimental approaches which could yield information on human eosinophil functions. Alternatively, mouse eosinophils can be obtained from transgenic mice overexpressing the eosinophil growth and differentiation factor, Interleukin (IL)-5 [19], or after in vivo stimulation with the immunoregulatory cytokine IL-33 [20]. However, transgenic mouse eosinophils may not be functionally representative of those in nontransgenic donors; furthermore, mouse eosinophil expansion in culture currently requires several rounds of stimulation with different cytokines which are costly and time-consuming [21]. Therefore, a chemically defined, small-molecule stimulant, capable of acting on nontransgenic mouse bone-marrow over a shorter period to expand murine eosinophil numbers, through an identifiable mechanism, might provide an attractive alternative for preparative purposes. The study of the effects of cysteinyl-leukotrienes (CysLT; comprising LTC_4, LTD_4, and LTE_4) on murine eosinophil production has provided us with an opportunity to explore this approach.

CysLT, end products of the 5-lipoxygenase [5-LO] pathway of arachidonate metabolism, are central mediators in allergic reactions and

account for bronchoconstriction [22–24] and excessive mucus secretion [25–27], two cardinal features of allergic asthma in humans and in animal models [28]. They also contribute to worm expulsion in animal models of helminth infection [29]. Mouse eosinophils respond to CysLT in vitro [30] and in vivo [31], concomitant with expression of their CysLT receptors [32]. Importantly, the production of eosinophils from murine bone-marrow progenitors and precursors is upregulated by CysLT [30,33]. Endogenous CysLT production mediates the stimulatory effects of cytokines (eotaxin, IL-13; [34]) and nonsteroidal anti-inflammatory drugs (indomethacin, aspirin; [30]) on eosinophil production by IL-5-stimulated murine bone-marrow cultures. The latter effect protects developing eosinophils from the proapoptotic effects of prostaglandin E_2 (PGE_2), which are mediated by inducible NO synthase (iNOS) acting upstream from the death receptor ligand CD95L and its receptor CD95 [35]. This proapoptotic mechanism is relevant in vivo in a murine model of asthma; as predicted, CysLT are required for the increase in eosinophil production by allergen challenge in this model [36,37]; furthermore, a leukotriene synthesis inhibitor, diethylcarbamazine, prevents challenge-induced eosinophilia by inhibiting this proapoptotic pathway [37]. Together, the evidences point to CysLT as endogenous mediators of the stimulatory effects of allergen challenge, cytokines, and drugs on the murine eosinophil lineage in various experimental settings.

To identify molecules able to increase eosinophil production from murine bone-marrow, we screened several compounds, including myriadenolide (12-*S*,16-dihydroxy-*ent*-labda-7,13-dien-15,16-olide; Myr). Myr is a labdane diterpene isolated from *Alomia myriadenia* Schultz-Bip.ex. Baker (Asteraceae), a herb native to the Brazilian Atlantic Forest [38]. Myr has previously been tested in several bioassays and is known to inhibit phytohaemagglutinin A-induced lymphocyte proliferation at μM concentration and to induce monocyte apoptosis without effect on T lymphocyte subset ratios [39–40]. Myr showed proapoptotic activity in some human cancer cell lines, as THP-1 and Jurkatt; depolarization of mitochondrial membranes and activation of caspases-8, -9 and -3 were shown [41]. We report here that Myr enhanced eosinophil production in a murine bone-marrow culture at very low concentrations and protects eosinophils from apoptosis mediated by the iNOS-CD95 pathway [30,42]. Analysis of the underlying mechanism of this potent stimulatory effect showed it is mediated by CysLT type 1 receptor ($CysLT_1R$) signaling, following activation of the 5-LO pathway.

2. Methods

2.1. Animals, ethical aspects

Naïve, wild-type mice of the BALB/c, C57BL/6, CBA, BP-2, 129S2/SvPAS (and their mutant lacking 5-LO (129S2/SvPAS-Alox5)), were provided by CECAL-FIOCRUZ, Rio de Janeiro, Brazil, were used at 6–8 weeks of age as a source of bone marrow cells. Animals were maintained in microisolador units, 7 mice maximum per cage, 12 h light/dark cycle, 23 °C, and received autoclaved mouse chow and water ad libitum. All animal and experimental procedures were approved by the Institutional Animal Ethical Committees (License numbers CEUA#L-010/04, CEUA#L-002/09, CEUA-CCS-UFRJ-181-2013) and followed the ARRIVE guidelines [43–44]. A total of 57 mice were used in the experiments described here. Euthanasia was performed under a CO_2/O_2 excess atmosphere.

2.2. Reagents

Myr (Fig. 1A) was isolated from an ethanol extract of aerial parts of *A. myriadenia* Schultz-Bip. ex. Baker (Asteraceae) (voucher code BHCB 42865) as previously described [38]. All spectral data (MS, IR, ^1H and ^{13}C NMR) of the crystals obtained for this study were in agreement with that published for myriadenolide. Myr was dissolved in DMSO, diluted in RPMI and added to the culture to attain the desired final concentrations. The control experiment used was performed using DMSO (< 0.05%).

RPMI 1640 medium and FBS were purchased from Hyclone (Logan, UT); rmIL-5, from R&D systems (Minneapolis, MN) or ProSpec (New Jersey, NJ) yielded comparable results; rmGM-CSF was from PeproTech (Rocky Hill, NJ); penicillin 100 U/mL (PEN-B), streptomycin 100 mg/mL, sodium nitroprusside (SNP), S-nitroso-*N*-acetylpenicillamine (SNAP), and grade V ovalbumin, were from Sigma-Aldrich (St Louis, MO); PGE_2, from Cayman Chemical (Ann Arbor, MI); alum [$AlNH_4(SO_4)_2x12H_2O$], from Synth (São Paulo, Brazil); and CFSE, from ThermoFischer (Waltham, MA).

