IL-4 activates equine neutrophils and induces a mixed inflammatory cytokine expression profile with enhanced neutrophil chemotactic mediator release ex vivo

Lavoie-Lamoureux A, Moran K, Beauchamp G, Mauel S, Steinbach F, Lefebvre-Lavoie J, Martin JG, Lavoie JP. IL-4 activates equine neutrophils and induces a mixed inflammatory cytokine expression profile with enhanced neutrophil chemotactic mediator release ex vivo. Am J Physiol Lung Cell Mol Physiol 299: L472–L482, 2010. First published July 16, 2010; doi:10.1152/ajplung.00135.2009.—Neutrophils are potent contributors to the lung pathophysiological changes occurring in allergic airway inflammation, which typically involve T helper type 2 (Th2) cytokine overexpression. We have previously reported that equine pulmonary endothelial cells are activated by the Th2 cytokine IL-4 and express chemotactic factors for neutrophils after stimulation. We have further explored the possible mechanisms linking Th2-driven inflammation and neutrophilia by studying the effects of recombinant equine IL-4, a prototypical Th2 cytokine, on peripheral blood neutrophils (PBN) isolated from normal animals and from horses with asthmatic airway inflammation (equine heaves). We found that IL-4 induced morphological changes in PBN, dose- and time-dependent expression of IL-8 mRNA, as well as the release of chemotactic factors for neutrophils in culture supernatants. Also, IL-4 induced a mixed inflammatory response in PBN from control and asthmatic animals with increased expression of proinflammatory IL-8 and TNF-α but a marked inhibition of IL-1β. IL-4 type I receptor (IL-4Rα) and CD23 (FcεRII) expression were also upregulated by IL-4. Importantly, disease as well as chronic antigenic exposure modified gene expression by PBN. Finally, we found that activation of equine neutrophils with IL-4 involved STAT6 phosphorylation and other Th2 cytokines either as activators (9, 10, 26) or inhibitors (41, 42, 46, 51) of neutrophil effector functions. The pharmaco-logical inhibitors, SB-203580 and LY-294002, respectively, significantly reversed IL-4-induced gene modulation in PBN. Overall, results from this study add to the link between Th2-driven inflammation and neutrophilia in the equine model and further extend the characterization of IL-4 effects on neutrophils.

cytokines; allergy; equine asthma

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Our results reveal that IL-4-activated equine neutrophils share features described for human neutrophils, suggesting that they behave similarly in a Th2-biased inflammatory milieu. Furthermore, we describe a mixed inflammatory response with both increases (IL-8 and TNF-α) and decreases (IL-1β) in proinflammatory cytokine expression by IL-4-stimulated neutrophils. We also demonstrate that PBN from both healthy and asthmatic horses are primed after chronic antigenic challenge and exhibit enhanced responsiveness to IL-4.

METHODS

Animals

Ten mixed-breed horses consisting of four control horses (495 ± 42 kg, 11 ± 2.9 yr old) with no history of respiratory disease and six heaves-affected (asthmatic) horses (499 ± 50 kg, 14.2 ± 3.8 yr old) from a research herd were studied. The two groups of horses were stabled together during the entire course of the study and were regularly vaccinated and dewormed. All animals had been pastured for >3 mo before the beginning of the study to ensure complete reversal of airway obstruction in asthmatic horses (remission). Antigenic challenge consisted of stabling the horses for 30 days in a barn with reduced ventilation where they were bedded on straw and fed hay and sweet feed twice a day. Blood was collected during clinical remission and after antigenic challenge. All experimental procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal.

Respiratory Function Tests

Respiratory mechanics were performed before and after the 30-day challenge as previously described (15). Briefly, pulmonary pressure was measured using a transducer attached to a balloon-tipped catheter inserted in the distal third of the esophagus and to a mask placed on the horse’s nose. Flow rates were obtained using a heated pneumotachograph fitted to the mask. Values of pulmonary elastance (E_L) and resistance (R_l) were calculated using dedicated computer software (RHT-Infodat, Montréal, Québec, Canada) by multiple regression analysis for the single-compartment model of the lung (8).

Neutrophil Isolation

Blood was drawn from the jugular vein into heparinized sterile tubes (BD Vacutainer). Two different methods were used to isolate neutrophils from the blood. Density gradient. The polymorphonuclear-rich cell layer was harvested following centrifugation of whole blood on a density gradient solution composed of sodium metrizoate and Dextran 500 (Lympholyte-poly; Cedarlane Laboratories). The remaining red blood cells were lysed by hypotonic treatment.

Immunomagnetic selection (MACS). This technique was used to achieve optimal levels of cell purity and repeatability as reported previously (34). Briefly, after sedimentation of blood for 1 h at room temperature, neutrophils were retrieved from the leukocyte-rich supernatant by sequential incubation with primary monoclonal antibody (cat. no. DH24A; VMRD) and secondary rat anti-mouse IgM antibody conjugated to paramagnetic microbeads (MACS; Miltenyi Biotec) before being loaded on a ferromagnetic LS separation column (MACS; Miltenyi Biotec).

