

Technical Note

A simple method for the in situ detection of eosinophils ☆

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Abstract

A simple and rapid method for identifying eosinophils in tissue sections is described. The assay is based on the incubation of frozen tissue sections with phenol red and the detection of cellular fluorescence. A one-to-one relationship was observed between cells exhibiting fluorescence following exposure to phenol red and eosinophils as identified by histochemical detection of eosinophil peroxidase (EPO) activity. Using the phenol red assay, eosinophils were detected in various eosinophil-infiltrated tissues, including uteri of rats from day 1 of pregnancy and uteri of prepubertal estrogen-treated rats. Intensity of the fluorescing eosinophils was dependent upon phenol red concentration and duration of incubation. The phenol red method of eosinophil identification was disrupted by co-incubation with resorcinol, an EPO inhibitor, or catalase, a hydrogen peroxide scavenger. The merits of this assay are its simplicity and compatibility with other procedures, such as histochemistry, immunocytochemistry, and in situ ligand–receptor binding. © 2002 Elsevier Science B.V. All rights reserved.

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Eosinophils are components of inflammatory responses and participate in immune surveillance against parasitic infections and solid tumors (Gleich, 2000). Eosinophils selectively infiltrate hormonally responsive tissues, such as the uterus and diseased tissues to exert their function. A variety of techniques have been employed to identify eosinophils, such as: size, nuclear morphology, and affinity for eosin (Gleich et al.,

1993), intracellular cyanide-resistant EPO activity (Horton et al., 1996), and surface expression of the C–C chemokine receptor-3 (CCR3; Grimaldi et al., 1999). In this report, we describe a simple and rapid eosinophil detection method utilizing phenol red that is compatible with other procedures, such as histochemistry, immunocytochemistry, and in situ ligand–receptor binding.

Uterine tissue sections (10 µm) obtained from rats on day 1 of pregnancy were incubated with Hank's balanced salt solution (HBSS; control) or HBSS containing 56 µM phenol red (0.002%, concentration commonly used in tissue culture medium) for 2 h at room temperature. Following incubation, the sections were washed extensively with HBBS and briefly with water. Tissue sections were then examined using an inverted Diaphot Nikon microscope equipped with

Abbreviations: HBSS, Hank's balanced salt solution; EPO, Eosinophil peroxidase.

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100 W Hg arc lamp and FITC–Texas red filter. Incubation of uterine sections with HBSS containing 56 μM phenol red resulted in fluorescence in a population of cells (Fig. 1, Panel B). Specificity of the phenol red interaction with uterine cells was further demonstrated by using HBSS in the absence of phenol red (Fig. 1, Panel E). Uterine cells showing fluorescence were similar to previous reports on the distribution of uterine eosinophils (Rytomaa, 1960; Bassett, 1962; Ross and Klebanoff, 1966; McMaster et al., 1992). The relationship between cells showing phenol red-dependent fluorescence and eosinophils was clarified by the cyanide-resistant eosinophil peroxidase (EPO) assay. Following incubation with HBSS containing phenol red or HBSS, the cyanide-resistant (8 mM KCN) EPO assay was performed on the same tissue section as previously described (Horton et al., 1996). Panels A and D of Fig. 1 show eosinophil-associated EPO staining in tissue sections that were previously incubated with or without phenol red.

Tissue sections dually processed for phenol red-dependent fluorescence and EPO activity showed that phenol red-dependent fluorescence and EPO are co-localized (Fig. 1, Panel C).

