In vitro evaluation of the effect of a novel immunosuppressive agent, FTY720, on the function of feline neutrophils

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Objective—To use in vitro assays to evaluate the effects of a novel immunosuppressive agent, FTY720, on biological functions (migration, phagocytosis, and production of reactive-oxygen species [ROS]) of feline peripheral neutrophils and determine the cytotoxic effects of FTY720 on feline peripheral neutrophils.

Sample Population—Peripheral neutrophils obtained from 8 healthy cats.

Procedure—Peripheral neutrophils were isolated from blood samples obtained from the 8 cats and exposed to the phosphorylated form of FTY720 (FTY720-P). A fluorescence-based in vitro evaluation of migration was performed. Phagocytosis of microbes and production of ROS were evaluated by use of a 2-color flow cytometry system. Samples of whole blood obtained from the cats were incubated with various concentrations of FTY720-P, fluorescein-labeled *Staphylococcus aureus*, and dihydroethidium. Cytotoxic effects were evaluated by use of propidium iodide staining.

Results—Addition of FTY720-P caused a slight nonsignificant decrease in phagocytosis and production of ROS by feline peripheral neutrophils. Migration activity of feline peripheral neutrophils was significantly increased by the addition of FTY720-P. Addition of FTY720-P at concentrations considered for clinical use did not increase the death rate of feline peripheral neutrophils.

Conclusions and Clinical Relevance—FTY720 does not inhibit critical functions of feline peripheral neutrophils in vitro. (*Am J Vet Res* 2006;67:588–592).

A novel immunosuppressive agent, FTY720, is a synthetic structural analogue of a fungal metabolite (myriocin). Studies[1,2] have revealed that the administration of FTY720 prolongs allograft survival by depleting lymphocytes in peripheral blood samples. When administered SC, FTY720 can reverse ongoing allograft rejection in dogs.[3] In addition, FTY720 has synergistic interactions with CsA or rapamycin (or both) to prolong allograft survival in nonhuman primates.[4] It has been reported[5] that phase III clinical trials of the use of FTY720 in humans undergoing renal transplantation are currently being conducted.

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Abbreviations

CsA	Cyclosporine A
FTY720-P	Phosphorylated form of FTY720
ROS	Reactive-oxygen species

Administration of FTY720 induces depletion of peripheral blood lymphocytes (up to 90%) as a result of lymphocyte migration to, and retention in, secondary lymphoid tissues.[3] In vivo, FTY720 is converted to FTY720-P through the actions of sphingosine kinase; FTY720-P then binds to sphingosine 1–phosphate receptors. Saturation of syphingosine 1–phosphate receptors on lymphocytes by FTY720-P inhibits lymphocytes from leaving the lymph nodes.[1] Furthermore, FTY720 influences lymphocyte migration without influencing cellular functions, such as proliferation of lymphocytes and production of cytokines. Therefore, immune function can be rapidly restored after cessation of FTY720 treatment.[1,3]

Because of the minimal adverse effects and wide margin of safety in research animals and humans,[2,6] FTY720 has the potential to be a safe and effective agent for the maintenance of cats with renal allografts. In addition to the dramatic depletion of lymphocytes from the blood, the authors have observed a severe depletion in the number of neutrophils in peripheral blood samples after oral administration of FTY720 to cats. In our experience, peripheral blood neutrophils were depleted by 70% after oral administration of a single low dose (0.05 mg/kg) of FTY720, whereas oral administration of a single high dose (1 mg/kg) of FTY720 prolonged the depletion of peripheral blood neutrophils for > 20 days. To our knowledge, such an FTY720-induced depletion of peripheral blood neutrophils has not been reported in other species. Because FTY720 has not had any effects on myeloid-lineage blood cells in other species, there is no information about the influence of FTY720 on the biological functions of neutrophils. Depletion of neutrophils may expose treated cats to a greater risk of infection. Therefore, it is necessary to understand the effects of FTY720 on the function of feline neutrophils before it can be used clinically to control rejection after kidney transplantation in cats. Migration, phagocytosis, and production of ROS are critical functions of neutrophils in the inflammatory response.

The objectives of the study reported here were to evaluate the effects of FTY720 on biological functions of feline peripheral neutrophils and determine the cytotoxic effects of FTY720 on feline peripheral neutrophils. We hypothesized that the biological functions of feline peripheral blood neutrophils would not be affected by exposure to FTY720.