2.3. Bone marrow studies

Bone marrow cells were collected from both femurs of naïve and challenged mice, washed, counted in a haemocytometer, seeded at a density of 10^6 cells/mL of RPMI 1640 medium, 10% FCS and rmIL-5 (1 ng/mL or as indicated; [45]) in 24-well plates, and incubated at 37 °C, 5% CO_2/95% air for 7 days (unless otherwise indicated). These conditions allow the addition of Myr at different times, unlike semi-solid culture, where proper mixing and diffusion of substances added after plating is restricted. Eosinopoiesis and its modulation by various agents were strictly dependent on IL-5 [45–47]. Where indicated, cultures received Myr (10^{-8}–10^{-16} M), together with PGE_2 (10^{-7} M), S-nitroso-*N*-acetylpenicillamine (SNAP; 10^{-3}–10^{-5} M), Sodium nitroprusside (SNP; 10^{-3}–10^{-5} M), MK886 (10^{-8} M), zileuton (10^{-8} M) or montelukast (MKT; 10^{-7} M). Unless otherwise indicated, each agent was added only once, immediately after IL-5, at the beginning of the culture period. Where indicated, Myr was added once at different times after initiation of the culture to define the period during which it must act to enhance eosinopoiesis. Cells present in 7-day cultures (or at the indicated times) were resuspended, collected, counted, cytocentrifuged and stained for eosinophil peroxidase (EPO; [46]). EPO+ cells were counted and photographed at 1000× magnification, under oil. Eosinophil numbers were calculated from total and differential counts.

In another set of experiments, bone marrow from BALB/c mice was used for semi-solid culture [47] in 35 mm culture plate, in triplicate. 1 mL of Iscove's Modified Dulbecco's medium, 20%FCS, containing 2×10^5 bone marrow cells and GM-CSF (2 ng/mL), was mixed with agar to 0,36% final concentration and plating. Colonies (> 40 cells) were scored at day 7 under the inverted microscope at 200× magnification. In additional controls, the frequency of pure eosinophil (Eos), pure neutrophil (G), pure monocyte (M), neutrophil-monocyte (GM), or granulocyte-monocyte (G/M/E) colonies was determined after staining for EPO and scored under 400–1000× magnification [37,45,47]. For flow cytometric analyses, 10^6 bone marrow cells were incubated with CFSE (1 mg/mL) in medium for 20 min, room temperature, washed and cultured. At the end of culture (7–10 days), cells were collected and submitted to flow cytometry in a FACSCalibur (Becton-Dickinson) with analysis by the SUMMIT software (v4.3, Dako), with gating in the granulocyte region, defined on the basis of forward and side scatter profiles.

2.4. Peroxidase assays

Where indicated, EPO activity was measured on 4×10^6 bone marrow cells plated in flat-bottom 96 well plates. Cells were washed twice (500 × g, 10 min) and resuspended in 50 μL of PBS. After plating in 50 μL per well, they were mixed with an equal volume of reaction mixture (0,15% OPD, 2% v/v H_2O_2 30%), with or without 6 mM KCN (mouse eosinophil peroxidase is cyanide-resistant, unlike other leukocyte peroxidases [47]) and were incubated for 5 min in the dark. The reaction was stopped with 50 μL 4 N H_2SO_4. Absorbance was measured at 490 nm.

2.5. Data analysis

Results were expressed as the mean ± SEM throughout and analyzed with SigmaPlot for Windows version 11.0 software. Comparisons of more than two means were carried out by factorial ANOVA with Bonferroni correction test. Were 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. Animals were randomized for experimental groups. The number of animals in each group is specified in the figure legends. All analyses were confirmed by an independent third-party.

3. Results

3.1. Increased eosinopoiesis in Myr- and cytokine-stimulated bone-marrow culture

We first examined the effects of Myr (structure shown in Fig. 1A) on murine myelopoiesis (including eosinopoiesis) by performing colony formation assays in semisolid culture, which evaluates the ability of progenitor cells to form colonies: bone marrow from naïve mice was cultured with GM-CSF (which is required for colony formation) over a 7-day period, in the absence or presence of Myr (Fig. 1). Myr (10^{-10} M) significantly upregulated colony formation, relative to controls containing GM-CSF only (B). The increase in colony numbers driven by Myr was accompanied by a significant increase in the percent of pure eosinophilic colonies (C), which in turn was reflected in an increased number of pure eosinophil colonies (D). No other colony type was significantly increased, indicating that the enhancing effect of Myr on the number of colonies is eosinophil-selective; importantly, a significant suppressive effect on pure macrophage colonies (CFU-M) was also observed in the same cultures. These lineage-selective enhancing and suppressive effects were detectable as changes both in % frequency of colony types (C) and in absolute number of eosinophil colonies (D).

Next, we examined the effect of Myr in liquid cultures established with IL-5, the eosinophil-selective differentiation-promoting cytokine. Myr significantly increased EPO+ cell numbers recovered by day 7, relative to IL-5 alone controls (E). This effect on EPO+ cell counts was paralleled by an increased EPO activity in the cultures, as detected by an assay for cyanide-resistant peroxidase activity (F). Together, this confirmed that an eosinophil-selective enhancing effect could be observed in well-established assays by both microscopic and biochemical assessment. This made it easier for us to further characterize this effect and probe the underlying mechanisms in liquid cultures, which allow us to separately add multiple reagents at different times.

