

Innate Immune Function of Eosinophils

From Antiparasite to Antitumor Cells

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Summary

Eosinophils are multifunctional leukocytes classically described as being involved in helminth parasitic infections and allergic diseases. Previously restricted to an exclusive role in the release of cytotoxic mediators, they are now also considered to be immunoregulatory cells and potential effectors in innate immune responses. Eosinophils are mainly found in tissues, so specific procedures are needed for their isolation from venous blood and for functional assays. Murine models are very useful for the dissection of eosinophil physiology *in vivo*. But murine eosinophils significantly differ from human ones. A complete understanding of eosinophil biology therefore requires comparative study of eosinophils from different mammalian species. We summarize here the main experimental protocols used to study human, mouse, and rat eosinophil biology. We focus on technical improvements of existing methods that optimize purification and *in vitro* functional studies of eosinophils.

Key Words: Eosinophil purification; hypodense; flow cytometry; activation markers; mediator release; cytotoxicity; heterogeneity; animal models; cell lines.

1. Introduction

Eosinophils have long been known to be involved in helminth parasitic infections and allergic diseases. Indeed, they have been characterized until recently by this functional duality. Today, eosinophils are considered rather as really multifunctional leukocytes, involved in inflammatory processes as well as in modulation of innate and adaptive immunity (*1*). Eosinophils express membrane receptors involved in adaptive immune responses like

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Fc receptors, class II major histocompatibility complex (MHC), or co-stimulatory molecules including CD86 and CD28 that allow interactions with lymphocytes and antigen-presenting cells (2). They also display an array of receptors involved in innate immunity, including lectin-type receptors and protease-activated receptors; Toll-like receptor (TLR)-specific mRNAs have also been detected (3). Triggering eosinophils by engagement of receptors for cytokines, immunoglobulins, or complement can lead to the secretion of numerous cytokines [interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and transforming growth factor (TGF) α/β], chemokines (RANTES, eotaxin), and lipid mediators (platelet-activating factor, leukotriene C4). We have demonstrated that there is a specific release of Th2 and Th1 immunoregulatory cytokines and cationic proteins, dependent on the nature of stimulus (4,5). Furthermore, eosinophils store in their specific granules great quantities of cytokines and cationic proteins, known to be highly cytotoxic mediators (6,7). Eosinophil granules contain a crystalloid core composed of major basic protein (MBP) and a matrix containing eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO) (8). They can also generate reactive oxygen species (ROS). This high cytotoxic potential can lead to deleterious effects in inflammatory and allergic processes, but it can also be deployed effectively to combat parasitic targets. We have in particular demonstrated that eosinophils are able to kill schistosomula, the young larvae of the helminth parasite *Schistosoma mansoni*, through an IgE-dependent antibody-dependent cellular cytotoxicity (ADCC) mechanism (9,10).

Eosinophils are able to integrate the different signals of their environment. They can respond to danger signals, including bacteria (11) or signals derived from stressed and necrotic cells (12). Moreover, eosinophil numbers have been documented to be elevated in peripheral blood and/or to infiltrate the tissues in some malignant disorders (13,14). Several studies have convincingly shown that tissue or blood eosinophilia was correlated with a significantly better prognosis (15). A putative tumoricidal activity of eosinophils is supported by recent *in vitro* (16) and animal studies (17,18). Not only efficient during helminthic infections, recent experimental results implicate the eosinophil cytotoxic potential in antitumor immunity. Eosinophils are also able to induce apoptosis of human tumor cells, and a new method has been developed to study this tumoricidal activity (submitted data).

Many arguments favor a primordial role for eosinophils in innate immune responses: the great storage potential of their granules, their prominent tissue localization at the interface with the external environment, their capacity for recognition and integration of danger signals, and their high and specific

cytotoxic content, which can be rapidly and selectively mobilized through finely regulated degranulation pathways.

In this chapter, we describe methods that can be needed to functionally characterize eosinophils. It is clear that we still have much to learn about this remarkable type of cell.

2. Materials

2.1. Isolation Protocols

1. Dextran solution: 4.5% (w/v) dextran T500 (DxT500, Amersham Pharmacia Biotech, Uppsala, Sweden) in normal saline.
2. Tyrode's gel: 0.1% (w/v) D-Glucose (Merck, Darmstadt, Germany), 0.1% (w/v) NaHCO₃, 0.02% (w/v) KCl, 0.8% (w/v) NaCl, 0.005% (w/v) Na₂HPO₄, 0.1% (w/v) gelatin, pH 7.5–7.8. First dissolve all the components except the gelatin in distilled water (smaller volume than desired final solution). Dissolve then the gelatin in a small volume of distilled water while heating slightly (50–60°C). Maintain the prepared medium at a warm temperature and add the dissolved gelatin (avoid solidification of the gelatin). Complete with water to the desired volume and sterilize the solution while heating slightly by filtering successively through cellulose acetate filters of 0.45- μ m and 0.22- μ m pore size. This solution can be stored at 4°C for several months. Just before use, add 0.003% (w/v) DNase I grade II from bovine pancreas (Boehringer Mannheim, Mannheim, Germany) to avoid DNA aggregates.
3. Metrizamide (Sigma-Aldrich, St. Louis, MO): Prepare a stock solution of metrizamide 30% (w/v) in Tyrode's gel. Sterilize through a 0.22- μ m filter and store at –20°C in the dark (*see Note 1*). This solution is stable for very long periods of time. Prepare working solutions by dilution in Tyrode's gel (as mentioned in **Table 1**). In order to be faster during the purification, we recommend preparing these gradients solutions 1 day before purification and storing them at 4°C.
4. Phosphate-buffered saline (PBS) 1 $\times$: always without calcium and magnesium.
5. Eosinophil purity: The cytocentrifuge (Shandon Cytospin 2) uses a unique sample chamber assembly that incorporates a plastic sample chamber, a filter card to act as a blotter, a microscope slide, and a unique stainless steel slide clip. Perform May-Grünwald Giemsa (MGG) coloration using RAL 555 (Rieux, France).
6. Percoll solution (d = 1.082 g/mL): In a graduated cylinder, add nine parts (v/v) of Percoll (d = 1.13 g/mL, Amersham Biosciences) to one part (v/v) of 9% (w/v) NaCl to have a solution adjusted to approximately 340 mOsm/kg H₂O. Transfer this 90% Percoll solution in a bottle and adjust density with physiological saline (0.9% NaCl). Calculate the required volume of normal saline (V) using the formula: $V = 1.7 \times V_0 - V_{90}$ with V_0 = volume of undiluted Percoll required; V_{90} = volume of total 90% Percoll (*see Note 2*). The solution is stable at 4°C for several weeks. As all the preparation should be made under a laminar flow hood in sterile conditions, filtering is not necessary.

Table 1
Preparation of Metrizamide Solutions

Number of gradients	2	4	6
Total volume (mL)	28	56	84
Prepared volume (mL)	30	60	90

Percentage	Density (g/mL)	Stock (mL)	Gel (mL)	Stock (mL)	Gel (mL)	Stock (mL)	Gel (mL)
18	1.105	3.0	2.0	6.0	4.0	9.0	6.0
20	1.115	3.35	1.65	6.7	3.3	10.05	4.95
22	1.125	3.65	1.35	7.3	2.7	10.95	4.05
23	1.130	3.85	1.15	7.7	2.3	11.55	3.45
24	1.135	4.0	1.0	8.0	2.0	12.0	3.0
25	1.140	4.15	0.85	8.3	1.7	12.45	2.55
Total		22	8	44	16	66	24

Stock: solution of metrizamide 30%; gel: Tyrode's gel.