Cytospin slides were prepared (Cytospin 2; Shandon) and stained with Protocol Hema 3 (Fisher Scientific, Pittsburgh, PA) for differential counting of >400 cells to assess neutrophil purity. Viability was determined by trypan blue exclusion. The purity and viability of neutrophils were 98.3 ± 0.2 and 98.0 ± 0.5%, respectively, when isolated using MACS and 95.8 ± 0.5 and 98.3 ± 0.5%, respectively, using density gradient.

Cell Culture

Neutrophils were suspended at 5 × 10⁶ cells/ml in culture medium RPMI 1640 supplemented with 10% heat-inactivated low-endotoxin FBS (GIBCO), 4 mM l-glutamine (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) and incubated in suspension cell culture plastic plates (Ultrident) for 5 or 18 h at 37°C, 5% CO₂, with different doses of recombinant equine (req) IL-4 (4, 40, or 400 U/ml) or with its vehicle as a negative control. This recombinant protein is produced by an insect cell line as described earlier (59) and has been previously shown to have biological activity on equine lymphocytes and monocytes. The vehicle consists of supernatant collected from nontransfected insect cells. Where indicated, a commercial source of reqIL-4 from Escherichia coli was used for comparison purposes (R&D Systems). A positive control for neutrophil activation consisted of incubating the cells with 100 ng/ml LPS (from E. coli 011:B4; Sigma-Aldrich, Oakville, Ontario, Canada) and 10 nM N-formyl-methionyl-l-leucyl-l-phenylalanin (fMLP; Sigma-Aldrich). The viability of agonist-stimulated cells was assessed. Photographs (Leica DFC320; Leica, Wetzlar, Germany) of the cells were taken using a phase-contrast inverted microscope (Leica DM IRM). Neutrophils were centrifuged at 5,600 × g (Eppendorf 5415C) for 5 min at 4°C. Culture supernatants were collected and frozen for further analysis (~80°C). Ten million neutrophils were homogenized in TRIzol reagent (Invitrogen) and immediately frozen at ~80°C for RNA extraction.

RNA Extraction and Reverse Transcription

RNA extraction was performed according to the manufacturer’s instructions using three-step nucleic acid precipitation with 0.2 volume of chloroform and 1 volume of isopropanol and 75% ethanol (TRizol reagent; Invitrogen). RNA pellets were air-dried, and total RNA concentration and purity were evaluated by spectrophotometry (GeneQuant Pro; Biochrom).

Five hundred nanograms of total RNA in 9 μl of DNase/RNase-Free Distilled Water (GIBCO) was heated at 70°C for 5 min (Standard Heat Block; VWR) for reverse transcription. One microliter of oligo(dT)12–18 (Invitrogen) primers was added, and the mixture was heated for 10 min at 70°C. Samples were incubated at 50°C for 1 h (5 of 1 MDL; Fisher Scientific) after adding 20 μl of a mastermix containing 0.01 M dithiothreitol (Invitrogen), 1.6 mM dNTP (Invitrogen), 2 U/μl RNA-Blocking Ribonuclease inhibitor (Ambion, Austin, Texas), and 1.2 U/μl AMV reverse transcriptase (Roche). The reaction was stopped by heating the samples at 100°C for 1 min. Samples were stored at −20°C.

Real-Time PCR

Real-time PCR was performed using QuantiTect SYBR Green PCR Kit (QIAGEN) according to the manufacturer’s instructions with minor modifications on the Rotorgene real-time centrifugal DNA amplification system (Corbett Research). Briefly, 1 μl of cDNA was used in 20-μl final PCR volume containing 0.5 μM each sense and antisense primers and 2.75 mM MgCl₂. Primers pairs were as follows: CD23 (sense) 5'-CCAGAACGTCTCTCAAGTTCC-3' and CD23 AS (antisense) 5'-CATCTGGTCTGCTAGTTCTGC-3' and IL-1β AS 5'-GACTCAGAAAGATACCTGTGGCCT-3' and IL-1β AS 5'-AGCAACAGCTGAGA- GCAGCCT-3'; IL-4 Ra AS 5'-AGACCTGCTGTTCCATCACCAG-3' and IL-4 Ra AS 5'-CAGCCACATTTCCAATCCTG-3' and IL-8 AS 5'-CTTCTGCGACTGCTTGTAAGG-3' and IL-8 AS 5'-GCAAGCCT- CAGCTCCTGTTGAC-3'; TLR4 AS 5'-TGGAGACTTCAAGGACTG- CAT-3' and TLR4 AS 5'-AGGTCC-AGGTTCTGTTAGGTG-3'; TNF-α AS 5'-CCTTGACTGTTGACCTTCCTTCCCT-3' and TLR-α AS 5'-CCTTGAGGAGAGGAGGTGAC-3'; GAPDH AS 5'-AAGTG- GATATAGTTGCGCCATCAAT-3' and GAPDH AS 5'-AATCTGC-
CATGGTGGAAATC-3’. Primers were designed to span exon-intron boundaries to prevent amplification of genomic DNA. Amplification conditions included a denaturation step of 10 min at 95°C followed by 40 cycles of denaturation, annealing, and elongation steps. For each gene, a serial dilution (10×) of known concentration of PCR products (QiAquick Gel Extraction Kit; QiAGEN) was performed and optimized to give a reproducible efficiency coefficient (>0.90). Quantification of gene expression was performed using imported standard curves and adjustment to a standard curve reference in the target run. Values were corrected with GAPDH as a housekeeping gene (37).