The specific association of phenol red-dependent fluorescence with eosinophils was further demonstrated by examining phenol red-dependent fluorescence in the uterus of estrogen-treated prepubertal rats and vehicle-treated control prepubertal rats. Estrogen is a known stimulator of uterine eosinophil infiltration (Bjersing and Borglin, 1964; King et al., 1981; Lee et al., 1989). Prepubertal rats (day 20 postnatal) were subcutaneously injected daily for 3 days with estradiol valerate (100 $\mu\text{g}/100 \mu\text{l}$; Bristol-Myers Squibb, Princeton, NJ) or vehicle (sesame oil). Twenty-four hours following the last injection, rats were sacrificed, uterine tissues dissected and frozen in dry-ice cooled heptane for subsequent analysis. Cells with phenol red-dependent fluorescence and possessing EPO activity dramatically increased in the uteri from the es-

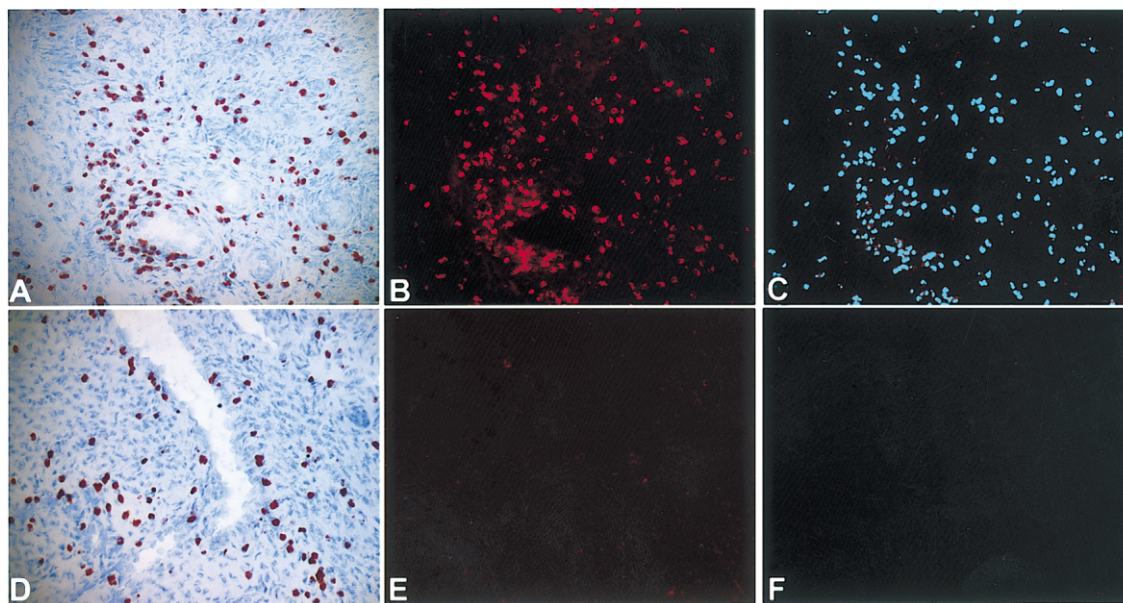


Fig. 1. Co-localization of phenol red-dependent cellular fluorescence and EPO in uterine tissues from day 1 pregnant rats. Uterine tissue sections were incubated with HBSS containing 56 μM phenol red (Panels A–C) or HBSS without phenol red (Panels D–F) for 2 h at room temperature. Following incubation, the sections were washed extensively with HBSS and briefly with water. Cyanide-resistant EPO was detected by histochemistry (Panels A and D). Tissue sections were then examined using an inverted Diaphot Nikon microscope equipped with 100 W Hg arc lamp and FITC–Texas red cube filter. Dual localization of phenol red-dependent fluorescence and EPO activity is shown in Panels C and F and is depicted as blue-staining cells. Please note the 1:1 relationship between phenol red-dependent fluorescence and EPO activity.

tradiol-treated rats versus the uteri of vehicle-treated control rats (Fig. 2). Reactivity of phenol red with tissue eosinophils was not restricted to the rat. Eosinophils from mouse tissues (lung and uterus) exhibited similar reactivity with phenol red (data not shown).

Relative fluorescence intensity of cells was estimated using image analysis software Optimas[®] 5.0 (Meida Cybernetics, Bothell, WA). In all experiments, a minimum of three different tissue samples was examined from three different animals and at least 100 cells were included in each analysis. Fluorescence intensity was directly correlated with phenol red concentration (Fig. 3A). The temporal profile of phenol red-dependent fluorescence in eosinophils was evaluated. Phenol red-dependent fluorescence in eosinophils increased as a function of incubation time, approaching saturation at 2 h (Fig. 3B).