7. Spinal needle (Terumo Corp., Tokyo, Japan).
8. Lysis buffer (isotonic ammonium chloride solution): 155 mM NH_4Cl , 10 mM NaHCO_3 , and 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. Sterile filtered through 0.45- μm and 0.22- μm filters. Stable for months at 4°C.
9. Anti-human CD16 and streptavidin-coated magnetic beads, CS and LD columns, and MACS system are from Miltenyi Biotec, Bergisch Gladbach, Germany.
10. Fraction V bovine serum albumin (BSA) (Eurobio, Les Ulis, France): 10% (w/v) stock solution in PBS, filter sterilized and stored at 4°C.
11. Biotinylated anti-mouse antibodies (anti-CD19, anti-CD90, and anti-CD8 α) and anti-rat antibodies (anti-CD45R, anti-mononuclear phagocytes and anti-CD8 α): Pharmingen, San Diego, CA.
12. Nylon filter (90 μm): PA 90/35 PW WH (SAATitech, Appiano Gentile, CO, Italy).

2.2. Cell Culture

1. The eosinophil culture medium (complete medium): RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 2 mM L-glutamine, 10 mM sodium pyruvate, and 10 $\mu\text{g/mL}$ of gentamycin. Use 100 IU/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin to replace gentamycin.
2. Recombinant human IL-5 (rhIL-5) (Peprotech, Rocky Hill, NJ): Reconstitute in water to a concentration of 100 $\mu\text{g/mL}$. Make small frozen aliquots. Prepare working solution at 5 $\mu\text{g/mL}$ by dilution in complete medium. Store at 4°C. Stable for a few months.

3. Peroxidase cyanide buffer: EPO is specifically detected by a technique based on the resistance of EPO enzyme to cyanide (see **Note 3**). Dissolve one tablet (10 mg) of the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, St. Louis, MO) in 15 mL of 0.1 M Tris-HCl, pH 7.6, containing 0.4 mg/mL potassium cyanide. This solution is stored at 4°C for 1 month. Just before use, add 3 μL of 3% (w/w) H_2O_2 solution to 1 mL of this buffer for a single use.

2.3. Analysis of Specific Markers and Activation Markers

2.3.1. Flow Cytometry Analysis

1. Saponin (Sigma): 10% (w/v) stock solution in PBS, filter sterilized and stored at 4°C.
2. Paraformaldehyde (Sigma): Prepare a 4% (w/v) solution in PBS. In order to dissolve it, add concentrated NaOH to increase the pH (up to 10–11) until the solution is completely clear and then readjust the pH to 7.5 with concentrated HCl. The solution is aliquoted and can be stored for a long time at –20°C and for 1 month at 4°C.
3. Fluorescence-activated cell sorting (FACS) membrane buffer (FACS buffer): 1% (w/v) BSA, 0.05% NaN_3 in PBS.
4. FACS intracellular buffer (IC buffer): 1% (w/v) BSA, 0.1% (w/v) saponin in PBS.
5. Antibodies: anti-mouse Fc γ R (2.4.G2), anti-human MBP, and anti-human EPO mAbs are from Pharmingen. The anti-human ECP (EG2) is from Pharmacia.

2.3.2. Immunocytochemistry

1. Tris-buffered saline (TBS): 0.05 M Tris-HCl, 154 mM NaCl, pH 7.6.
2. Dako pen, the APAAP detection system (rabbit anti-mouse immunoglobulins and mouse APAAP complex), New Fuchsin kit, and Mayer's hematoxylin (ready-to-use aqueous solution) are from Dako (Glostrup, Denmark).
3. Immumount (Shandon, Pittsburgh, PA).
4. Stratalinker UV 2400 (Stratagene, La Jolla, CA).
5. Human serum albumin (HSA) (Sigma): 10% (w/v) stock solution in PBS, filter sterilized and stored at 4°C.
6. Fluoromount G (Southern Biotechnology Assoc., Birmingham, AL).

2.4. Functional Tests

2.4.1. Mediator Release Assays by Chemiluminescence

1. White 96-well cell culture plate with flat bottom and individual lid (Nunc, Roskilde, Denmark).
2. RPMI 1640 without phenol red (Cambrex Bio Science, Rockland, ME) (see **Note 4**).

3. Luminol (Sigma) is dissolved at 5 mg/mL in dimethyl sulfoxide (DMSO) and stored in single use aliquots: volume of 15 μ L for measurement of ROS and 80 μ L for measurement of EPO. Aliquots are stored at -20°C in the dark.
4. ROS buffer: 15 mM Tris-HCl, 150 mM NaCl, pH 7.4.
5. EPO buffer: 15 mM Tris-HCl, pH 6.0.
6. D-Luciferin (Roche Diagnostics, Basel, Switzerland): 0.16 mM in EPO buffer. Twenty-microliter aliquots are stored at -20°C for single use (*see Note 5*).
7. Stimulus: rhIL-5 at a final concentration of 20 ng/mL.
8. Luminometer: Victor²™ Wallac (PerkinElmer, Shelton, CT) allows the measurement of the intensity of light released by a chemical reaction (chemiluminescence). Data analysis is performed with Wallac 1420™ software.

2.4.2. Cytotoxicity Assays

1. Hank's medium and Modified Eagle's Medium (MEM) (Cambrex).
2. 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark).
3. PKH-26 red fluorescent cell linker kit (Sigma): Store at room temperature or refrigerate. Keep dye stock tightly capped to prevent evaporation.
4. Fluorescein isothiocyanate (FITC)-conjugated annexin V (Pharmingen).
5. Binding buffer (1 $\times$): 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2 . Sterilize through 0.45- μm filter. Store at 4°C .
6. Colorectal adenocarcinoma cell line, Colo-205 (ATCC LGC Promochem, Middlesex, UK).

3. Methods

Eosinophils, which are mainly found in tissues, only represent 1–5% of circulating leukocytes in normal donors. Specific experimental procedures are thus needed for eosinophil purification from peripheral blood. Two main protocols of purification can be used to isolate eosinophils. The first to be described, using metrizamide discontinuous gradients, is based on the specific cell density of eosinophils and allows one to obtain separately eosinophils with normal density (normodense) and with lower density (hypodense), the latter representing the activated phenotype. The second technique, more efficient regarding the eosinophil purity and the most commonly used today, consists of blood centrifugation on a Percoll gradient followed by a negative immunomagnetic selection by anti-CD16-coated microbeads and the MACS system. Even if eosinophils are end-stage cells, they can be maintained overnight in culture. This rest, after the stress of purification, allows turnover of membrane receptors. Eosinophils are characterized by their phenotypical and functional heterogeneity, strongly dependent upon eosinophil donors. Studying phenotype is possible by immunofluorescent staining using two efficient and complementary techniques like flow cytometry (FCM) and immunocytochemistry

(ICC). Indeed, techniques such as enzymatic or fluorescent ICC analysis can be used to confirm the membrane or intracellular expression of the proteins of interest observed by FCM analysis. Moreover, ICC on cytospin preparations can complement results obtained by FCM by localizing the storage sites of the molecules studied (granular, cytoplasmic, or membrane-like staining).

To understand the effector roles of eosinophils, it is necessary to characterize the functional properties of these cells. We describe here two functional assays: mediator release and cytotoxicity assays. Release of several cytotoxic mediators, either neo-synthesized (e.g., ROS) or preformed as granule cationic proteins (e.g., EPO), can be measured by chemiluminescence. After activation, release of ROS generated by NADPH-oxidase can be measured by luminol-dependent chemiluminescence. The ROS produced by eosinophil activation oxidize luminol, and this oxidation results in an excited state of luminol, which fluoresces and releases photons as it decays to a lower energy level. Released photons are then collected by a luminometer (*see Note 6*). EPO, which constitutes $\sim 25\%$ of the total protein mass of specific granules, catalyzes the oxidation reactions to form highly ROS and reactive nitrogen metabolites. As EPO is released during degranulation by activated eosinophils, it can be considered to be a marker of eosinophil activation and degranulation. EPO release can also be measured by chemiluminescence. The cytotoxicity assays presented here are based on protocols measuring *in vitro* beneficial cytotoxic properties of eosinophils toward two different targets, helminthic parasite larvae and tumor cell lines. First, parasite killing by purified human eosinophils is an IgE-dependent ADCC mechanism and seems restricted to hypodense eosinophils present only in highly hypereosinophilic patients. Concerning antitumor activity of eosinophils, several *in vitro* arguments allow one to postulate that eosinophils are also able to use effectively their cytotoxic potential toward human tumor cell lines by inducing their apoptosis (submitted data).