Chemotaxis Assays

PBN from healthy horses were obtained by density gradient centrifugation as described above. Neutrophils were suspended in PBS containing 0.2 M Ca²⁺, 0.265 M Mg²⁺, and 0.5% BSA to obtain 1.5 × 10⁵ neutrophils/55 μL. This volume of cell suspension was added to the upper wells of the 48-Well Micro Chemotaxis Chamber (Neuro Probe). Supernatants (30.5 μL) retrieved from reqIL-4 or vehicle-treated neutrophils were added to the lower wells in triplicate or quadruplicate. CXC chemokine receptor CXCR2 antagonists were used to evaluate the role of CXC chemokines in the chemotactic activity of culture supernatants. Dose-response assays monitoring the inhibition of equine neutrophil migration were first performed with SB-225002 (10⁻³ to 10⁻⁸ M in DMSO; Tocris Bioscience) and SB-265610 (10⁻⁴ to 10⁻⁶ M in DMSO; Tocris Bioscience) toward reqIL-8 (Endogen; Thermo Fisher Scientific), a prototypical CXCR2 ligand. SB-225002 and SB-265610 are nonpeptide allosteric antagonists of the diaryl urea class (18) shown to have multispecies cross-reactivity. Briefly, neutrophils were pretreated on ice with the CXCR2 antagonists for 30 min (final concentration of DMSO: 0.1%) before migration toward reqIL-8 (10 ng/ml in PBS containing Ca²⁺/Mg²⁺ and BSA). Dose titration studies were performed to determine doses of drugs required for inhibition of neutrophil migration toward supernatants from IL-4-stimulated neutrophils (40 U/ml, 5 h). In addition, the contribution of the protein fraction to neutrophil chemotaxis was evaluated by heating the supernatants for 30 min at 100°C (denaturation of proteins vs. lipids). Negative control for chemotaxis consisted of PBS alone. Neutrophils were allowed to migrate through an 8-m pore size cellulose nitrate filter (Neuro Probe) placed between the supernatants from IL-4 or vehicle-stimulated neutrophils (100 ng/ml; R&D Systems) or with isotype control antibodies in similar concentrations (goat IgG; Vector Laboratories) for 30 min at room temperature and washed. Slides were further incubated for 30 min with Vectastain Elite ABC peroxidase (Vector Laboratories) supplemented with 0.1% saponin, washed, subjected to diaminobenzidine (DAB Peroxidase Substrate Kit; Vector Laboratories) for 8.5 min, rinsed with deionized H₂O, counterstained using hematoxylin and lithium carbonate, dehydrated, and mounted in Permount® Mounting Medium (Fisher Scientific). The expression of IL-1β by neutrophils was similarly ascertained using rat monoclonal anti-equine IL-1β (MAB3340; R&D Systems) and goat anti-rat IgG (1:100; Vector Laboratories) in total blood leukocytes stimulated for 5 h with LPS (5 μg/ml) in the presence of brefeldin A.

Flow Cytometry

Intracellular IL-1β expression was evaluated in neutrophils cultured for 5 h with reqIL-4 (100 ng/ml; R&D Systems) or its vehicle in the presence of brefeldin A (10 μg/ml). Following culture, cells were washed twice in PBS, counted, and stained for extracellular neutrophil marker (15 μg/ml DH24A; VMRD) for 30 min (10³/100 μl). All antibody incubation steps were performed at 4°C. Cells were washed 3 times in staining buffer [SB; Dulbecco’s PBS (Invitrogen), 3% FBS (GIBCO), and 0.09% wt/vol sodium azide (Invitrogen), pH 7.4] and incubated with phycoerythrin-coupled goat anti-mouse IgM antibody (1:100 in wash buffer; CLCC31505; Cedarlane Laboratories) for 30 min. From this moment, all procedures were performed in the dark. Cells were washed twice with SB and fixed for 15 min in 4% paraformaldehyde (wt/vol in PBS, pH 7.4) and permeabilized in wash buffer (SB supplemented with 0.1% saponin) for 15 min at 4°C. Cells were incubated with anti-equine IL-1β (5 μg/ml) for 30 min, washed 3 times in wash buffer, and incubated 30 min with secondary FITC-coupled anti-rat IgG (ab’) antibody (1:1000 in wash buffer; Rockland). Cells were then washed twice in wash buffer and suspended in 400-μL SB before flow cytometry acquisition of 200,000 events and analysis using CellQuest Pro software on a FACSCalibur instrument (BD Biosciences). Isotype-matched control antibodies (mouse IgM and rat IgG) as well as single marker-stained cells were used to set photomultipliers (PTM) voltage and compensation parameters for fluorescence detection in FL-1 and FL-2 channels.