To investigate the underlying mechanism regulating the phenol red-dependent fluorescence in eosinophils, an inhibitor of EPO and a hydrogen peroxide

scavenger were used. Resorcinol, a potent inhibitor of EPO (Schneider and Issekutz, 1996), inhibited phenol red-dependent fluorescence in eosinophils in a dose-dependent manner (Fig. 4A). Addition of catalase, a hydrogen peroxide scavenger (Fridovich, 1999), at concentrations ranging from 10 to 2500 U/ml resulted in a decrease in phenol red-dependent fluorescence in eosinophils (Fig. 4B). The catalase inhibition of phenol red-dependent eosinophil fluorescence on tissue sections from the uterus of the day 1 pregnant rat is shown in Fig. 5. These findings suggest that phenol red-dependent eosinophil fluorescence is influenced by the presence of hydrogen peroxide.

In the preceding experiments, we demonstrated that the phenol red method of eosinophil identification is compatible with a histochemical assay for EPO. In the following experiments, we evaluated whether the phenol red method is compatible with other *in situ* methods designed to evaluate eosinophil behavior. Eosinophil behavior was assessed by expression of the F4/80 surface antigen, a marker of both monocyte and eosi-

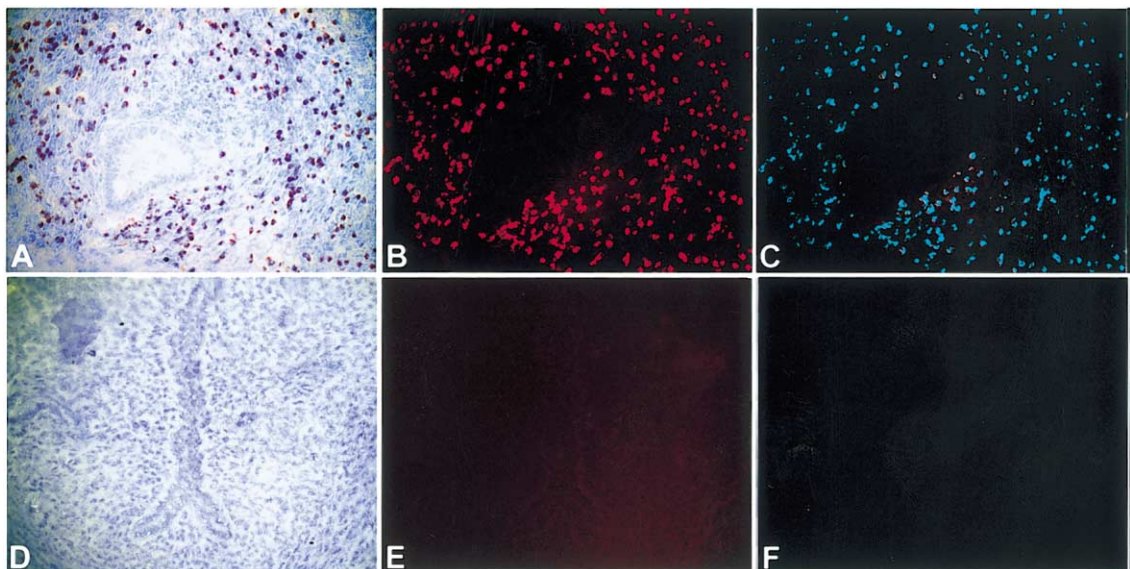


Fig. 2. Co-localization of phenol red-dependent cellular fluorescence and EPO in uterine tissues from estradiol (Panels A–C) or oil (Panels D–F) treated prepubertal rats. Uterine tissue sections were incubated with HBSS containing 56 μ M phenol red for 2 h at room temperature. Following incubation, the sections were washed extensively with HBBS and briefly with water. Cyanide-resistant EPO was detected by histochemistry (Panels A and D). Phenol red-dependent fluorescence was examined as described in the legend for Fig. 1 (Panels B and E). Dual localization of phenol red-dependent fluorescence and EPO activity is shown in Panels C and F and is depicted as blue-staining cells. Please note the 1:1 relationship between phenol red-dependent fluorescence and EPO activity.