The effect of Myr in liquid culture (Fig. 2) was concentration-dependent, significantly enhancing eosinophil production in the presence of IL-5, at 10^{-10}–10^{-14} M final Myr concentrations (A). At 10^{-8} M, the effect of Myr was nonsignificant, which we consider to be evidence of high-dose inhibition, the mechanism of which has not yet been defined (see below in Results and Discussion). At the other extreme of the concentration-response curve (10^{-16}–10^{-18} M), no significant effect was observed. Hence, Myr is active in this assay at the concentration range of 10^{-10}–10^{-14} M, provided IL-5 is present at 1 ng/mL. At this concentration range, and in the presence of IL-5 concentrations 10- or 100-fold lower, Myr effects remained significant but of lesser magnitude. Over the entire range of IL-5 concentrations, no response was observed with Myr above 10^{-10} M, or below 10^{-14} M (B). To define the kinetics of eosinophil differentiation in the presence or absence of Myr, bone marrow was cultured with IL-5 and eosinophil numbers were determined daily up to 7 days of culture. Control cultures established in the presence of IL-5 alone showed continuous growth in eosinophil numbers until day 4, and stabilized between days 5 and 7. By contrast, in the presence of Myr, eosinophil numbers were significantly higher than in IL-5-only controls by day 1 of culture and remained significantly higher throughout the observation period of 7 days (C). Myr had similar enhancing effects on eosinophil production by cultured bone-marrow of mouse strains with widely different genetic makeup (BALB/c, C57BL/6, CBA, and BP-2). In all of them, Myr significantly enhanced IL-5-stimulated eosinophil production; in no case, Myr had an effect in the absence of IL-5 (D). Importantly, the effectiveness of Myr depended on its addition to the bone marrow culture during a short period, which begins at the establishment of the culture and lasts up to day 2 (E); the addition of Myr at later times did not have a significant effect relative to the IL-5-only controls. We further evaluated by flow cytometry whether an increase in the number of cell divisions in Myr-exposed cultures accounted for the longer duration of the proliferative phase relative to IL-5-only controls (F). The dilution of the labeling with CFSE indicated that Myr kept inducing cell proliferation between days 7 and 10 of culture. By contrast, the IL-5 control culture showed no dilution of the labeling between days 7 and 10 (F). The morphology of eosinophils in control and in Myr-exposed cultures presented a mixture of mature (small, with abundant peroxidase-containing granules, and donut-shaped nucleus) and immature (larger, with fewer peroxidase-containing granules, and larger and incompletely segmented nuclei) eosinophils. Besides, clusters of immature eosinophils containing 4–8 cells of a rather uniform appearance by day 7 were a common finding in Myr-exposed (but not in control) cultures (G).

3.2. Pharmacological interactions of Myr with exogenously added PGE_2 and NO-releasing chemicals

In addition to demonstrating the eosinophil-selective differentiation-promoting action of Myr, it was important to define whether it was cytoprotective for immature eosinophils. Cytoprotective effects are important in the pathogenesis of allergic eosinophilia [34] and in counteracting the effects of cytokines [48] and drugs [49] which induce apoptosis in immature eosinophils. The prototypic agent for this effect in bone-marrow culture is exogenously added Prostaglandin E_2 (PGE_2), which induces immature eosinophil apoptosis through a pathway dependent on both inducible nitric oxide synthase (iNOS) and the death ligand/receptor CD95L/CD95 pair [42]. This pathway is also operative in vivo, where it mediates the antiallergic effects of the leukotriene synthesis inhibitor, diethylcarbamazine, on murine bone-marrow. Myr increased eosinophil generation in IL-5-stimulated cultures; as expected, exogenous PGE_2 decreased it (Fig. 3, Panel A). When added together, Myr and PGE_2 acted in opposite ways. As a result of the *pharmacological* (as opposed to *chemical*) interaction between Myr and PGE_2 added together, eosinophil counts were brought back to the same level as in IL-5-only controls (A). This has previously been observed with other agents that protect the eosinophil lineage from PGE_2-induced apoptosis [30,46]. The morphology of eosinophils in IL-5-only controls and in Myr-exposed cultures (B, upper left and upper right images, respectively) was like that shown in Fig. 2 (G, left and right images, respectively). By contrast, the morphology of cultures exposed to IL-5 plus PGE_2 (B, lower left image) and IL-5 plus PGE_2 and Myr (B, lower right image) differed markedly from the controls shown in B, upper left and upper right. In the absence of Myr, PGE_2-exposed cultures showed both apoptotic eosinophils and macrophages containing EPO+ granules. Both features were previously described [42] and reflect apoptotic death and ingestion of eosinophils by macrophages in the cultures. In the presence of Myr, PGE_2-exposed cultures lacked these features and displayed morphologically preserved mature eosinophils. Overall, this provides evidence of: a) a cytoprotective effect of Myr against PGE_2; b) a decrease in the eosinopoietic effect of Myr due to its pharmacological interaction with PGE_2.

To further define the relationship of the cytoprotective effect of Myr to the iNOS-CD95 pathway, we examined whether Myr was able to counteract the suppressive effects of the NO-releasing chemicals, SNAP and SNP, on eosinophil production in bone-marrow culture. Both chemicals can dose-dependently suppress eosinophil production by iNOS-deficient bone-marrow, but not by CD95-deficient bone-marrow [42],