Western Blot

An antibody directed against the phosphorylated form of human STAT6 was used to detect the activation of signaling pathways associated with the IL-4 receptor. The equine translated nucleotide sequence surrounding the targeted phosphorylated amino acid Tyr641 shows 100% homology with the human sequence, according to the available equine sequences in National Center for Biotechnology (NCBI) Horse Genome Resources (National Institutes of Health PubMed acc. no. XM_001488414). Briefly, neutrophils were isolated using density gradient centrifugation and suspended in Ca²⁺/Mg²⁺-containing PBS supplemented with 10% FBS at 5 × 10⁶ cells/ml. They were incubated for 15 min at 37°C in a water bath with insect reqIL-4 (40 U/ml), E. coli reqIL-4 (100 ng/ml), LPS (100 ng/ml), and IFNγ (10 ng/ml) or left unstimulated. Stimulation with LPS and IFNγ is not typically associated with STAT6 activation, and therefore this was used as a reference for antibody specificity. Following incubation, neutrophils were centrifuged at 2,500 g for 10 min at 4°C. Cell pellets were homogenized in M-PER lysis buffer (Pierce) supplemented with 1:100 Halt Phosphatase Inhibitor Cocktail (Pierce) using a tuberculin syringe and centrifuged at 14,000 g at 4°C for 15 min. Where...
indicated, a protease inhibitor cocktail (1:100; Sigma-Aldrich) was added to the lysis buffer. Protein content of the supernatants was evaluated using the Bradford method (AMRESCO) and BSA as a standard reference. Equal amounts of proteins (25 μg/lane) were mixed with loading buffer containing 2-mercaptoethanol, heated for 5 min at 100°C, and then subjected to 10% SDS-PAGE. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Hybond-P; GE Healthcare) and blotted against phospho-STAT6 (Tyr641) primary antibody (1:1,000 in 1× TBS, 0.1% Tween 20, with 5% wt/vol nonfat dry milk; Cell Signaling Technology) overnight at 4°C with gentle agitation. Membranes were washed with 0.1% (vol/vol) Tween 20 in TBS (pH 7.5) and incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 2 h at room temperature. Protein bands were visualized using ECL Plus Western Blotting Detection system (Amersham, GE Healthcare) according to the manufacturer’s instructions. Digitalized images were obtained by scanning the membrane under blue light (Storm 840; Molecular Dynamics).

Inhibition of IL-4-Induced Gene Expression Using Pharmacological Inhibitors

In separate experiments, MAPK and phosphatidylinositol 3-kinase (PI3K) inhibitors were used to identify the signaling pathways involved in IL-4-induced mRNA regulation. SB-203580 (10 μM) and U-0126 (2.5 μM; BioSource, MediCorp, Montréal, Québec, Canada) were used to block p38 MAPK, JNK, and MEK1/2, respectively. Wortmannin (50 nM) and LY-294002 (10 μM) were used to block PI3K (Sigma-Aldrich). Effective doses of MAPK inhibitors were selected according to their ability to inhibit IL-8 mRNA upregulation by total equine blood leukocytes stimulated with LPS (100 ng/ml) and fMLP (10 nM) (data not shown). Wortmannin and LY-294002 doses were chosen according to the manufacturer’s recommendation and concentrations previously reported to suppress IL-8 mRNA expression (31). Our previous unpublished PCR experiments showed the presence of IL-4R mRNA in equine neutrophils, suggesting that they could respond to this cytokine. Figure 1 shows that, indeed, the neutrophils underwent striking morphological changes following culture with reqIL-4 compared with vehicle-treated cells. The IL-4–stimulated cells were elongated and/or exhibited cytoplasmic prolongations characteristic of cellular activation and cytoskeletal reorganization. Furthermore, titration experiments showed a dose- and time-dependent effect of reqIL-4 on gene expression by equine neutrophils. Thus, following incubation for 5 h (Fig. 2A), IL-8 transcription in equine neutrophils was significantly enhanced by 40 and 400 U/ml reqIL-4 and 100 ng/ml LPS + 10⁻⁸ M fMLP (P < 0.05, Dunnett test; n = 4). After 18 h (Fig. 2B), neutrophils showed lower levels of IL-8 transcription compared with 5-h stimulation (10- to 100-fold less; P = 0.0008; n = 4). At this time point, only stimulation by bacterial products resulted in a significant increase in IL-8 mRNA expression (P = 0.035) over negative control (vehicle).