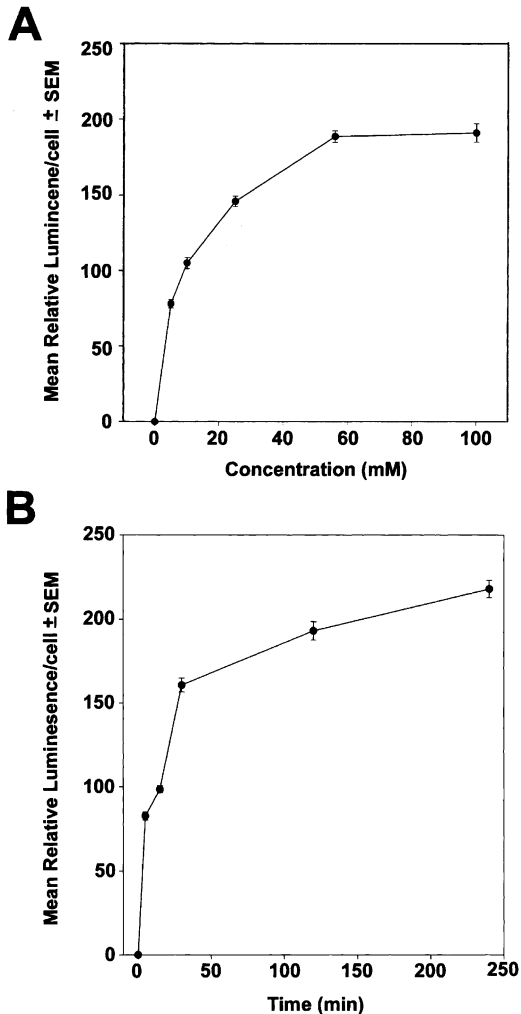


Fig. 3. Effect of phenol red concentration (A) and time (B) on eosinophil-associated fluorescence. (Panel A) Uterine tissue sections (day 1 of pregnancy) were incubated with HBSS containing 0–100 μ M phenol red for 2 h at room temperature. Following incubation, the sections were washed extensively with HBBS and briefly with water. Relative fluorescence intensity per cell was measured using image analysis software, Optimas[®] 5.0. A minimum of three different tissue samples was examined from three different animals and at least 100 cells were used for each concentration tested. (Panel B) Uterine tissue sections were incubated with HBSS containing 56 μ M phenol red for various time intervals (0–4 h) at room temperature and relative fluorescence was estimated as described for Panel A.