Materials and Methods

Sample population—Venous blood samples were obtained from 8 cats. Samples (5 mL) were collected into heparin-coated (10 U of heparin/mL of blood) syringes and kept on a rotator. The study was approved by an institutional animal care and use committee.

Experimental design—Cats are easily stressed by physical manipulations, and blood cell homeostasis of cats is highly influenced by psychologic stress. Therefore, we designed a series of in vitro tests to evaluate migrating ability, phagocytosis, and production of ROS by feline peripheral neutrophils in response to exposure to FTY720.

On the basis of preliminary pharmacokinetic and pharmacodynamic studies of in vivo blood concentrations of FTY720 in cats, we selected 3 concentrations of FTY720-P[a] for evaluation in the in vitro study reported here. We selected 0.04µM FTY720-P because it is equal to the maximum concentration of FTY720-P in the blood of cats after a single oral administration of FTY720 at a dosage of 0.05 mg/kg and 0.2µM FTY720-P because it is equal to the maximum concentration of FTY720-P in the blood of cats after a single oral administration of FTY720 at a dosage of 0.3 mg/kg; 8µM FTY720-P was selected as a high dose.

Phagocytosis and production of ROS—Phagocytosis of microbes and production of ROS were evaluated by use of a 2-color flow cytometry system.[7,8] N-ethyl-maleinacidamide[b] was used as a negative control sample for phagocytosis. Phorbol myristate acetate[c] was used to stimulate the production of ROS and served as a positive control sample, whereas PBS solution was used as a negative control sample for ROS production. Cells in whole blood were incubated with an oxidative sensitive dye, dihydroethidium,[d] at 37°C for 10 minutes. Various concentrations of FTY720-P (0 [vehicle], 0.04, 0.2, and 8µM) were added, and cells were then incubated at 37°C for 15 minutes. An aliquot (90 µL) of whole blood treated with dihydroethidium and FTY720-P was transferred to a 5-mL polystyrene tube,[e] and 25 µL of fluorescein isothiocyanate–labeled *Staphylococcus aureus*[f] was added to stimulate ROS production and to act as a target for phagocytosis. After incubation (with shaking) in a water bath at 37°C for 15 minutes, the process was stopped by the addition of 1M ice-cold N-ethyl-maleinacidamide and use of ice to cool the mixture. Phagocytosis of bacteria was detected by measurement of a green fluorescent signal. Dihydroethidium was oxidized to emit red fluorescence; thus, ROS production was detected by measurement of a red fluorescent signal. Purity of the neutrophils was confirmed by staining with a neutrophil-specific monoclonal antibody and red phycoerythrin–conjugated secondary antibody.[g]

Migrating ability of neutrophils—A fluorescence-based in vitro evaluation of migration was performed by use of fluorescence-labeled feline peripheral blood neutrophils.[9] A gradient system[h] was used to separate lymphocytes from heparinized whole blood. Neutrophils were isolated by lysis of RBCs.[10] Purity and viability of isolated neutrophils were confirmed by performing a differential count (by use of Wright's stain) and a trypan-blue dye exclusion assay. Isolated feline neutrophils were labeled with a fluorescent dye,[i] and an in vitro migration study was performed in a 96-well chemotaxis chamber with a pore size of 3 µm.[j] A yeast-based compound[k] was used to activate feline serum; the activated feline serum served as the chemoattractant for neutrophils. The quantity of migrating cells was measured by use of a fluorescent reader.[l]

Cytotoxic effects—Staining with propidium iodide was used for the evaluation of cytotoxic effects of FTY720 on feline peripheral neutrophils. Propidium iodide is excluded by viable cells; therefore, only dead or dying cells will be penetrated and labeled by propidium iodide. Isolated feline peripheral neutrophils were cultured for 24 or 48 hours with various concentrations of FTY720-P (0 [vehicle], 0.04, 0.2, 4, and 8µM). Two microliters of propidium iodide solution[m] (0.5 mg/mL) was added to each sample at the end of the culture period. The percentage of propidium iodide–labeled cells was determined by measurement of red fluorescence by use of a flow cytometer.

Statistical analysis—A 1-way ANOVA was used for statistical analysis. Data were compared among groups of neutrophils treated with the various concentrations of FTY720-P. Values were considered significant at $P < 0.05$.

Results

Phagocytosis—Neutrophils were selected on the basis of forward- and side-scatter characteristics by use of flow cytometry. Purity of the feline neutrophils in the gating area was > 99%, which was confirmed by use of the neutrophil-specific monoclonal antibody and conjugated secondary antibody (**Figure 1**).