IL-4 Stimulates the Release of CXCR2 Ligands from Neutrophils in Culture Supernatants

As chronic airway inflammation involves the ongoing recruitment to and activation of neutrophils in the airway lumen, we studied whether IL-4 could mediate this phenomenon. As shown in Fig. 3A, there was a qualitative dose-dependent effect of reqIL-4 on the release of chemotactic factors by neutrophils in culture supernatants. Separate experiments indicated that reqIL-4 itself was not responsible for the chemotactic activity...
of the supernatants, since freshly diluted reqIL-4 in supplemented RPMI, from both insect (40 U/ml) and E. coli (100 ng/ml), failed to induce neutrophil migration relative to their vehicles in similar concentration (Fig. 3B).

We hypothesized that IL-8 release by stimulated neutrophils might be responsible for the chemotactic activity of culture supernatants. As neither blocking antibodies to equine IL-8 nor equine IL-8 receptors were commercially available, we used nonpeptide CXCR2 antagonists to evaluate the nature of the chemotactic mediators present in IL-4-stimulated neutrophils culture supernatants. As shown in Fig. 3C, SB-225002 completely inhibited neutrophil migration toward reqIL-8 (10 ng/ml) at 10⁻⁵ M, whereas 10⁻⁶ and 10⁻⁷ M concentrations were less effective. SB-265610 showed a clearer dose-response effect with almost complete inhibition of chemotaxis at 10⁻⁶ M. Figure 3D shows that both CXCR2 inhibitors as well as heat treatment of supernatant reduced the number of cells migrating toward IL-4-stimulated neutrophil supernatants, suggesting that mainly CXCR2 ligands, including IL-8, are involved.

**IL-4-Induced Gene Expression by Neutrophils is Modulated by Disease State and Antigenic Exposure**

We next compared gene expression induced by IL-4 in neutrophils from animals affected with chronic airway disease and healthy animals while they were in clinical remission (pasture) and exacerbation (antigen challenge). We sought to determine whether disease-associated alterations could contribute to Th2-driven neutropenia in heaves-affected horses such as enhanced responsiveness (30) or increased receptor expression (52) to IL-4 and reconcile conflicting ideas surrounding the effect of Th2 cytokines on neutrophils. Respiratory mechanics were performed to compare the degree of airway obstruction. As predicted, no difference in \( R_l \) (0.62 ± 0.22 vs. 0.70 ± 0.18 cmH₂O·l⁻¹·s⁻¹; Fig. 4) and \( E_L \) (0.71 ± 0.38 vs. 0.66 ± 0.31 cmH₂O/l) was observed between control and asthmatic horses during remission. Stabling induced pulmonary obstruction in asthmatic horses as indicated by a significant increased in \( R_l \) (0.46 ± 0.22 vs. 2.45 ± 0.18 cmH₂O·l⁻¹·s⁻¹; \( P = 0.0005 \); Fig. 4) and \( E_L \) (0.74 ± 0.38 vs. 4.05 ± 0.31 cmH₂O/l; \( P = 0.0007 \)) in asthmatic animals only. There were also other clinical signs of airway disease such as cough, nasal discharge, and increased respiratory effort in these horses.

Based on results from our dose- and time-titration experiments, neutrophils were stimulated with 40 U/ml reqIL-4 for 5 h to induce a significant signal while avoiding saturation of the response. We studied proinflammatory cytokines (IL-1β, IL-8, and TNF-α) as well as receptor gene expression [IL-4Rα, the low-affinity receptor for IgE (FceRII) CD23 and TLR4] by purified neutrophils to evaluate the potential outcomes of IL-4-induced neutrophil activation in a Th2-biased inflamma-
Values are expressed as means ± SE. *Significant differences between the means (P < 0.05).

The role of neutrophils in allergic inflammation

Figure 4. Asthmatic horses but not healthy control horses develop airway obstruction following antigenic challenge. Respiratory mechanics were performed on control (n = 4) and asthmatic (n = 6) horses to assess pulmonary function while the animals were at pasture (remission) or stalled in the presence of moldy hay (30-day antigen challenge). Pulmonary resistance (Rl) was obtained from measuring airflow rates and transpulmonary pressures. Values are expressed as means ± SE. *Significant interaction between group and stimulation (P = 0.04). Thus PBN from asthmatic horses following chronic antigenic challenge expressed lower levels of IL-4Rα compared with neutrophils from control horses exposed to the same environmental conditions. P values from statistical analysis are listed in Supplemental Table S1 (available in the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site).