To complete our description of methods for eosinophil study, we describe here isolation protocols for mouse and rat eosinophil purification. The main experimental procedures detailed for human eosinophils—immunostaining (FCM and ICC) and mediator release and cytotoxicity experiments toward parasite targets—can be used with murine eosinophils. Protocol modifications are then mentioned in notes if needed. Although murine models are advantageous to study eosinophil physiology *in vivo*, several differences observed between murine and human eosinophils have to be seriously considered (19). First, mouse eosinophils are poorly granulated, in contrast to rat and human eosinophils (*see Fig. 1c* and *Fig. 2*), and therefore granule release is more

Table 2
Different Expression of Ig Receptors in Human, Mouse,
and Rat Eosinophils (18, 19)

Receptors	Ligand	Human	Mouse	Rat
FcεRI	IgE	Yes ^a	No ^b	Yes
CD89	IgA	Yes	No	mRNA
pIgR	IgA/IgM	Yes	No	No
FcαμR	IgA/IgM	No	mRNA	No
Tf-R	Tf/IgA	Yes (Tf-R1)	Yes (Tf-R1, -R2)	No

Tf, transferrin.

^a Only expressed on activated cells.

^b In human IL-5/FcεRIα transgenic mouse, eosinophils express FcεRI with a distribution similar to human cells.

difficult to induce. Second, differences in IgE and IgA membrane receptor expression have been described, as summarized in **Table 2 (20)**.

3.1. Isolation Protocols

3.1.1. Purification of Human Eosinophils

3.1.1.1. BLOOD SAMPLING

1. Collect human venous blood from subjects in vacutainers containing heparin or sodium citrate as anticoagulant. Be careful of taking a minimum of 80–100 mL of venous blood if it is a normal donor in order to have enough purified eosinophils. The donors should not have received any corticosteroids or histamine antagonists at the time of the study. It is better they not received such therapy in the preceding weeks.
2. Maintain fresh blood at room temperature and use it as soon as possible (maximum 3–4 h).

3.1.1.2. METRIZAMIDE DISCONTINUOUS GRADIENTS

1. Maintain all the solutions used before erythrocyte lysis at room temperature. Perform dextran sedimentation by adding five volumes of heparinized blood to one volume of the dextran solution under a laminar flow hood at room temperature. When the level of sedimentation is stabilized, collect the leukocyte rich supernatant, wash it in Tyrode's gel, and centrifuge at $500 \times g$ for 8 min (*see Note 7*).
2. Remove the contaminating erythrocytes by hypotonic lysis by adding one volume of 0.2% (w/v) ice-cold NaCl solution to the cell pellet for 10 s while mixing. Restore the physiological osmolality by addition of one volume of 1.6% (w/v) NaCl

solution. Centrifuge immediately at 4°C for 8 min at $350 \times g$. Repeat once the lysis step.

3. Wash the cells with Tyrode's gel containing DNase (*see Note 8*). Centrifuge at 4°C for 8 min at $700 \times g$. Resuspend the preparation of mixed leukocytes at 25×10^6 to 50×10^6 cells per mL in Tyrode's gel.
4. Prepare the discontinuous metrizamide gradient (this step can be performed during dextran sedimentation) by using metrizamide solutions prepared 1 day before as mentioned in **Subheading 2.1., step 3**. With a transfer pipet carefully layer 2 mL of each metrizamide solution (from 1.140 to 1.105 g/mL density) in a 15 mL tube (*see Note 9*) and then layer on the top 2 mL of leukocytes suspension in Tyrode's gel. The details of the metrizamide gradient are presented in the table in **Fig. 1**.
5. Centrifuge at $1200 \times g$ for 45 min at 20°C (without brake) and recover the different layers of the metrizamide gradient. Wash the cell suspensions three times with PBS [centrifugation respectively at $700 \times g$ (*see Note 10*), $550 \times g$, and $450 \times g$].
6. Determine the eosinophil purity: cytoцентрифуге the cell suspension for 2 min at $10 \times g$ (prepare a dilution in PBS to have 10^5 cells/100 μL /cytoспin) and then stain cytoспin with RAL 555 coloration. Typical results are presented in **Fig. 1**. One can see morphological changes such as cytoplasmic vacuoles and reduced numbers and size of granules in activated eosinophils, called "hypodense." We recommend counting a minimum of 300 cells per cytoспin for a good estimation. The contaminating cells are mainly neutrophils (*see Note 11*).

3.1.1.3. CD16-COATED BEADS AND MACS SYSTEM

1. The first step consists of separation of polynuclear and mononuclear cells using a Percoll gradient (*see Note 12*). Distribute fresh blood in 50-mL tubes (maximum 15 mL per tube) and dilute with PBS (1:1 v/v). After mixing, add gently 15 mL Percoll solution ($d = 1.082 \text{ g/mL}$) at the bottom of the tube using a spinal needle and centrifuge at room temperature, $700 \times g$ for 20 min without brake.
2. After centrifugation, the pellet contains red blood cells and granulocytes and the thin layer at the interface of Percoll and plasma contains mononuclear cells. After removal of excess plasma, collect carefully the peripheral blood mononuclear cell (PBMC) layer. Remove all the Percoll and transfer the pellet to new 50-mL tubes (no more than 10 mL pellet volume per tube) (*see Note 13*).
3. Perform a hypotonic lysis to remove the contaminating erythrocytes by adding 40 mL of ice-cold lysis buffer directly onto the remaining pellet, mix vigorously, and leave on ice for 15 min. After centrifugation at $500 \times g$, 4°C for 15 min, pool all the pellets in one tube and repeat a second lysis step for 10 min on ice (*see Note 14*).
4. Resuspend the granulocytes in cold PBS, count them, and determine the percentage of neutrophils and eosinophils by staining cytoцентрифуге preparation with RAL 555 coloration. We recommend counting a minimum of 300 cells per cytoспin for a good estimation.

14 mL		Layer	Eosinophil	Major cells
12 mL	Cells	1	Hypodense	Monocyte
10 mL	18 %	2	Hypodense	Lymphocyte
8 mL	20 %	3	Intermediate	Neutrophil
6 mL	22 %	4	Normodense	Eosinophil
4 mL	23 %	5	Normodense	Eosinophil
2 mL	24 %	6	Normodense	Eosinophil
	25 %			

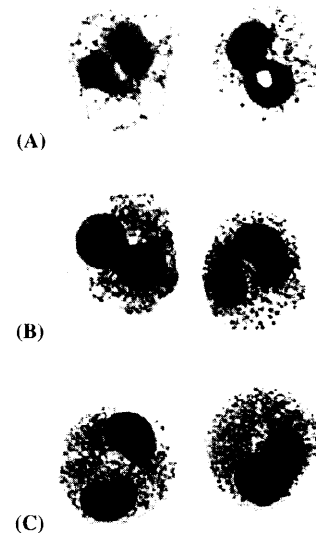


Fig. 1. Human eosinophils purified on metrizamide gradients. Cells are centrifuged on microscope slides and stained with RAL 555 as described in **Subheading 3**. Notice the bilobated nucleus and numerous orange granules. (A) Layer 2: hypodense eosinophils, (B) Layer 3: eosinophils with intermediate density, (C) Layer 4: normodense eosinophils ($\times 100$). (D) Representation of the metrizamide gradient layers and cellular composition.