The percentage of phagocytosing neutrophils was used to evaluate the effect of FTY720 on phagocytosis. Assessment of the vehicle (control) treatment was designated as the baseline result and assigned a value of 100%. The percentage of phagocytosing neutrophils was slightly increased by FTY720-P, compared with the value for the control group. Mean ± SD values were 103.8 ± 9.1%, 103.8 ± 8.8%, and 103.9 ± 15.8% for

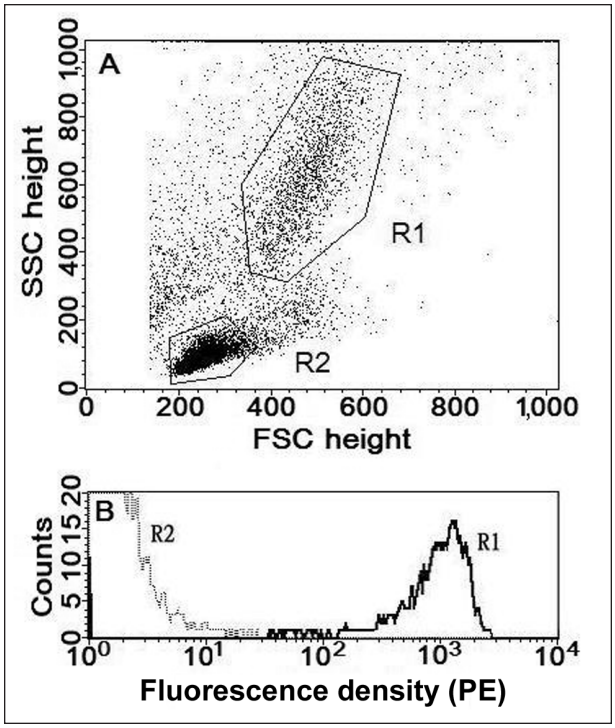

Figure 1—Selection of feline peripheral blood neutrophils on the basis of size and granularity of cells for forward-scatter (FSC) and side-scatter (SSC) characteristics by use of flow cytometry (A) and counts for cells stained by use of an antineutrophil antibody (B). Neutrophils were located in the R1 area, whereas lymphocytes were located in the R2 area. Notice that > 99% of cells in the R1 area were stained by the antineutrophil antibody. Phycoerythrin (PE) is a fluorescence material conjugated to antibody.

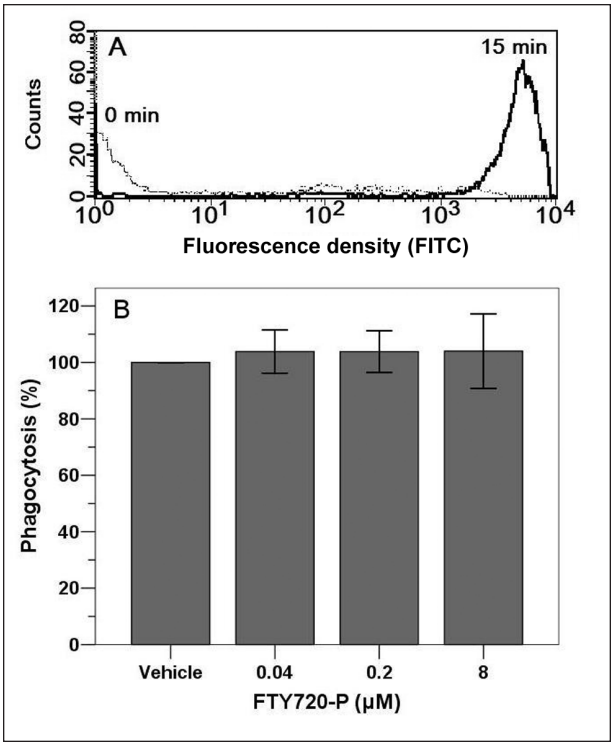

Figure 2—Measurement of green fluorescence by use of flow cytometry in feline peripheral blood neutrophils obtained from 8 cats after addition of fluorescein isothiocyanate–conjugated (FITC) *Staphylococcus aureus* and mean ± SD percentages of neutrophils with staining evident of phagocytosis after incubation with various concentrations of FTY720-P (B). Notice that > 80% of neutrophils had green fluorescence 15 minutes after addition of the FITC-labeled *S aureus*. Assessment of the vehicle treatment (0μM FTY720-P) was designated as the baseline result with a value of 100%.