ICC allowed detecting for the first time the protein expression of TNF-α and IL-1β in equine neutrophils. TNF-α+ neutrophils were found in IL-4-supplemented cultures (Fig. 6C), however, they were present in very low numbers. Preliminary experiments using flow cytometry suggest that <1% of neutrophils express TNF-α (range 0.06–0.90%) at both 6- and 18-h time points when stimulated with IL-4 (n = 1, data not shown). IL-1β+ neutrophils were detected in a mixed leukocytes population following stimulation with LPS (Fig. 6E). We next used flow cytometry to evaluate the effect of IL-4 on IL-1β expression by double-stained neutrophils. IL-1β was also found to be expressed in a small proportion of neutrophils (0.04–0.08%). Nevertheless, Fig. 7 shows that the percentage of IL-1β+ neutrophils was ~12-fold decreased after stimulation with IL-4 in primary cell cultures from 2 horses.

Intracellular Pathways Involved in IL-4-Induced Gene Expression in Equine Neutrophils

We used Western blotting to detect the activated form of STAT6 in equine neutrophils using an anti-phospho-STAT6 antibody (Tyr641). This transcription factor is recruited to the cytoplasmic portion of the IL-4Rα subunit where it is phosphorylated through JAK1 activation and redirected to the nucleus to mediate specific gene transcription (45). Hence, the detection of its phosphorylated form represents the functional consequence of IL-4 binding to its receptor on equine neutrophils. Figure 8A shows that phospho-STAT6 could only be detected in reqIL-4-stimulated neutrophils (15 min) indepen-

Fig. 5. Differential mRNA expression in peripheral blood neutrophils from asthmatic and control horses following culture with reqIL-4 for 5 h. Peripheral blood from each horse was collected at time of remission and following a 30-day antigenic challenge. Neutrophils were purified using paramagnetic microbeads (MACS) and incubated with the vehicle (~) or insect reqIL-4 (40 U/ml) for 5 h at 37°C, 5% CO2. Following culture, gene expression of proinflammatory cytokines (IL-8, IL-1β, and TNF-α; A) and receptors [IL-4 type I receptor (IL-4Rα), CD23, and Toll-like receptor 4 (TLR4); B] was quantified using real-time PCR and gene-specific primers. Absolute values were corrected relative to GAPDH expression. Ratios are expressed as means ± SE. *Significant effect of IL-4 over vehicle (P < 0.05). †Significant difference between groups (P < 0.05). #Significant effect of antigen challenge (P < 0.05). ⊗, Outlier value.

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dently of the source. Furthermore, activated STAT6 was not found in protein extracts from nonstimulated or LPS + fMLP-stimulated neutrophils (15 min). In contrast to a previous report (4), detection of phosphorylated STAT6 in neutrophils was unaffected by the absence of protease inhibitors in lysis buffer.

Various kinases are activated in human neutrophils stimulated by IL-4 (50). Here, we used pharmacological inhibitors to assess the specific involvement of MAPKs, PI3K, as well as the transcription factor NF-κB in the IL-4-induced regulation of gene expression by neutrophils. Figure 8B shows that preincubation of neutrophils with LY-294002, an inhibitor of PI3K, as well as SB-203580 (p38 MAPK inhibitor) significantly inhibited the upregulation of CD23 mRNA by IL-4 stimulation (5 h). Moreover, CD23 mRNA level was significantly lower in LY-294002-pretreated neutrophils compared with negative control neutrophils, suggesting that PI3K plays a role in the regulation of basal CD23 mRNA expression. IL-1β expression is significantly downregulated by IL-4 stimulation in neutrophils. Only LY-294002 pretreatment of neutrophils significantly reversed this inhibition by IL-4 (LY-294002/IL-4- vs. DMSO/IL-4-treated neutrophils). Here, uncorrected mRNA data are reported because we were unable to find a single housekeeping gene unaffected by all of the inhibitors (GAPDH by LY-294002 and AS602868; β-actin by AS602868; β-glucuronidase by SB-203580; data not shown).

**DISCUSSION**

Neutrophils are recruited to the airways of human asthmatics and in animal models of asthma after allergen inhalation (38, 43, 60). Importantly, severe asthma phenotypes are associated with substantial recruitment of neutrophils to the airways (62), suggesting a role of these cells in the pathology. However, the pathways linking neutrophilic inflammation to Th2-mediated allergic responses remain to be clarified.

A considerable amount of published work aiming at characterizing the effects of IL-4 on neutrophils has been performed using neutrophils primed with microbial compounds or proinflammatory cytokines (13, 41, 42, 46). In most of these reports, a predominant anti-inflammatory effect was observed questioning whether Th2 cytokines contribute to neutrophil activation during the allergic response. In the present study, we have shown that IL-4 induces a mixed inflammatory profile in unprimed equine neutrophils with a combination of increased (IL-8 and TNF-α) and decreased (IL-1β) proinflammatory cytokine expression. We also found prospective markers of IL-4-activated equine neutrophils as the IL-4Rx subunit and CD23 mRNA expression were upregulated by IL-4. Furthermore, we demonstrated a dose and time dependence of both IL-8 mRNA expression by IL-4-stimulated neutrophils and their release of chemotactic factors in culture supernatants. This suggests that IL-4 may contribute to the recruitment and activation of neutrophils during allergic inflammation. In an attempt to further characterize the pathways involved in IL-4-driven gene transcription in neutrophils, we found that both PI3K and p38 MAPK were specifically involved in up- and downregulation of mRNA expression. Finally, although statistical limitations arise from the small sample size in the present study, these preliminary data suggest that antigen challenge altered gene expression by PBN in response to IL-4 in both asthmatic and healthy horses.