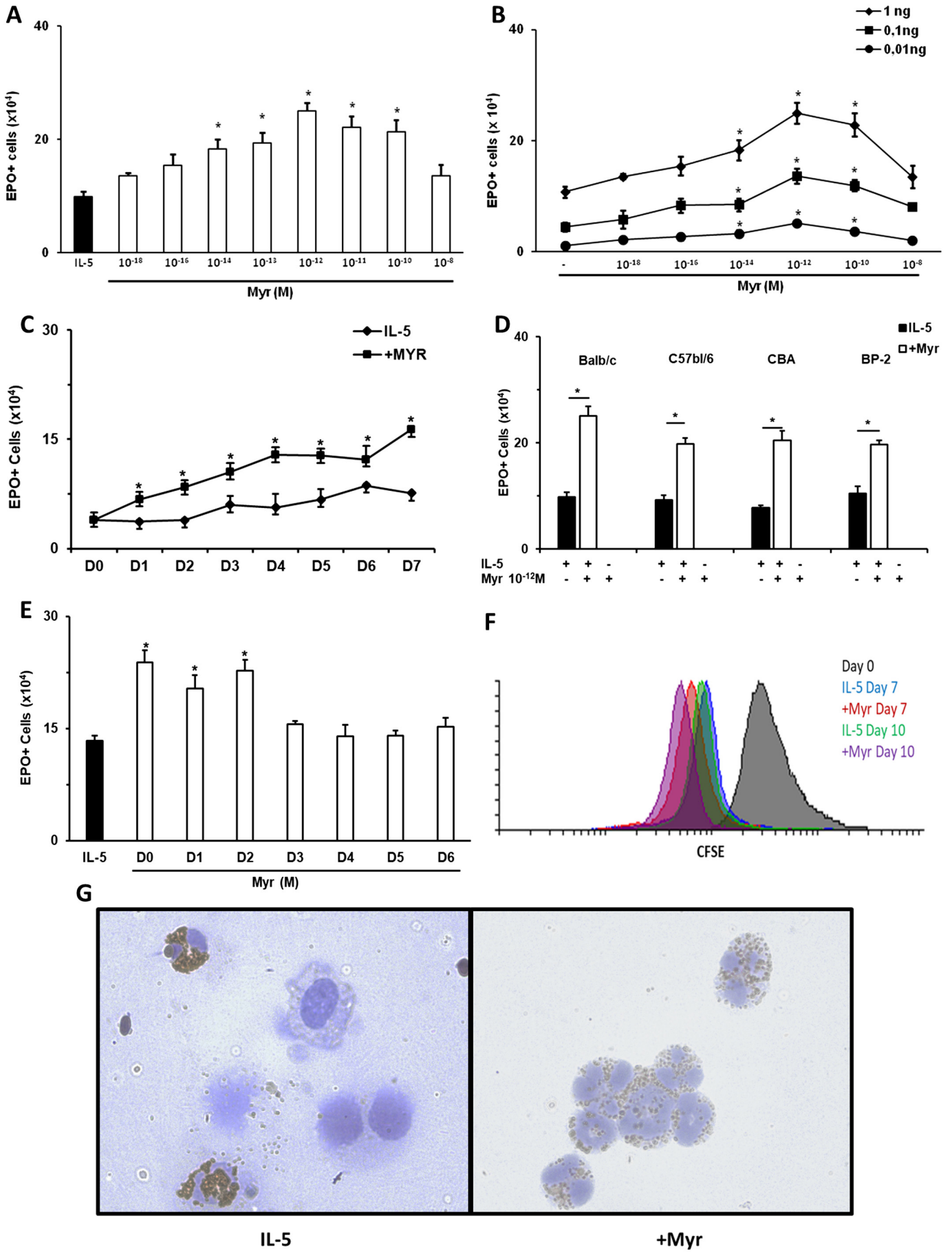

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Fig. 2. Kinetics of Myr-induced eosinopoiesis. Cultures were established in presence of IL-5 with (open bars in A, D; closed lozenge in C) or without (closed bars in A, D; closed square in C) Myr [10^{-12} M], from naive Balb/c (A–G) or C57BL/6, CBA and BP-2 donor mice (D). Data from liquid cultures established from freshly collected bone marrow for the indicated periods are Mean (+SEM) of total EPO+ cell numbers. In A, B, C, D and G, IL-5 and Myr were added in the first day of culture, and the cultures were harvested at day 7 (A, B, D, E, and G) only, or daily up to day 7 (C). In B, IL-5 was used at 1 ng/mL (closed lozenges), 0.1 ng/mL (closed squares) or 0.01 ng/mL (closed circles). In E, Myr was added once, at the indicated days of culture. In F, CFSE analysis from cultures from Balb/c mice bone-marrow established in presence of IL-5 with (red and purple curves) or without (blue and green curves) Myr [10^{-12} M], and harvested at day 7 (blue and red curves) or day 10 (green and purple curves). The grey curve in F represents CFSE signal at day 0. In G, the morphology of bone marrow cells in 7-day cultures (1000× magnification) is illustrated by representative images: to the left, a culture established with IL-5 alone; to the right, a culture established with IL-5 and Myr. *, significant differences relative to the indicated controls ($p < 0.05$). n = 5 in A, B and C, n = 5–13 in D, n = 3 in E, n = 5 in F. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

indicating that NO acts as a chemical messenger to activate the downstream death receptor-mediated step, rather than as a directly cytotoxic agent. Both SNAP and SNP at 10^{-3} M strongly suppressed IL-5-driven eosinophil production (C and D, respectively). This effect was not prevented by Myr, suggesting that the cytoprotective effect of Myr against PGE_2 depends on preventing generation of NO by iNOS, rather than on blocking downstream signaling by NO. Interestingly, SNAP and SNP at suboptimal concentrations (10^{-4} and 10^{-5} M) failed to suppress eosinophil production in the absence of Myr (as previously reported [47]) but did prevent the enhancing effect of Myr on eosinophil production (C and D, respectively). This suggests that suboptimal concentrations of SNAP and SNP do not kill eosinophils, but interfere with the effectiveness of Myr as an enhancer of eosinophil production.