- The second part of the purification procedure consists of a negative selection of eosinophils using the MACS system. Resuspend granulocytes in 500 μ L ice-cold filtered PBS-BSA 0.5% and incubate with anti-CD16-coated magnetic beads (80 μ L beads for 100×10^6 polynuclear neutrophils) for 30 min in the fridge (less cold than on ice) with intermittent gently mixing (*see Note 15*).
- Equilibrate the column with cold PBS-BSA 0.5% (it is recommended to perform this step during incubation with the magnetic beads). For CS column, proceed as follows: Flush gently the column from the side with 10 mL of buffer to remove air bubbles, and then add 15 mL buffer at the top. Just before adding the granulocyte suspension (in 4 mL), add 3 mL of ice-cold PBS-BSA 0.5%. Eosinophils are eluted following passage through the field of a permanent magnet. After reaching 25 mL of eluate, flush the column again from the side and continue to collect eluate until 50 mL. Centrifuge the eluted cells at $350 \times g$, 4°C , and count them in PBS.
- Determine the purity of eosinophils on cytocentrifuged preparations after RAL 555 coloration. A purity $>98\%$ is usually obtained. One really needs to be careful during all the procedure to avoid contaminating cells. Contaminating cells are mainly lymphocytes (*see Note 16*).

3.1.2. Purification of Mouse Eosinophils

Mouse eosinophils are purified from IL-5 transgenic mice by negative selection using the MACS system (21). These transgenic mice have the advantage to exhibit massive eosinophilia in blood and different organs like spleen, bone marrow, and peritoneal exudate.

- To obtain peritoneal cells, flush the peritoneal cavity with ice-cold PBS. Perform gentle dissociation of the spleen in ice-cold PBS to obtain splenocytes. Isolate bone marrow cells from femur and tibia of mice by flushing the bone marrow cavities with ice-cold PBS.
- Perform a hypotonic lysis to remove erythrocytes by adding ice-cold lysis buffer on the pellet (5 mL for spleen cells and 2–3 mL for bone marrow cells). Mix and leave for 10 min (spleen cells) or 5 min (bone marrow cells) on ice (*see Note 17*). Centrifuge for 10 min at $350 \times g$, 4°C .
- Remove aggregates by filtering the cell suspensions onto a 90- μ m nylon filter. Place a piece of sterile filter on the top of a 50-mL tube. Prewet the filter with two drops of PBS and then gently layer the cells and let them go through. Centrifuge for 10 min at $350 \times g$, 4°C .
- Resuspend the cells at 1×10^8 cells/mL in cold PBS containing 0.5% BSA and 2 mM EDTA, and incubate for 30 min at 4°C with biotinylated anti-CD19, anti-CD90 (Thy1.2), and anti-CD8 α (Ly-2). These antibodies will remove B cells, macrophages, and T lymphocytes, respectively.
- After washing with PBS, resuspend the cells at 1×10^8 cells/mL in PBS + 2 mM EDTA and incubate for 15 min at 4°C with streptavidin magnetic beads.
- Equilibrate the LD column with cold PBS + 0.5% BSA + 2 mM EDTA.
- Collect the eosinophils by passage through the field of a permanent magnet. Collect the eluate and centrifuge at $350 \times g$, 4°C for 10 min.
- Count the cells and estimate the cell purity on cytopspin preparation (*see Subheading 3.1.1*). Typical results are presented in **Fig. 2**. Notice the morphological changes of mouse eosinophils compared with human eosinophils, such as low granularity and circular nucleus. Cell purity is usually 95–97%.

3.1.3. Purification of Rat Eosinophils

Rat eosinophils are obtained from the peritoneal cavity of LouM rats by negative selection using the MACS system (22). In order to induce a cell influx in the peritoneal cavity, we injected rats intraperitoneally with 30 mL of 0.9% (v/w) NaCl 48 h before killing.

- To obtain peritoneal cells, flush the peritoneal cavity with 40 mL of ice-cold PBS.
- Remove aggregates by filtering the cell suspensions onto a 90- μ m nylon filter, as described in **Subheading 3.1.2**.

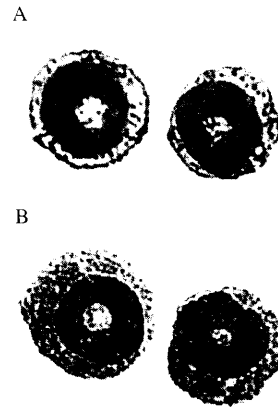


Fig. 2. Murine eosinophils stained with RAL 555 as described in **Subheading 3.1.2.** Notice the circular nucleus of a mouse (**A**) and a rat (**B**) eosinophil. Notice the low granularity (dark spots) in mouse eosinophils compared with human and rat ones ($\times 100$).

3. Perform a hypotonic lysis to remove erythrocytes by adding 5 mL ice-cold lysis buffer on the pellet, mix and leave for 5 min on ice. Centrifuge for 10 min at $350 \times g$, 4°C .
4. Resuspend the cells at 2×10^6 cells/mL in culture medium and culture for 90 min at 37°C in order to allow macrophages to adhere to the surface of the culture flask.
5. Collect supernatants and centrifuge at $350 \times g$, 4°C for 10 min.
6. Resuspend the cells at 1×10^8 cells/mL in PBS containing 0.5% BSA and 2 mM EDTA.
7. Incubate the cells for 30 min at 4°C with biotinylated anti-CD45R, anti-mononuclear phagocytes (1C7), and anti-CD8 α . These antibodies will remove B cells, macrophages, and T cells.
8. After washing with PBS, resuspend the cells at 1×10^8 cells/mL in PBS + 2 mM EDTA and incubate for 15 min at 4°C with streptavidin magnetic beads.
9. Collect eosinophils, count them, and estimate the cell purity as described in **Subheading 3.1.2., steps 7 and 8.** The cell morphology of rat eosinophils is presented in **Fig. 2**. Cell purity obtained with rat eosinophils is usually 85–90% (see **Note 18**).

3.2. Culture Conditions

3.2.1. Eosinophils

1. Purified human eosinophils can be cultured overnight at 2×10^6 per mL in complete medium. Increased adhesion of activated eosinophils and mortality of these end-stage cells explain a usual output of 50–75% (see **Note 19**).

2. IL-5 (1–2.5 ng/mL) can be added to the culture medium to improve eosinophil survival. Higher concentrations of IL-5 could activate eosinophils and induce apoptosis (not recommended).
3. As the purification protocol can generate some cellular stress and increase the basal activation state of eosinophils, overnight culture with complete medium is recommended and allows turnover of membrane receptors for phenotype study and eosinophils to rest before functional assays.
4. Purified mouse and rat eosinophils are cultured up to 4 days in culture medium supplemented with 2.5 ng/mL rhIL-5.