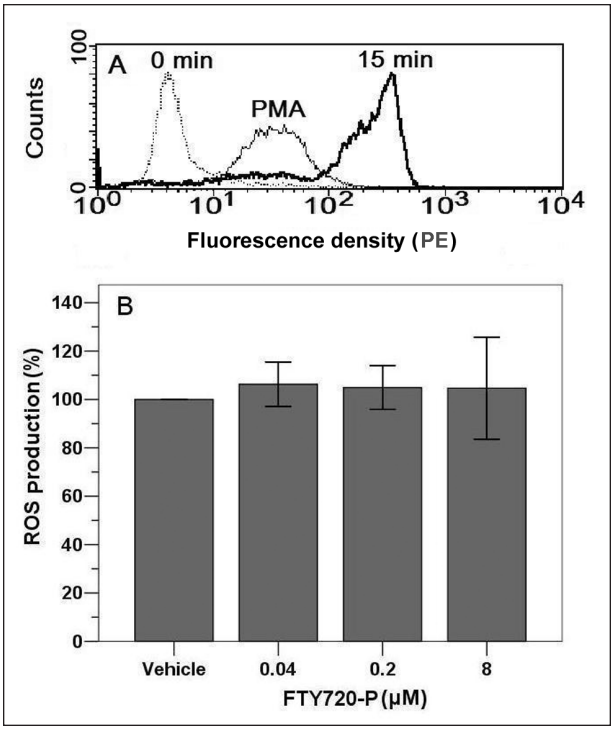

Figure 3—Measurement of ROS production by use of flow cytometry in feline peripheral blood neutrophils obtained from 8 cats (A) and mean ± SD value for ROS production in feline peripheral blood neutrophils stimulated by the addition of phorbol myristate acetate (PMA) and incubation with various concentrations of FTY720-P (B). Notice that production of ROS in neutrophils can be detected 15 minutes after onset of stimulation with PMA. Assessment of the vehicle treatment (0μM FTY720-P) was designated as the baseline result with a value of 100%.

0.04, 0.2, and 8μM FTY720-P, respectively; however, these values did not differ significantly, compared with the baseline value (**Figure 2**).

Production of ROS—Intensity of mean red fluorescence was used to evaluate ROS production. Assessment of the vehicle (control) treatment was designated as the baseline result and assigned a value of 100%. At the beginning of the stimulation (0 minutes), no positive cells could be detected (**Figure 3**). Treatment with FTY720-P slightly increased ROS production by feline neutrophils. Mean ± SD values were 106.3 ± 11%, 104.9 ± 10.8%, and 104.7 ± 25.1% for 0.04, 0.2, and 8μM FTY720-P, respectively; however, these values did not differ significantly, compared with the baseline value.

Migration of neutrophils—Intensity of green fluorescence, which was highly correlated ($r > 0.99$) with the number of cells, was used to measure the number of neutrophils migrating through the membrane. Assessment of the vehicle (control) treatment was designated as the baseline result and assigned a value of 100%. Addition of PBS solution was used as a chemotactic factor for the negative control sample. Migration of feline neutrophils was significantly increased in a dose-dependent manner by treatment with FTY720-P. Mean ± SD values for neutrophils obtained from 5 cats

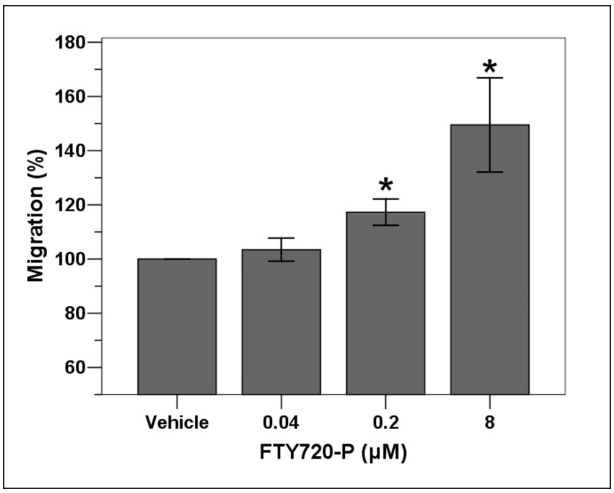

Figure 4—Mean ± SD values for in vitro migration of isolated feline neutrophils obtained from 5 cats after treatment with various concentrations of FTY720-P. *Value differs significantly ($P < 0.05$) from the value for the vehicle (0μM FTY720-P) treatment.

were 103.4 ± 3.4%, 117.2 ± 3.9%, and 149.5 ± 14.1% for 0.04, 0.2, and 8μM FTY720-P, respectively (**Figure 4**).