3.3. Myr-increased eosinopoiesis is mediated by CysLT

The ability of Myr to protect eosinophils from PGE_2 but not from NO-releasing chemicals resembles the actions previously reported for CysLT [50]. While Myr is structurally quite different from leukotrienes (see Fig. 1A for Myr chemical structure; for comparison, see https://upload.wikimedia.org/wikipedia/commons/d/d2/Leukotriene_D4.svg), it is possible that it acts through leukotriene production followed by leukotriene signaling, as has been previously shown for NSAID (indomethacin and aspirin [50]) and proallergic cytokines (eotaxin and IL-13 [34]). If so, its effects would be preventable by pharmacological or genetical approaches targeting the 5-LO pathway, which produces leukotrienes; it would also be preventable by targeting $CysLT_1R$ with montelukast (MKT), which has been shown to duplicate the in vivo effect of diethylcarbamazine, a leukotriene synthesis inhibitor that activates the iNOS-CD95 pathway [37]. As shown in Fig. 4, the blockade of the 5-LO pathway by 5-lipoxygenase activating protein (FLAP) inhibitor MK886 prevented the increase in eosinophil production induced by Myr (A); in the absence of Myr, MK886 had no significant effect on eosinophil numbers, showing that the 5-LO pathway is not required for baseline eosinophil production, but for its upregulation by Myr, as well as by drugs [50] and cytokines [34]. As further shown in Panel B of the same Figure, bone marrow cultures from 5-LO-deficient mice (open bars) did not respond to Myr, although they had responses to IL-5 alone which were very similar to those of the wild-type controls. Furthermore, zileuton, a leukotriene synthesis inhibitor which acts directly on 5-LO rather than on FLAP, duplicated the effects of MK886 (shown in A), by preventing the effect of Myr on IL-5-stimulated eosinophil production. In the absence of Myr, zileuton had no significant effect (C). MKT, a selective inhibitor of CysLT1R, which has no effect on the operation of the 5-LO pathway but interferes with the effectiveness of its end products (CysLT), duplicated the effects of MK886 and zileuton. This shows that interference with both the generation and the action of CysLT abolishes the effectiveness of Myr. This observation is compatible with a mechanism of action for Myr based on activation of the 5-LO pathway and leading to CysLT production; it is, however, incompatible with a mechanism of action for Myr on the $CysLT_1R$ alone, which would not account for the effectiveness of knocking out 5-LO (B), or of blocking the 5-LO pathway (A, C). We further addressed directly the possibility that loss of Myr effects observed at 10^{-8} M and higher concentrations in our assay (Fig. 2A) was due to high-concentration inhibition by CysLT acting at $CysLT_1R$. Unlike Myr, LTD_4 at higher (10^{-6}–10^{-7} M) concentrations did not cause inhibition of eosinophil differentiation relative to the standard concentration we used in our cultures (10^{-8} M; Fig. 4E).

The ability of PGE_2 to induce apoptosis in developing eosinophils, as well as the ability of Myr, like COX inhibitors indomethacin and aspirin, to prevent the effects of exogenously added PGE_2 (Fig. 3), raised the issue of whether Myr acted through COX inhibition. COX inhibition is not sufficient for enhancing eosinopoiesis, since indomethacin loses the ability to do so if leukotriene synthesis is blocked; however, it is conceivable that inhibition of COX by Myr might play a role in its effects. We have directly addressed this issue through the following rationale: agents which act in similar ways through the same mechanism may compete with each other; alternatively, they may have additive effects when both are below the concentration needed to achieve the maximal effect. However, they are not expected to show *synergism*, as defined by the appearance of a joint effect when each is present in a concentration known to be ineffective. To distinguish between these two modes of pharmacological interaction, bone-marrow cultures were established with indomethacin and Myr, separately or in combination, at effective concentrations, namely 10^{-10} M Myr and 10^{-7} M indomethacin (Fig. 5A); alternatively, the same agents were present separately or in combination, at lower concentrations, shown to be ineffective, namely 10^{-16} Myr and 10^{-9} M indomethacin (Fig. 5B). In effective concentrations, we found no evidence of competition, or of additive effect between Myr and indomethacin (A). When Myr and indomethacin were present together at ineffective concentrations, a significant enhancement of eosinophil production was observed (B). This suggests that Myr does *not* behave as expected from an inhibitor of COX, the known pharmacological target of NSAID.

4. Discussion

This is the first report of the potent enhancing effects of Myr on eosinopoiesis. The increase in eosinophil colony formation following Myr exposure is sufficient to account for the positive effects of Myr on total colony counts. Hence, the intense eosinopoietic effect of Myr is not accompanied by a broad stimulation of other myeloid lineages in the same cultures, even though the latter also respond to GM-CSF, a multilineage hemopoietin. In liquid culture, Myr requires IL-5 (lineage-specific growth factor). This indicates that Myr modifies the eosinopoietic actions of IL-5, but does not replace this lineage-specific growth factor [45].

One striking feature of this effect of Myr is the unusually low concentration range over which it is detectable (between 10^{-10} M and 10^{-14} M). For comparison, other agents well-known to have a similar effect (and a similar $CysLT_1R$-dependent mechanism of action) act at a substantially higher concentration range: for both indomethacin and LTD_4, activity is detectable from 10^{-7} M to 10^{-9} M [33]. Despite its high potency, Myr activity is no longer detectable, when it is diluted down to 10^{-16} M and lower concentrations (not shown). On the upper end of this effective concentration range (at or above 10^{-8} M Myr), the eosinopoietic effect is also undetectable. This clearly departs from other effects previously described for the same agent, which has been shown to be active around the μM range in other assays [39–41] that involve