3.2.2. Eosinophilic Cell Lines

Three eosinophilic cell lines are available:

1. YY-1: The YY-1 cell line was kindly provided by Dr. J. Chihara (Akita University, Akita, Japan) (23). This eosinophilic cell line is derived from the promyelocytic leukemia cell line HL-60. Cells are maintained in culture in complete medium and induced to further differentiate into eosinophil-like cells using 0.3 mM butyric acid as shown in **Fig. 3**.
2. AML14.3D10: The AML14.3D10 cell line was kindly provided by Dr. Cassandra Paul (Wright State University, Dayton, OH) (24). This cell line is a fully differentiated eosinophilic myelocyte subline of the AML14 cell line that continues to proliferate and maintain a differentiated eosinophil phenotype without cytokine supplementation. It displays many of the characteristics of mature peripheral blood eosinophils, including constitutive expression of the granule cationic proteins (MBP, EPO, EDN, and ECP) and Charcot-Leyden crystal (CLC) protein as shown in **Fig. 3**. AML14.3D10 cells are maintained in culture in complete medium containing 5×10^{-5} M β -mercaptoethanol.
3. Eol-1: The Eol-1 cell line was kindly provided by Dr. J. Chihara (Akita, Japan) (25). This cell line is derived from the blood of a 33-year-old man with acute eosinophilic leukemia following hypereosinophilic syndrome. Although the Eol-1 cells do not show visible granules, they express ECP and the fusion protein FIP1L1-PDGFR α , which has been recently shown to be associated to the myeloproliferative form of the hypereosinophilic syndrome and to chronic eosinophilic leukemia (26).
4. YY-1 and AML14.3D10 cell lines are cultured at 37°C in 5% CO_2 , up to a maximum density of 10^6 cells/mL. Cultures are diluted in fresh medium twice a week. Fresh cultures are started from frozen stocks after a maximum of 30 passages to minimize genetic drift and phenotypic changes. The Eol-1 cell line is maintained in culture in complete medium at 37°C in 5% CO_2 and is diluted in fresh medium twice a week.

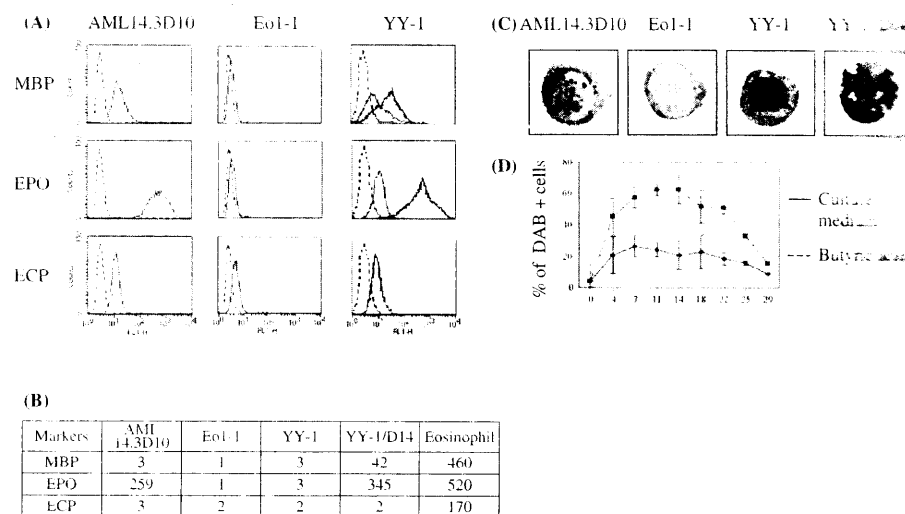


Fig. 3. Analysis by flow cytometry (A, B) and by immunocytochemistry (C, D) of cationic proteins contained in the eosinophilic cell lines, AML14.3D10, Eo1-1, and YY-1. (A) Fluorescein isothiocyanate (FITC)-conjugated anti-cationic protein (solid line) or isotype-matched antibody (dashed line). Staining obtained with YY-1 cells differentiated for 14 days under butyric acid (YY-1/D14) is represented in bold line. (B) Mean fluorescence intensity values of staining obtained for the cationic proteins in the eosinophilic cell lines in comparison with blood peripheral eosinophils. (C) Eosinophil peroxidase (EPO) detection using 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining of cytopsin ($\times 100$). (D) Kinetic of YY-1 differentiation by butyric acid as determined by the percentage of DAB+ cells.

3.3. Analysis of Specific Markers and Activation Markers

3.3.1. Flow Cytometry Analysis

Eosinophils are highly granular cells. Forward scattered/side scatter (FSC/SSC) criteria are represented by a dot plot in Fig. 4A.

1. Membrane staining: To avoid the turnover of membrane receptors, perform the whole experiment on ice (see Note 20). After two washes in PBS, resuspend the eosinophils at $4 \times 10^6/\text{mL}$ in FACS buffer. Add 50 μL of the cell suspension in round-bottom 96-well plates (2×10^5 cells/well). For direct staining, incubate with the labeled antibody 30–45 min in the dark and then wash the cells twice with FACS buffer (200 $\mu\text{L}/\text{well}$) (see Note 21). At the end, resuspend the cellular pellet in 100 μL PBS, transfer samples in appropriate FACS tubes, and add 100 μL of

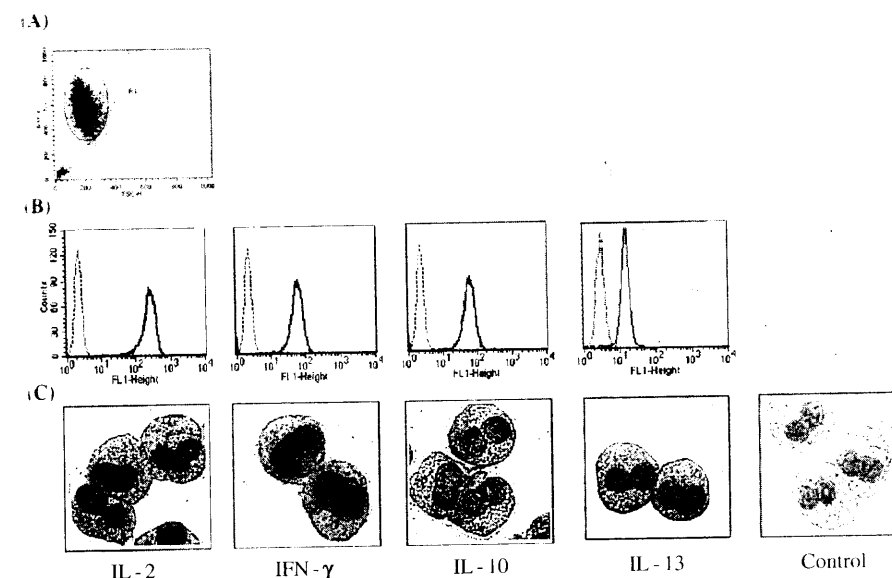


Fig. 4. Flow cytometry and immunocytochemistry (ICC) analysis of eosinophils. (A) Dot plot representation of freshly purified eosinophils. (B, C) Intracellular staining of Th1 and Th2 cytokines analyzed by immunofluorescence cytometry and ICC. (B) Fluorescein isothiocyanate (FITC)-conjugated anti-cytokine (bold line) or isotype-matched antibody (dashed line). Dot plot and histograms are obtained using a FACSCalibur equipped with the CellQuest software. (C) ICC staining on cytopsin eosinophils revealed using APAAP detection system and New Fuchsin coloration ($\times 100$). (B) and (C) are reproduced from ref. (2) (IL-2, IFN- γ , IL-10) and ref. (5) (IL-13).

FACS buffer. Staining with directly-labeled antibody is highly recommended and preferred to indirect staining (see Note 22). For indirect staining, incubate the cells with the primary antibody for 30 min, wash twice with FACS buffer (200 $\mu\text{L}/\text{well}$), and then add the secondary antibody for 20 min in the dark. If necessary, non-specific binding of secondary antibody can be previously blocked by the addition of serum (10%) of the same species directly on the cell pellet during 10 min at 4°C before incubation with the labeled antibody. Analyze the samples within 2 h (see Note 22).