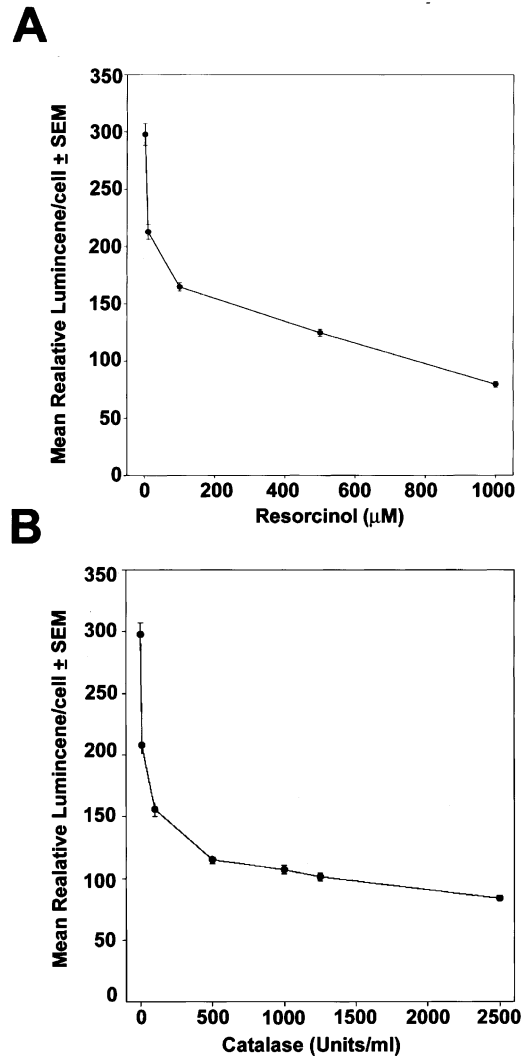


Fig. 4. Inhibition of phenol red-dependent fluorescence intensity per eosinophil by resorcinol (A) and catalase (B). Uterine tissue sections (rat, day 1 of pregnancy) were co-incubated with HBSS containing 56 μ M phenol red and either resorcinol (0–1000 μ M; A) or catalase (0–2500 U/ml; B) for 2 h at room temperature. Following incubation, sections were washed extensively with HBBS and briefly with water. Tissue sections were then examined as described in the legend for Fig. 3.

nophil lineages (Austyn and Gordon, 1981; McGarry and Stewart, 1991), and decidual/trophoblast prolactin-related protein (d/tPRP) binding, a ligand for eosinophils (Wang et al., 2000). Frozen rat uterine tissue sections from day 1 of pregnancy were pre-incubated

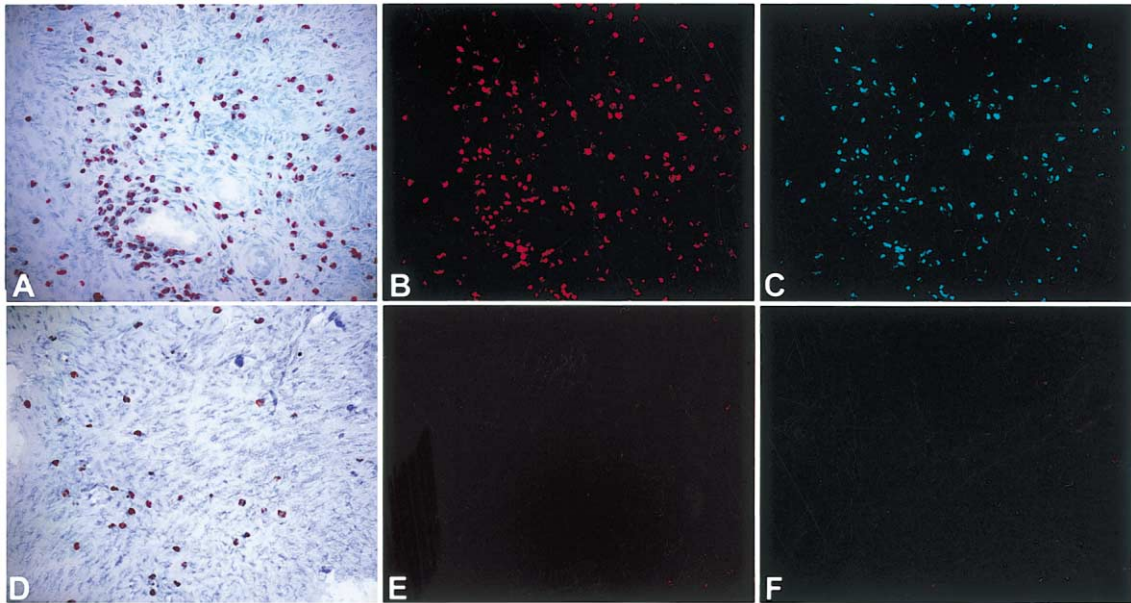


Fig. 5. Catalase inhibition of phenol red-dependent fluorescence of eosinophils. Uterine tissue sections (rat, day 1 of pregnancy) were incubated with HBSS containing 56 μM phenol red (Panels A–C) or with HBSS containing 56 μM phenol red and 2500 U/ml of catalase (Panels D–F) for 2 h at room temperature. Following incubation, the sections were washed extensively with HBBS and briefly with water. Cyanide-resistant EPO was detected by histochemistry (Panels A and D). Phenol red-dependent fluorescence was examined as described in the legend for Fig. 1 (Panels B and E). Dual localization of phenol red-dependent fluorescence and EPO activity is shown in Panels C and F and is depicted as blue-staining cells. Please note that catalase inhibited phenol red-dependent cellular fluorescence. EPO activity was unaffected because catalase was washed out before EPO assay.