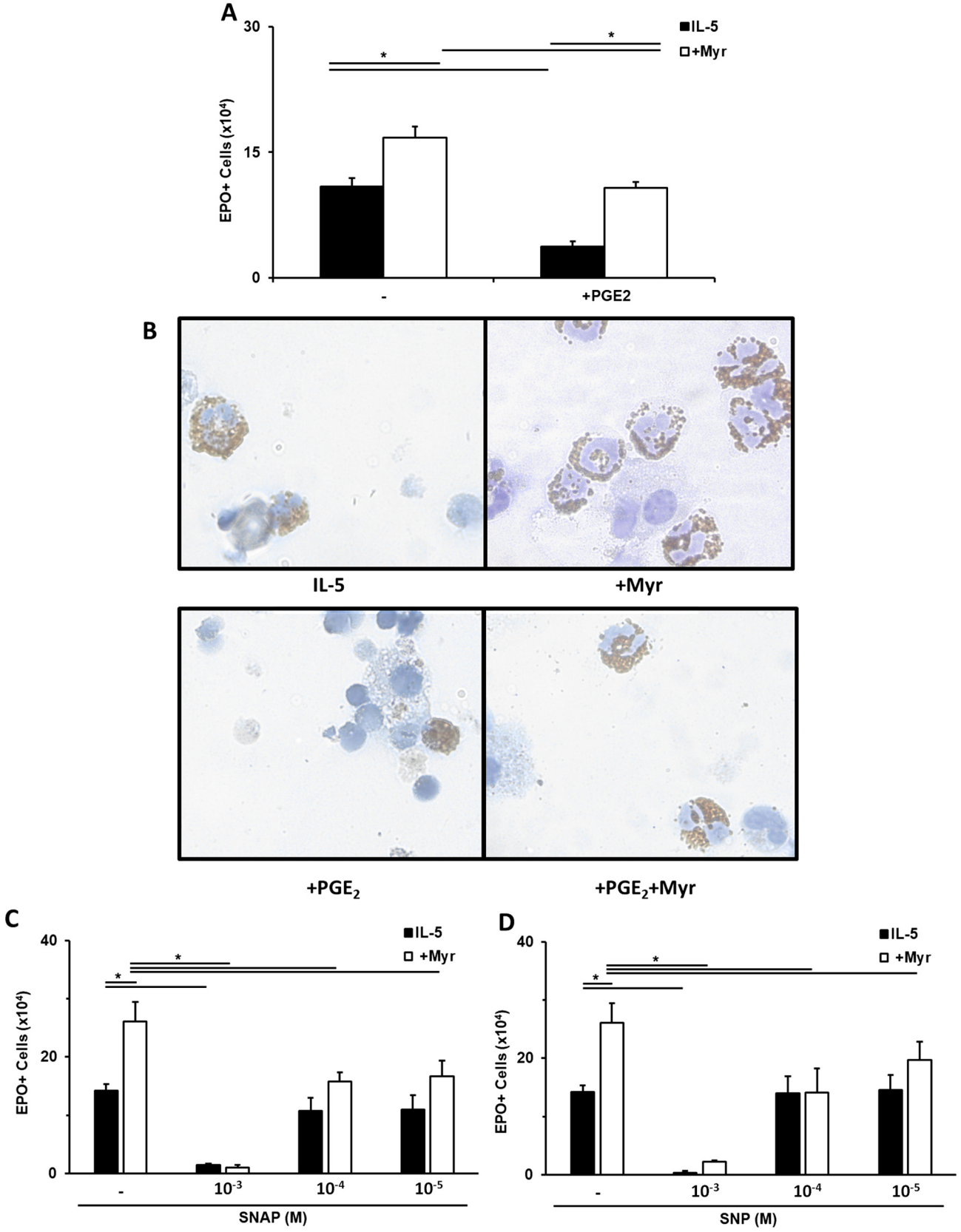

Fig. 3. Myr antagonizes the effects of PGE2 and NO-releasing chemicals on eosinophils from bone marrow cultures. Cultures were established in the presence of IL-5 with (open bars) or without (closed bars) Myr [10^{-10} M], from bone-marrow of naïve BALB/c donor mice. Data are Mean (+SEM) of total EPO+ Cells (A, C and D) 7-day liquid cultures established from freshly collected bone marrow, in combination with PGE2 (A), SNAP (C) ou SNP (D). In B, the morphology of bone marrow cells in 7-day cultures (1000× magnification), is illustrated by representative images of cultures established respectively with: upper left, IL-5 alone; upper right, IL-5 and Myr; lower left, in left-bottom, IL-5 and PGE2; lower right, IL-5, PGE2 and Myr. *, significant differences relative to the indicated controls ($p < 0.05$). n = 5 in all panels.