2. Intracellular staining: After fixation of the cells, the experiment can be performed at room temperature. To fix the cells, resuspend eosinophils at $4 \times 10^6/\text{mL}$ in PBS and then add the same volume of 4% paraformaldehyde for 10 min at 4°C . After washing in PBS and centrifugation for 7 min at $350 \times g$, permeabilize the cells ($4 \times 10^6/\text{mL}$) for 10 min in IC buffer. Add directly 50 μL (2×10^5) of the cell suspension

- to round-bottom 96-well plates. For direct staining, first block non-specific binding by incubating the cells for 10 min with 10% of serum from the species of your antibody and then add the labeled antibody for 30 min in the dark. For indirect staining, incubate the cells with the primary antibody for 30 min, wash twice with IC buffer, block non-specific binding with serum (10%), and incubate with the secondary antibody for 20 min in the dark (*see Note 23*). Wash the cells twice with IC buffer (200 μ L) and once with PBS in order to remove saponin. Resuspend the pellet in 200 μ L PBS + 0.5% BSA and analyze within 2 h (*see Note 24*).
3. Acquisition step: A total of 10^4 events are usually acquired for each sample. Analyze on a FACSCaliburTM using the CellQuestTM software (Becton Dickinson, Mountain View, CA) (*see Note 25 and Fig. 4*).

3.3.2. Immunocytochemistry

For the two procedures described below, perform cytopins from freshly purified eosinophils as described above or from eosinophils cultured overnight in complete medium with rhIL-5 (2.5 ng/mL).

3.3.2.1. ENZYMATIC IMMUNOCYTOCHEMISTRY

The cytospin preparations presented in **Fig. 4** are processed for using the APAAP method, followed by counterstaining with hematoxylin.

1. Encircle eosinophil spot with Dako pen to provide a barrier to liquids. Perform each step of incubation by adding around 100 μ L of solution on the spot, and after each incubation or washing step, tap off excess liquid (*see Note 26*).
2. Fix in cold acetone/methanol (1:1 v/v) for 10 min and rehydrate in TBS for 10 min (*see Note 27*).
3. Each incubation step, wash cytopins for 3×10 min in TBS containing 0.1% BSA (*see Notes 28 and 29*).
4. After blocking unspecific sites with TBS + 3% BSA for 30 min, incubate cytopins with unlabeled mouse anti-human cytokine or isotype control monoclonal antibodies at 40 μ g/mL in TBS + 3% BSA overnight at 4°C.
5. Incubate the slides with rabbit anti-mouse immunoglobulins (1:25) in TBS + 3% BSA for 1 h at room temperature, followed by incubation with the APAAP complex (1:40) for 1 h (*see Note 30*).
6. After an additional wash for 2×10 min in TBS, the reaction is developed with New Fuchsin substrate (*see Note 31*).
7. Counterstain slides with Mayer's hematoxylin for 4 min (nuclear blue staining) and apply coverslips with Immumount.

3.3.2.2. IMMUNOFLUORESCENCE

1. After **step 1** (*see Subheading 3.3.2.1.*), fix eosinophil cytopins in 4% paraformaldehyde for 10 min and rehydrate in 0.05 M PBS, pH 7.4 (*see Note 32*).

2. Quench endogenous fluorescence by exposition to UV (3×2 min at 0.12 joules) using a Stratalinker, followed by incubation with 0.1 M glycine for 4 min and incubation with 50 mM NH₄Cl pH 7.4, for 15 min. After each incubation step, wash cytopins for 10 min in PBS under soft agitation (*see Note 33*).
3. To prevent non-specific binding, incubate the slides for 30 min with PBS + 3% BSA supplemented with 5% HSA. After one additional wash in PBS, incubate cytopins with primary antibodies overnight in the fridge in a wet chamber.
4. After washing 2×10 min in PBS + 0.1% BSA, incubate the slides with secondary antibodies in PBS + 3% BSA + 5% HSA for 2 h and then with streptavidin-Texas red in PBS + 3% BSA for 1 h.
5. Perform a final step of washes with PBS + 0.1% BSA + 0.02% Tween 20 for 2×10 min and with PBS for 10 min. Cytopins are mounted with Fluoromount G and analyzed by fluorescence microscopy using an oil immersion objective. Texas red fluorophores require excitation at 543 nm; select the fluorescence emissions wavelengths pass bands between 581 and 621 nm in accordance with the emission spectral analysis.

3.4. Functional Tests

3.4.1. Mediator Release Assays by Chemiluminescence

3.4.1.1. ROS PRODUCTION

1. Pre-incubate purified eosinophils (3×10^5 cells per well) in 25 μ L RPMI 1640 without phenol red in white 96-well plate for 30 min at 37°C in 5% CO₂ (*see Note 34*).
2. Program the luminometer and prewarm it at 37°C.
3. Five minutes prior measurement, prepare luminol solution (0.015 mg/mL) with one 15- μ L aliquot in 3 mL ROS buffer.
4. Add 25 μ L of stimulus solution to the 25 μ L of eosinophil suspension immediately before running measurements in the Victor²TM Wallac luminometer. Measure ROS production immediately by the addition of 50 μ L of luminol (in injector 1) (*see Note 35*). Perform kinetics at 37°C over 60-min time period, and for each time-point, count chemiluminescence for 5 s. Results are expressed in counts per second (CPS).

3.4.1.2. MEASUREMENT OF EPO FOR ASSESSMENT OF DEGRANULATION

1. Incubate purified eosinophils (2×10^5) in 50 μ L RPMI 1640 without phenol red either alone or with 50 μ L stimulus (e.g., rhIL-5 at a final concentration of 20 ng/mL) in 96-well plates at 37°C in 5% CO₂ (final volume of 100 μ L).
2. After 2 h stimulation, centrifuge the plate 10 min at $300 \times g$ and transfer 50 μ L of supernatant in a white 96-well plate. Keep the plate on ice.
3. Prepare the following solutions and keep them on ice in the dark:

- a. Luminol (80- μ L aliquot) and D-luciferin (20- μ L aliquot) in 4 mL EPO buffer.
- b. H_2O_2 solution: 15 μ L of 30% (w/w) H_2O_2 solution (stored in the dark at $+4^\circ C$) in 3 mL EPO buffer.

These solutions should be used immediately and cannot be stored.

4. Program the luminometer.
5. Add the luminol/D-luciferin solution in injector 1 and H_2O_2 solution in injector 2 (see **Note 36**). Start running the protocol: 100 μ L luminol/D-luciferin and 50 μ L H_2O_2 will be added per well (see **Note 37**) to the supernatants. Only one measure is taken and the results are expressed in CPS.

3.4.2. Cytotoxicity Assays

3.4.2.1. CYTOTOXIC ACTIVITY OF EOSINOPHILS TOWARD PARASITIC TARGETS

1. *Schistosoma mansoni* schistosomula are the targets of the cytotoxicity assays and their obtention is based on the transformation of the infective larvae, the cercariae into schistosomula after skin penetration, which is enhanced by light illumination. Schistosomula are prepared according to the skin-penetration procedure: under sterile conditions, shave with caution the abdomen of an anesthetized normal albino OF1 mouse and cut off the shaved skin. Degrease the inner leaflet of this skin sample with a gauze to facilitate the transpenetration of the cercariae, the invading form of *S. mansoni*. Fill the bottom part of a glass collector with Hank's medium. Place then the thin skin sample on the top of the collector (without air bubbles underneath) and assemble the upper part of the chamber. Fix the system with pliers and put the cercaria suspension on the skin. Protect the top of the collector from the light to allow cercariae to be attracted by the light at the bottom and to cross the skin barrier. Incubate the bottom collector for 2 h in a water bath at $37^\circ C$. During skin penetration, cercariae mature to become larvae called schistosomula. After separation of the two parts of the collector, recover then the larvae-containing medium and centrifuge it at $100 \times g$ for 3 min. Keep schistosomula overnight in 96-well flat-bottomed plate at $37^\circ C$, 5% CO_2 , in MEM with 1% normal serum before use in the cytotoxicity assay. They can be kept longer in medium culture. Only schistosomula obtained 3 h after skin penetration are susceptible targets for cytotoxicity (see **Note 38**).
2. Because eosinophils need to be activated by antibodies to become cytotoxic in this ADCC procedure, use unheated serum samples from *S. mansoni*-infected patients (IgE-rich) as a source of IgE antibodies (see **Note 39**).
3. For IgE-mediated eosinophil-dependent cytotoxicity, add 50 μ L of the schistosomula suspension containing 50 targets (1000/mL) in MEM supplemented with 1% heat-inactivated FCS in flat-bottomed microtiter plates. Then add 50 μ L of unheated serum from *S. mansoni*-infected patients or normal subjects as controls at a final dilution of 1:32 together with 100 μ L of effector cells at an effector/target ratio of 5000/1 (see **Note 40**) (9).