with phenol red (56 μM) before F4/80 immunocytochemistry or concurrently in an in situ ligand–receptor binding assay using an alkaline phosphatase-d/tPRP fusion protein (AP-d/tPRP; Wang et al., 2000). A subset of F4/80 positive cells was positive for phenol red-dependent fluorescence consistent with the known reactivities of F4/80 antibodies with both monocytes and eosinophils (Fig. 6, Panels A–C; McGarry and Stewart, 1991). AP-d/tPRP binding exhibited a 1:1 relationship with cells displaying phenol red-dependent fluorescence (Fig. 6, Panels D–F). The phenol red assay for detecting tissue eosinophils was not compatible with certain fixatives, including, Bouin's and formalin, nor was it compatible with paraffin processing, presumably due to the inactivation of the enzyme(s) required for the phenol red-associated fluorescence.

In conclusion, we have described a simple and effective method for the in situ identification of tissue eosinophils. The method is compatible with other

procedures for evaluating the function of eosinophils and should prove to be useful in future analyses of eosinophils in physiological and pathological states.

References

- Austyn, J.M., Gordon, S., 1981. F4/80: a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* 11, 805–811.
- Bassett, E.G., 1962. Infiltration of eosinophils into the modified connective tissue of oestrous and pregnant animals. *Nature* 194, 1259–1261.
- Bjersing, L., Borglin, N.E., 1964. Effect of hormones on incidence of uterine eosinophilia in rats. *Acta Pathol., Microbiol. Scand.* 60, 353–364.
- Fridovich, I., 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. N.Y. Acad. Sci.* 893, 13–18.
- Gleich, G.J., 2000. Mechanisms of eosinophil-associated inflammation. *J. Allergy Clin. Immunol.* 105, 651–663.
- Gleich, G.J., Adolphson, C.R., Leiferman, K.M., 1993. The biology of the eosinophilic leukocyte. *Annu. Rev. Med.* 44, 85–101.