Our (15) previous findings suggest that the airway neutrophilia occurs concurrently with the expression of Th2 cytokines in equine heaves, a common disease of horses that shares pathophysiological features with human asthma. Using in situ hybridization, we (15) found higher numbers of bronchoalveo-
lar lavage cells expressing Th2 cytokine mRNA including IL-4 in these horses compared with healthy horses under similar environmental conditions. Notably, neutrophilic inflammation also occurs in the airways of healthy horses on antigen challenge but tends to resolve over time. Based on these observations, we hypothesized that the interaction between Th2-type cytokines and neutrophils may contribute to the development and/or persistence of airway inflammation in the asthmatic lung. Cytokines of the IL-17 family (IL-17A, IL-17F, and IL-22) (61) are potent drivers of neutrophilic recruitment during chronic inflammation and may contribute to the presence of neutrophils in human asthma (12) as well as in equine heaves (19). However, Th1 and Th2 profiles may not be mutually exclusive but rather sequentially expressed in the airways. Thus their specific contribution to neutrophil recruitment and activation in these diseases remains to be established. Our first objective was to validate the agonistic activity of reqIL-4 in equine PBN before studying its effect in the context of allergic inflammation. We found several features that are commonly induced by IL-4 in both human and equine neutrophils, suggesting that they may share similar behavior in an allergic context. These include cellular polarization, functional activation of STAT6, dose-dependent regulation of IL-8 expression, as well as the involvement of similar signaling pathways (MAPK p38 and PI3K) (26, 50). Other reported effects of IL-4 on human neutrophils include enhanced adherence and survival (26), improved phagocytosis, degranulation and respiratory burst activity (10), as well as enhanced leukotriene B4 (LTB4) production via increased LTA4 hydrolase expression and activity (66).

We observed in the present study that equine neutrophils underwent morphological changes after IL-4 stimulation, together with enhanced mRNA expression of IL-8, a glutamate-leucine-arginine (ELR)+ motif CXC chemokine that has potent chemotactic effects on neutrophils. Moreover, we found that supernatants from IL-4-activated neutrophils, but not IL-4 itself, possessed chemotactic activity toward freshly isolated equine neutrophils. We postulated that an autocrine pathway involving IL-4-induced chemotactic factor release by neutrophils is responsible for the above-mentioned changes in cellular shape and migration. This is supported by the fact that IL-13, a Th2 cytokine sharing biological homology with IL-4 (40), does not induce human neutrophil chemotaxis but mediates IL-8 release by these cells (27). Similarly, equine neutrophil migration toward IL-4-treated neutrophil culture supernatants was reversed by CXCR2 antagonists, reinforcing the hypothesis that IL-8, and maybe other CXCR2 ligands such as MIP-2 (34), are rapidly released by neutrophils following activation by IL-4. Our results indicate that priming of peripheral neutrophils by antigen challenge further potentiates IL-4-induced IL-8 mRNA expression by these cells, a phenomenon accentuated in asthmatic horses (P = 0.06). Interestingly, IL-8 has been implicated in airway neutrophilia in equine heaves (23) and in severe asthmatic patients (57), in which neutrophils were shown to be the primary source of IL-8 in sputum samples (25). Taken together, these observations suggest that IL-4 could mediate neutrophilia in the asthmatic lung, i.e., where Th2 cytokines are being expressed, via an indirect pathway involving the secretion of chemotactic factors by neutrophils themselves.

Neutrophils express proinflammatory cytokines, including IL-1β, IL-8, and TNF-α, which exert paracrine/autocrine effects on their cellular functions (14) and contribute to promoting ongoing inflammation. We demonstrate here for the first time the direct effect of IL-4 on differential modulation of the expression of these cytokines in neutrophils, IL-1β being strongly inhibited, whereas IL-8 and TNF-α were significantly upregulated. This pattern of expression is considerably different from proinflammatory agonists such as LPS or IFN-γ, which lead to upregulation of these three cytokines in both
human and equine neutrophils (14, 34). Using immunohistochemistry and available species-specific antibodies, we also report for the first time that TNF-α and IL-1β are expressed by equine neutrophils at early time points following culture. Results obtained using flow cytometry suggest that transcriptional changes induced by IL-4 on IL-1β expression are reflected at the protein level, as shown also for IL-8 protein expression in the present study using indirect methods in migration assays (CXCR2 blockers). It remains to be determined whether further posttranscriptional regulatory pathways apply to TNF-α production by neutrophils, as the protein expression of this cytokine is tightly regulated in other species (5).