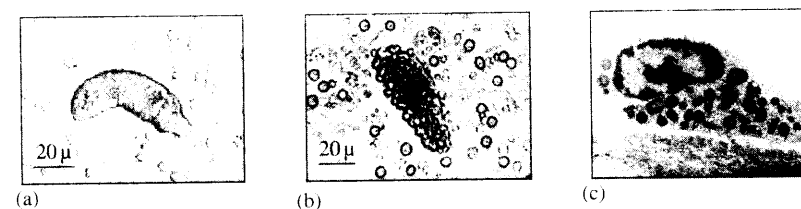


Fig. 5. Eosinophil cytotoxicity toward *Schistosoma mansoni* schistosomula. (A) Living schistosomule. (B) After 48-h coculture, eosinophils firmly adhered to schistosomula. Photographs by light microscopy ($\times 100$). (C) Adhesion of an hypodense eosinophil to schistosomula by electron microscopy.

4. Evaluate the percentage of schistosomula with adherent eosinophils and the percentage of cytotoxicity (dead schistosomula) by optical microscopic examination after 24- and 48-h culture at $37^\circ C$, 5% CO_2 . After 24 h, eosinophil adherence to schistosomula can be easily observed and quantified. After 48 h, the percentage of dead schistosomula can be counted (see **Fig. 5**). No additional staining is required to differentiate dead or alive schistosomula, because live parasites are transparent and dead larvae are becoming black and are surrounded by numerous adherent eosinophils.

3.4.2.2. CYTOTOXIC ACTIVITY OF EOSINOPHILS TOWARD TUMOR CELL LINES

1. Determine eosinophil-mediated cytotoxicity against tumor cell lines by using PKH-26 and Annexin V-FITC stainings (see **Note 41**).
2. After overnight culture in complete medium, test for eosinophils' (effector cells; E) cytotoxicity against carcinoma cell lines (target cells; T). Culture eosinophils in 96-well U-bottom plates at 2×10^6 per mL (4×10^5 eosinophils per well).
3. Stain target cells with 10 μ M PKH-26 according to the manufacturer's instructions. Perform all steps at room temperature. Place 2×10^6 cells in a conical bottom polypropylene tube and wash once in PBS. After centrifugation for 5 min at $350 \times g$, carefully aspirate the supernatant leaving no more than 25 μ L on the cell pellet. Resuspend the cells at 10^7 per mL in diluent C (supplied with the kit), pipetting to ensure complete dispersion. Do not vortex. Prior to staining, prepare a 20 μ M PKH-26 dye solution using diluent C. Add an equal volume of this PKH-26 solution to the cell suspension and immediately mix by pipetting. Incubate for 5 min. Stop the reaction by adding an equal volume of FCS. After 1 min incubation, dilute with 10 mL PBS. Centrifuge at $400 \times g$ for 10 min (see **Note 42**). After three washes, resuspend stained cells in complete medium and add the target cells (1.6×10^4 per well) to the plate containing eosinophils (E:T ratio of 25:1).
4. After 6-h coculture, measure apoptotic cell death by FCM using FITC-conjugated Annexin V. Recover the cells from the plate and wash the samples twice with cold

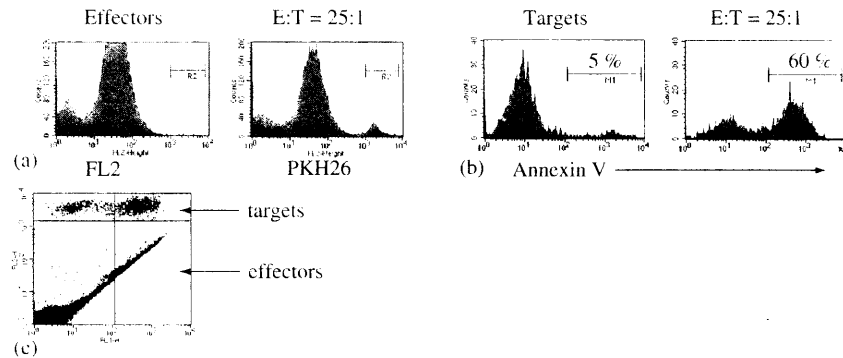


Fig. 6. Eosinophil tumoricidal activity after 6-h coculture with Colo-205. (A) FL2 fluorescence of effectors (eosinophils) and effectors plus targets cells. A high PKH26 staining of targets allows to gate them on R2 (FL2 high cells). (B) Annexin V-fluorescein isothiocyanate (FITC) staining (FL1) on PKH26+ cells, gated on R2. Eosinophil coculture induces apoptosis of Colo-205 (55%). (C) Dot plot representation, not gated. Observe the good separation of the cell populations. Histograms and dot plot are obtained using a FACSCalibur equipped with the CellQuest software.

PBS. Resuspend cells in binding buffer at 2×10^6 /mL and incubate with Annexin V-FITC (5 μ L per test) for 15 min at room temperature in the dark (see Note 43). Analyze by FCM as soon as possible (within 1 h).

5. Acquisition step: A total of 5000 PKH-26+ cells are usually acquired per experimental condition and analyzed on a FACSCaliburTM using the CellQuestTM software. Eosinophil-mediated cytotoxicity against tumor cells is determined by evaluation of the number of PKH-26+ Annexin V+ cells. A representative experiment performed with a colon carcinoma cell line is shown in Fig. 6.

4. Notes

1. Metrizamide is not autoclavable; therefore the solution has to be sterilized by filtration. This is an important point because metrizamide solutions are susceptible to bacterial degradation.
2. Percoll has to be used at room temperature to avoid errors of measurement caused by volume retraction. The coefficient 1.7 used in the formula comes from the following equation: $(d_0 - 1)/(d - 0.1d_{10} - 0.9)$, where d_0 = density of Percoll undiluted (1.13 g/mL); d = desired density of final working solution (1.082 g/mL); and d_{10} = density of 1.5 M NaCl (1.058).
3. Detection of EPO by ICC using DAB staining and by FCM are two complementary protocols: DAB solution allows one to detect only mature EPO whereas anti-EPO

antibody used for FCM can detect mature and immature forms of the eosinophil peroxidase.