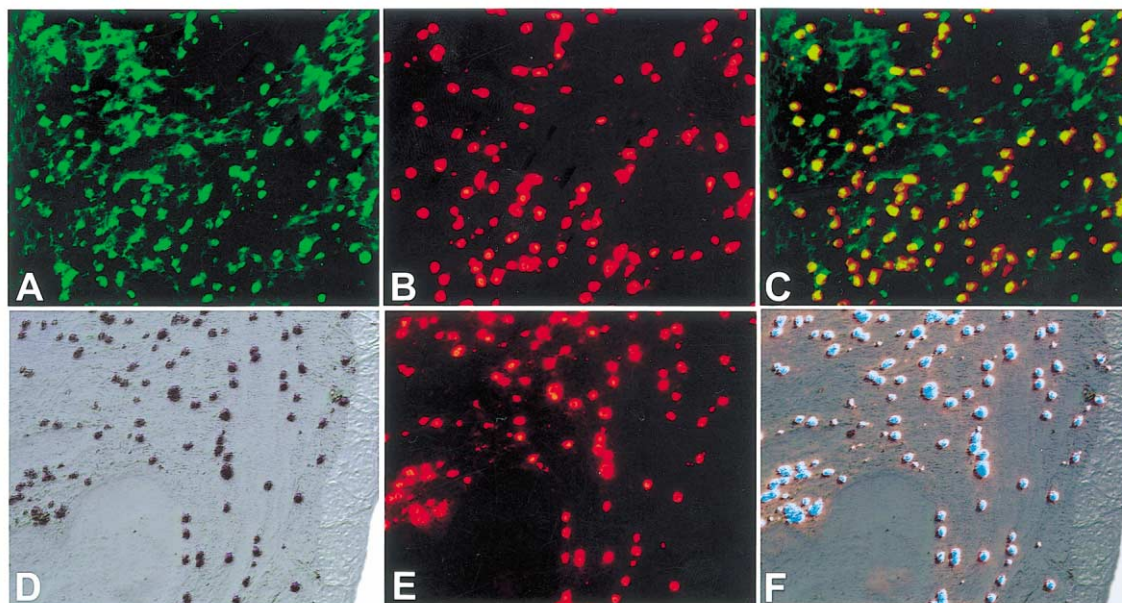


Fig. 6. Compatibility of the phenol red method for detecting eosinophils with other methods for detection of eosinophil behavior. Uterine tissue sections (mouse, day 1 of pregnancy) were incubated with HBSS containing 56 μ M phenol red for 2 h at room temperature. Following incubation, the sections were washed extensively with HBBS and briefly with water and the same tissue section was then processed for either indirect immunofluorescence with F4/80 antibody and FITC-conjugated secondary antibody or AP-d/tPRP binding. F4/80 immunofluorescence detects both monocytes and eosinophils (Panel A), whereas phenol red method detected only eosinophils (Panel B). Dual localization of both F4/80 immunoreactive cells and phenol red-dependent fluorescing cells (shown as cells staining yellow; Panel C). AP-d/tPRP binding (Panel D) exhibited a 1:1 relationship with cells displaying phenol red-dependent fluorescence (Panel E). The dual localization of both AP-d/tPRP and phenol red-dependent fluorescence is shown in Panel F and is depicted as blue-staining cells.

- Grimaldi, J.C., Yu, N.X., Grunig, G., Seymour, B.W., Cottrez, F., Robinson, D.S., Hosken, N., Ferlin, W.C., Wu, X., Soto, H., O'Garra, A., Howard, M.C., Coffman, R.L., 1999. Depletion of eosinophils in mice through the use of antibodies specific for C-C chemokine receptor 3 (CCR3). *J. Leukocyte Biol.* 65, 846–853.
- Horton, M.A., Larson, K.A., Lee, J.J., Lee, N.A., 1996. Cloning of the murine eosinophil peroxidase gene (mEPO): characterization of a conserved subgroup of mammalian hematopoietic peroxidases. *J. Leukocyte Biol.* 60, 285–294.
- King, W.J., Allen, T.C., DeSombre, E.R., 1981. Localization of uterine peroxidase activity in estrogen-treated rats. *Biol. Reprod.* 25, 859–870.
- Lee, Y.H., Howe, R.S., Sha, S.-J., Teuscher, C., Sheehan, D.M., Lyttle, C.R., 1989. Estrogen regulation of an eosinophil chemotactic factor in the immature rat uterus. *Endocrinology* 125, 3022–3028.
- McGarry, M.P., Stewart, C.C., 1991. Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80. *J. Leukocyte Biol.* 50, 471–478.
- McMaster, M.T., Newton, R.C., Dey, S.K., Andrews, G.K., 1992. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. *J. Immunol.* 148, 1699–1705.
- Ross, R., Klebanoff, S.J., 1966. The eosinophilic leukocyte. Fine structure studies of changes in the uterus during the estrous cycle. *J. Exp. Med.* 124, 653–659.
- Rytomaa, T., 1960. Organ distribution and histochemical properties of eosinophil granulocytes in rat. *Acta Pathol., Microbiol. Scand., Suppl.* 140, 11–118.
- Schneider, T., Issekutz, A.C., 1996. Quantitation of eosinophil and neutrophil infiltration into rat lung by specific assays for eosinophil peroxidase and myeloperoxidase. Application in a Brown Norway rat model of allergic pulmonary inflammation. *J. Immunol. Methods* 198, 1–14.
- Wang, D., Ishimura, R., Walia, D.S., Müller, H., Dai, G., Hunt, J.S., Lee, N.A., Lee, J.J., Soares, M.J., 2000. Eosinophils are cellular targets of a novel uteroplacental heparin-binding cytokine, decidual/trophoblast prolactin-related protein. *J. Endocrinol.* 167, 15–29.