In agreement with our results, it had been shown that IL-4 stimulation of human neutrophils leads to production of IL-1ra (16), an endogenous antagonist of IL-1β. This effect of IL-4 on neutrophils could represent a protective mechanism against uncontrolled inflammation during immune responses and therefore tends to support the concept of Th2 cytokines having anti-inflammatory effects. Interestingly, we found that PBN from asthmatic horses have a significantly decreased expression of IL-1β compared with controls. This phenomenon has been reported in mononuclear cells of patients with atopic dermatitis (49) and thus might be more generally related to Th2-driven chronic inflammation. Such “alternative” phenotype featuring a contrasting inflammatory profile is relevant to immune responses leading to excessive tissue repair. Likewise, neutrophils are increasingly recognized as important components of the Th2-driven immune response elicited during parasitic infections (6) in which the aim of inflammation combines fighting the invading microorganism and preserving tissue integrity.

Recent findings demonstrate that IL-4 also regulates the expression of the high-affinity receptor for IgEs (FcεRI) on human neutrophils (3), suggesting that neutrophils could intervene in IgE-mediated inflammatory events. Our finding that CD23, the low-affinity IgE receptor, is also upregulated by IL-4 in equine PBN further supports a role of neutrophils in allergic inflammation. This molecule, in both its membrane-bound (mCD23) and soluble form (sCD23), is implicated in the regulation of IgE synthesis, antigen processing by antigen-presenting cells, and inflammatory cytokine production (54). Interestingly, it has been suggested that neutrophil proteases could mediate cleavage of mCD23 (11). Hence, increased sCD23 level is a general feature of chronic inflammation (7), including asthma (39). Neutrophils are thus likely to contribute to sustaining inflammation through an autoregulatory pathway involving CD23. This is of relevance to heaves as local production of allergen-specific IgE in the airways of heaves-affected horses has been reported (28, 56). In addition, we demonstrate here for the first time that IL-4 induces the upregulation of its own receptor in neutrophils, perhaps again creating a positive feedback mechanism promoting chronic Th2-driven inflammation. In contrast to our expectations, we observed a slight but significant decrease in the level of IL-4Rα mRNA expression in PBN from asthmatic horses when exposed to allergen. In both human asthmatics and equine heaves, the expression of other Th2 cytokine receptors (IL-5 and IL-9) were shown to be increased on PBN (1, 20). We postulate that there may have been a reduction in a subset of IL-4Rα-expressing neutrophils in peripheral blood of these horses consequent to their recruitment to target organs. Alternatively, inhibitory feedback mechanisms affecting the JAK-STAT pathways such as suppressor of cytokine signaling (SOCS) proteins (29) could have been initiated following Th2 cytokine stimulation of leukocytes in the systemic compartment of asthmatic horses, which decreased IL-4Rα at the mRNA level. Results from the present study allowed us to infer biological activity of IL-4 on equine neutrophils and to show an effect of disease and environmental changes on transcriptional regulation of proinflammatory cytokines and receptors in these cells.

Identification of novel therapeutic targets to control Th2-driven inflammation is an area of intense research. Recent in vivo experiments performed by our group suggest a role for MAPK pathway in Th2-driven neutrophilic airway inflammation, as the administration of a MAPK p38 inhibitor delayed the onset of symptoms and reduced neutrophil recruitment to the airways of asthmatic horses (36). Our in vitro experiments support and extend previous studies indicating a role of the MAP kinases in allergic inflammation by showing that p38 is implicated in the upregulation of CD23 mRNA expression in equine neutrophils. We did not find, however, agreement between the two different PI3K inhibitors LY-294002 and wortmannin in our experiments. Other studies have shown divergent effects of PI3K inhibitors (63) where calcium signaling was inhibited by LY-294002 and wortmannin had no effect.

In conclusion, the results of these ex vivo studies suggest that IL-4, a Th2 cytokine, may contribute to the activation of neutrophils during allergic inflammation. This phenomenon involves the regulation of chemokine and cytokine expression by neutrophils including increased IL-8 and TNF-α expression combined with decreased IL-1β. Hence, IL-4 generated a mixed inflammatory profile in equine neutrophils that may correspond to the signature of “alternative activation” in Th2-dominated inflammatory states. Furthermore, using a natural animal model of chronic airway diseases, we have shown that neutrophils are systemically activated following allergen exposure and that they have increased responsiveness to IL-4, one of the cytokines shown to be overexpressed in the airways of affected animals. These preliminary findings direct future work aimed at verifying the in situ effects and contribution of IL-4 on airway neutrophils and how the commonly used corticosteroid treatment affects this interaction and contributes to decrease disease severity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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