4. The use of RPMI 1640 without phenol red, rather than PBS, allows an optimal activation of eosinophils. Neither FCS nor BSA should be present in the medium because these proteins give a high background in chemiluminescence measurements.
5. Contrary to D-luciferin obtained from Sigma, we have found D-luciferin from Roche Diagnostics to be excellent for measurement of eosinophil peroxidase release.
6. This protocol has the advantage of detecting general synthesis of ROS (superoxide, oxygen radicals, and peroxide), and of not being restricted to superoxide anion generation as are methods based on cytochrome c (27).
7. Dextran sedimentation step allows removing of erythrocytes, platelets, and some small lymphocytes. This step stands generally for around 20–30 min.
8. The use of DNase is important to remove all dead cells from the cell suspension before performing the metrizamide separation.
9. Use polystyrene tube (transparent) and not polypropylene tube.
10. Metrizamide is a slightly hypertonic medium and can alter the cells, so high-speed centrifugation is needed to remove all the metrizamide.
11. There is a cell density variation with the physiological state of the eosinophils (28). In normal donors, eosinophils are found mainly in layer 4 and a few in layer 3. By contrast, in eosinophilic donors, hypodense eosinophils are principally found in layers 1 and 2.
12. Percoll solution has to be used at room temperature for good separation of polynuclear and mononuclear cells. Ficoll solution ($d = 1.077$ g/mL) has also been tested and compared with Percoll ($d = 1.082$ g/mL). Ficoll is less efficient because more contaminating lymphocytes are found in the polynuclear cell pellets.
13. To avoid lymphocyte or monocyte contamination, we recommend very careful removal of the PBMC layer without touching the tube wall.
14. It has been observed that the type of buffer used for erythrocyte lysis can influence some functional studies, as shown in mouse (1). For example, compared with NaCl lysis, the use of ammonium chloride buffer can interfere with mouse eosinophil antigen-presentation activity, because ammonium chloride is an inhibitor of lysosome acidification needed for antigen presentation.
15. Anti-human CD16-coated magnetic beads are used for negative selection to separate neutrophils from eosinophils, which do not usually express CD16. A subpopulation of CD16+ eosinophils can, however, be found in some patients (29). The proportion of this minor population can differ between patients.
16. Compared with metrizamide, Percoll (not hypertonic) is less toxic for the cells. Percoll gradients and negative selection by anti-CD16 is routinely used because this procedure allows one to increase the yield and the degree of purity and is faster than metrizamide gradients. It leads, however, to the obtention of the whole

- eosinophil population and does not allow the comparison of normodense and hypodense activated eosinophils.
17. For peritoneal cells, no lysing step is necessary as no erythrocytes are found in the peritoneal exudate.
 18. For higher cell purity (95–98%), rat eosinophils can be obtained by sorting peritoneal cells according to their typical forward and side scatter.
 19. We have noticed that culture yield is increased when RPMI without phenol red is used when making complete medium.
 20. During all the procedure, do not vortex eosinophils. Just shake tubes or tap plates to avoid stress and activation of the cells.
 21. For mouse eosinophils, it is absolutely necessary to block Fc receptors by anti-Fc γ receptor antibody (2.4.G2) before primary antibody incubation.
 22. We recommend using directly-labeled monoclonal antibodies for eosinophil membrane receptor detection as we have noticed that weakly expressed receptors are usually not detected by indirect staining (21).
 23. Do not fix the eosinophil samples with paraformaldehyde for further analysis, rather leave them on ice and analyze them within 2 h. We have observed staining alterations after fixation.
 24. An important step is blocking of non-specific binding by serum pre-incubation particularly for intracellular staining. Although a blocking step is not really necessary for membrane staining on freshly purified eosinophils, one does need to use blocking after overnight culture, otherwise you might observe a second fluorescent peak.
 25. It could be interesting to compare membrane and intracellular staining on freshly purified eosinophils and after overnight culture. This will allow one to observe whether expression or increased expression of molecule of interest at the plasma membrane correlates with decreased expression of the intracellular pool, or whether culture allows neo-synthesis of the protein with, or without, membrane expression. Eosinophils are able to express several receptors or cytokines and to store them in intracellular pools. Very often intracellular expression is higher than membrane expression (21).
 26. The acquisition step has to be performed the day of the experiment because eosinophils, contrary to lymphocytes, cannot be fixed for overnight analyses.
 27. Avoid drying the spot during all the procedure. If you have to suspend the protocol for a moment, add TBS onto the cells. Ready-to-use degreased microscope glass slides are needed.
 28. By performing fixation in acetone/methanol, one can keep the cytopsin preparations for 1 year at -20°C . For this procedure, let dry the cytopsins in a hood after fixation and freeze individually in aluminium. When needed, let thaw at room temperature for 30 min and then rehydrate in 0.05 M TBS, pH 7.4, for 15 min.
 29. TBS is a suitable diluent for this protocol. Phosphate buffers must be avoided as phosphate is a substrate for alkaline phosphatase and, therefore, competes with the

- chromogenic substrate. Additionally, phosphate in the dilution buffer for APAAP will form an insoluble precipitate with magnesium.
30. Perform each step of washing in a bath containing around 200 mL of TBS + 0.1% BSA under gentle agitation.
 31. The APAAP method consists of using soluble immune complexes of calf intestinal alkaline phosphatase and anti-alkaline phosphatase. This method is preferred for staining eosinophils, as this leukocyte cell type is rich in endogenous peroxidase and furthermore it adds an amplification step (alkaline phosphatase–anti-alkaline phosphatase).
 32. One can increase the intensity of the reaction by performing a second round of APAAP reaction. It is the case for interferon- γ and IL-10 detection, presented in Fig. 4C.
 33. Contrary to the intracellular procedure, after fixation in paraformaldehyde for membrane staining, one cannot freeze the cytopsins. The fixed cytopsin preparations can, however, be stored for 1 week at 4°C in a humid chamber.
 34. Unstained human eosinophils exhibit marked autofluorescence in comparison with other leukocytes due to granule-associated fluorescent substances. Eosinophil endogenous fluorescence is more pronounced in green than in red wavelengths. Ultraviolet exposition and glycine and NH_4Cl incubations are required for quenching the autofluorescence. Add 100 μL of PBS during UV exposition to avoid drying spot.
 35. We noted that, before activation, a pre-incubation (30 min) of eosinophils with serum-free medium effectively decreases cellular stress and background.
 36. Use of injectors allows one to perform a homogenous and synchronous addition of luminol, D-luciferin, or H_2O_2 solutions directly in the wells of the plate and to start measurements rapidly.
 37. To avoid the degradation of EPO in supernatants and the loss of signal, the measure of chemiluminescence must be taken immediately. Keep supernatants and solutions on ice until measurement. Here, the released peroxidase catalyzes the oxidation of luminol by H_2O_2 , which gives the excited molecule 3-aminophthalate. As the reactant H_2O_2 is added in excess, photons collected are proportional to the quantity of catalyst, which is EPO. The reaction is amplified by D-luciferin.
 38. Mouse strain: OF1. Avoid taking young mice with too thin skin. Be careful during skin cleaning to avoid making holes in sample that could allow passive passage of cercaria. Use first sterile gauze soaked with culture medium to put out skin muscles and fatty tissues and then wipe with dry cotton.
 39. Unheated sera from infected patients have to be used to allow evaluation of IgE-dependent cytotoxicity (heat-labile).
 40. Effector cells are hypodense eosinophils, purified by metrizamide discontinuous gradients. The cytotoxic activity of eosinophils purified from hypereosinophilic patients is increased when compared with eosinophils from normal donors. Moreover, there is a heterogeneity of eosinophil cytotoxic function among

different subpopulations of eosinophils purified from the same patient; hypodense eosinophils are more cytotoxic than normodense eosinophils (28).

41. Annexin V-FITC is used to quantitatively determine the percentage of cells undergoing apoptosis, by detecting the phosphatidylserine (PS) exposed on the cell membrane of apoptotic cells. In the early phases of apoptosis, PS translocates from the inner leaflet of the plasma membrane to the outer leaflet.
42. PKH26 staining is a primordial parameter for this experiment. High and homogenous PKH26 staining allows a good discrimination of the two cell populations and consequently a quantification of the percentage of apoptotic targets, by gating on R2. As one can see in **Fig. 6A and B**, eosinophil fluorescence in FL2 channel does not interfere with FL2 fluorescence of stained targets.
43. Because of the Ca^{2+} -dependent affinity of Annexin V for PS, the cell staining with FITC-conjugated Annexin V has to be performed in a Ca^{2+} -enriched binding buffer.

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