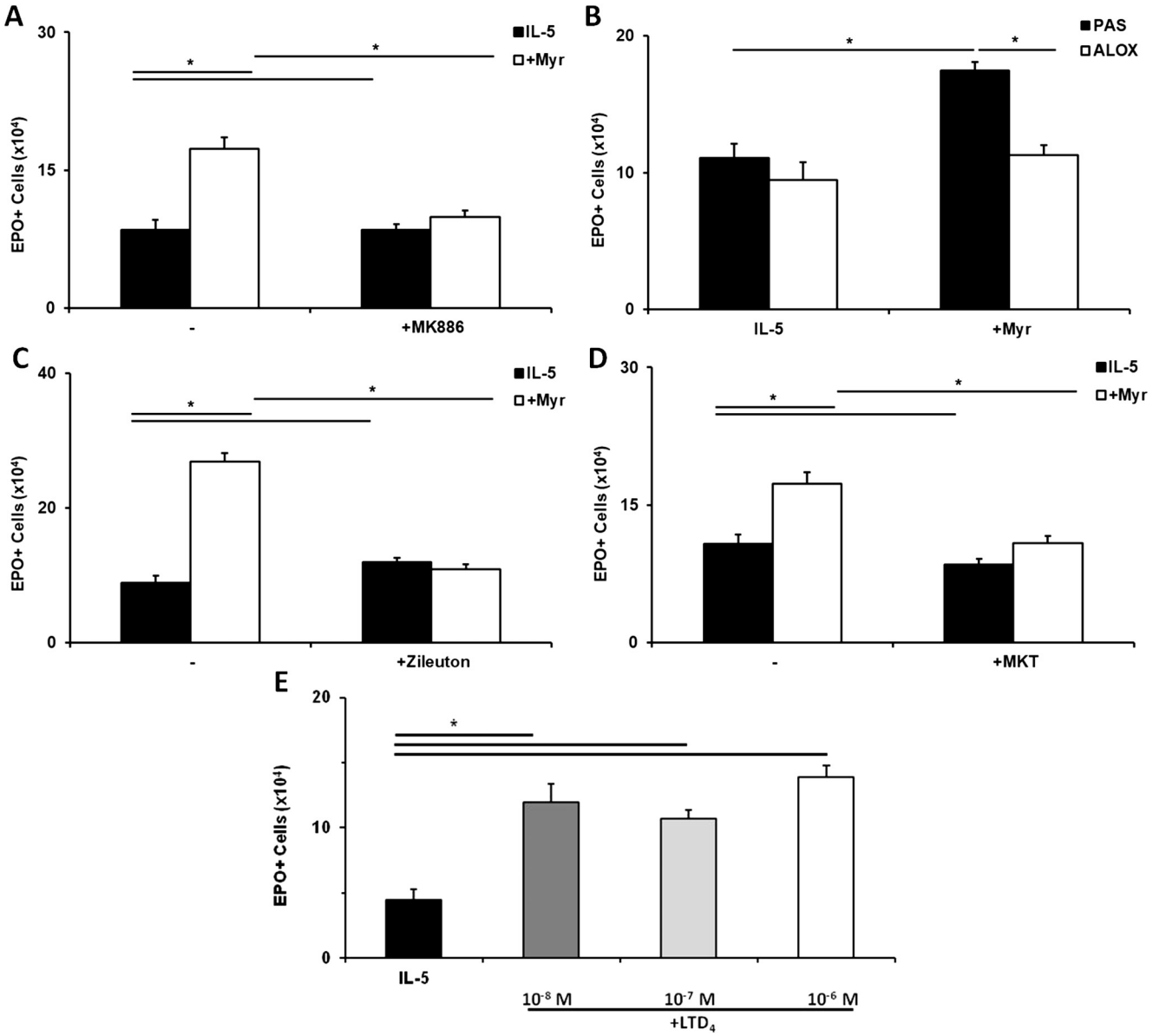

Fig. 4. Myr-induced enhanced eosinopoiesis depends on the integrity of the 5-LO pathway and on CysLT1 receptors. Cultures were established in presence of IL-5 with (open bars) or without (closed bars) Myr [10^{-10} M] (A, C and D) or LTD4 [10^{-8} M – dark grey bar; 10–7 M - light grey bar; 10^{-6} M – Open bar] (E), from bone-marrow of naïve Balb/c (A, C, D and E) or PAS (closed bars) and ALOX (open bars) donor mice (B). Data are Mean + SEM of total EPO + cells in 7-day liquid cultures established from freshly collected bone marrow. Cultures were established with MK886 (A), Zileuton (C) or Montelukast (D - +MKT). *, significant differences relative to the indicated controls ($p < 0.05$). n = 5 in all panels.

other cellular targets. This dose-response relationship shift towards lower concentrations is useful to distinguish this proliferation/differentiation-promoting effect from the previously described effects, which were, for the most part, cytotoxic. It also suggests that the mechanism through which Myr enhances eosinopoiesis is different from the mechanism(s) involved in these previously described assays. The lack of activity at concentrations of Myr higher than 10^{-8} M is consistent with high-dose inhibition, one of the mechanisms through which cells are known to protect themselves from the untoward effects of excessive stimulation. High-dose inhibition prevents secondary damage to other cells, arising from the release of potentially damaging soluble mediators and intracellular molecules from overstimulated cells [51]. One possibility is that high-dose inhibition in Myr-exposed cultures results from overstimulation of $CysLT_1R$, a G protein-coupled receptor. We have directly ruled out this possibility by showing that $CysLT_1R$ does not show high-dose inhibition in the presence of its physiological ligand, LTD_4. Other possibilities could not be excluded, such as an effect on post -receptor signaling by $CysLT_1R$, analogous to the mechanisms involved in protecting target cells from excessive stimulation by tetradecanoyl-phorbol acetate, which involve dissociation of the regulatory and catalytic subunits of Protein Kinase C, making cells insensitive to this activator [52].

Myr enhances eosinopoiesis in liquid culture by acting during a narrow time window, that is, up to the second day of culture. Its effect lasts a much longer time, however, since in Myr-exposed cultures a significant increase in eosinophil counts is demonstrable from day 1 to day 7 (Fig. 3, Panel C). Flow cytometry data indicate that Myr-exposed cultures remain proliferative up to day 10 at least. This differs strikingly from our observations in control cultures with IL-5 alone, which show significant growth between days 3 and 7, and a progressive decline thereafter. Other substances well-known to enhance eosinopoiesis act during a similar window of opportunity, as is the case of

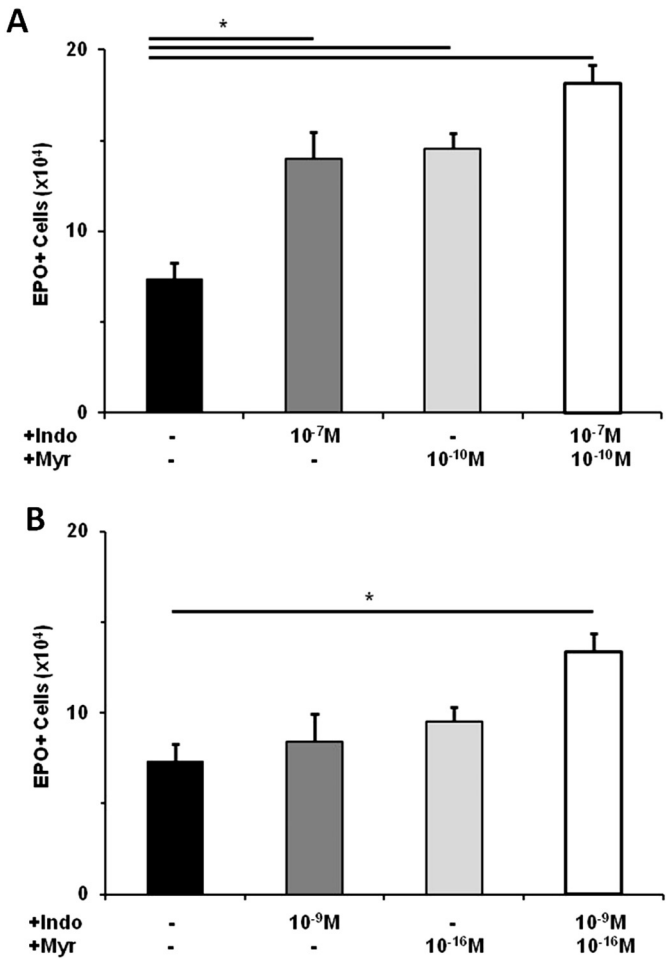

Fig. 5. Effect on Myr and Indo cultures in sub-optimal concentrations. Cultures were established in presence of IL-5 alone (closed bars), or with Myr alone (light grey bars) at 10^{-10} M (A) or 10^{-16} M (B), Indomethacin alone (dark grey bars) at 10^{-7} M (A) or 10^{-9} M (B), combination from both (open bars), from bone-marrow of naïve Balb/c donor mice. Data are Mean + SEM of total EPO + cells in 7-day liquid cultures established from freshly collected bone marrow. *, significant differences relative to the indicated controls ($p < 0.05$). n = 5 in all panels.

dexamethasone [47]. Unlike Myr, however, dexamethasone induces incomplete differentiation, with a large proportion of immature and aggregated forms [47].

We provide several lines of evidence that the eosinopoiesis-promoting effect of Myr is closely related, but not identical, to that previously described for CysLT [30,53] and for drugs [50] and cytokines [34] indirectly acting through CysLT production and signaling. The effectiveness of Myr is abolished: a) by pharmacological blockade of leukotriene production with MK886 and zileuton; b) on bone-marrow from 5-LO-deficient mice (ALOX); c) by pharmacological blockade of $CysLT_1R$-mediated signaling with montelukast. Hence, the effectiveness of Myr requires the functional integrity of the FLAP/5-LO/CysLT1R axis, and blockade of any of its steps is enough to abolish Myr effects on eosinopoiesis.

Myr has a very different structure from that of the CysLT and is, in our view, unlikely to be directly acting on $CysLT_1R$. Importantly, its effects are prevented by the absence or blockade of the leukotriene-generating 5-LO pathway, showing that the intact $CysLT_1R$, in the absence of leukotriene generation, is not sufficient for Myr activity. Hence Myr is assumed to be primarily an inducer of CysLT production.

Similarities between Myr and CysLT effects further extend to their shared ability to protect developing eosinophils from apoptosis induced by PGE_2 [30]. Myr prevents the effects of PGE_2, both on the number of eosinophils in culture, and on the morphology of these eosinophils [42]. The effects of PGE_2 are mediated by activation of an iNOS-CD95-dependent pathway, in which NO produced by iNOS acts as a diffusible signal, and the effector step promoting apoptosis is dependent on the CD95L/CD95 pair function [35]. Hence, it is important to define whether Myr acts downstream of NO to prevent apoptosis induction, or upstream of iNOS to prevent generation and signaling through NO. We approached this issue by examining the ability of Myr to protect against concentrations of two different NO-releasing chemicals that are able to induce apoptosis in developing eosinophils (10^{-3} M); we further examined the actions of Myr in the presence of concentrations of the same chemicals that do not induce apoptosis in the same targets (10^{-4} M e 10^{-5} M). The results show that Myr cannot protect eosinophils from effective concentrations of SNAP or SNP, thus favoring the view that it acts upstream of iNOS to counteract the actions of PGE_2. The use of subthreshold concentrations of both chemicals had, as expected, no suppressive (i. e., apoptotic) effect; unexpectedly, however, it had an effect on Myr, which lost its ability to enhance eosinophil production after exposure to subthreshold concentrations of NO. This suggests that either Myr or its cellular target is very sensitive to NO. Since NO is known to react with a variety of proteins, we favor this second possibility, which might prove useful in the future characterization of a receptor for Myr [42].

Finally, Myr may prove a valuable tool in the production of murine eosinophils in numbers large enough to allow their purification and use in biochemical studies. In comparison to other agents which act through a similar mechanism, Myr represents a more potent and longer-acting alternative, which can be anticipated to provide higher yields. Alone, or in combination with other pharmacological agents known to prime bone-marrow in vivo for increased eosinophil production, such as dexamethasone [47] and all-trans retinoic acid [54], Myr may provide useful insights of eosinophil differentiation in different biochemical environments.

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Author contributions

M. I. Gaspar-Elsas, C. L. Zani and P. Xavier-Elsas conceived the study; T. Alves and C. L. Zani provided purified Myr and revised the paper; M. I. Gaspar-Elsas provided the inbred mouse strains, wild-type and mutant, verified data and statistical analyses and revised the paper; B. M. Vieira and M. C. Souza dos Santos separately carried out experiments which are equally essential to the final contents of the manuscript, and should, therefore, be considered as joint first authors. D. Masid-de-Brito carried out experiments in collaboration with B. M. Vieira and T. Queto; B. M. Vieira and P. Xavier-Elsas wrote the paper.

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