Cytotoxic effects—After incubation for 24 hours, percentages of propidium iodide–labeled neutrophils for all treatment groups remained low, and there was no significant difference in labeled neutrophils

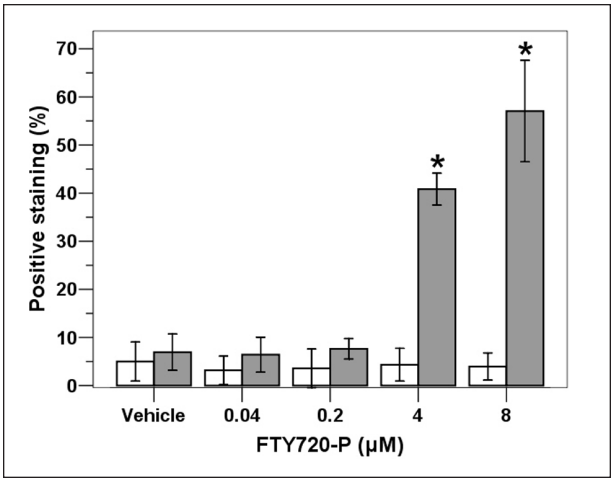

Figure 5—Mean ± SD percentages of dead feline peripheral blood neutrophils after blood neutrophils were obtained from 5 cats and incubated with various concentrations of FTY720-P for 24 (white bars) or 48 (black bars) hours.

obtained from 5 cats among the various concentrations of FTY720-P. Mean ± SD values were 4.99 ± 3.27%, 3.16 ± 2.39%, 3.59 ± 3.22%, 4.33 ± 2.74%, and 3.96 ± 2.27% for 0 (vehicle), 0.04, 0.2, 4, and 8μM FTY720-P, respectively (**Figure 5**). After incubation for 48 hours, mean ± SD percentages of propidium iodide–labeled neutrophils remained low for the 0 (vehicle), 0.04, and 0.2μM treatments (6.93 ± 3.04%, 6.42 ± 2.93%, and 7.63 ± 1.71%, respectively). However, percentages of propidium iodide–labeled neutrophils increased significantly ($P = 0.01$) for treatment with 4μM FTY720-P (40.83 ± 2.67%) and 8μM FTY720-P (57.05 ± 8.49%).

Discussion

Use of fluorochrome-conjugated microorganisms and oxidization-sensitive dyes for the evaluation of phagocytosis and ROS production has been described. For most protocols,[7,8,11,12] 30 minutes of activation time is recommended to achieve the maximum rate of phagocytosis and ROS production. However, more rapid activation of feline peripheral blood neutrophils was detected in the study reported here because phagocytosis and ROS production reached a peak at 10 to 15 minutes.

A calcineurin inhibitor, CsA, is the basic component of immunosuppressive treatment currently used for cats that have received transplanted organs. Although CsA is extremely effective in preventing rejection, it can also cause adverse effects that injure tissues and may result in the death of human and feline transplant patients.[13-17] Because of these adverse effects, there is a movement to develop calcineurin inhibitor–free protocols for human transplant patients.[18,19] During the past 10 years, several new drugs have been developed for use in humans and evaluated in vitro in cats.[20] Unfortunately, all of those drugs have been too toxic for use in cats.

However, FTY720 is an immunosuppressive agent with a unique mechanism of action, wide safety margin, and minimal adverse effects. It is metabolized by cytochrome P450 4F in the liver, rather than the cytochrome P450 34A system, which renders it free from pharmacokinetic interactions with CsA. Furthermore, there are only modest variations among animals in blood concentrations of FTY720 for rats, dogs, and nonhuman primates.[1,6] Therefore, therapeutic drug monitoring is not likely to be needed. In addition, FTY720 has the ability to prevent cancer progression induced by CsA in vitro. Low concentrations (0.1μM) of FTY720 prevent CsA-induced alterations in morphologic characteristics and cell motility, and 5μM FTY720 induces apoptosis in CsA-treated cancer cells.[21]

The dose of FTY720 needed to control rejection of a transplanted organ in animals is higher than the dose that induces lymphocyte depletion in peripheral blood samples in nontransplanted subjects. Higher doses of FTY720 may induce longer periods of neutropenia in cats. The possible mechanisms of neutrophil depletion include cytotoxic effects, inhibition of production, or segregation of cells in some area or areas of the body. In vivo, cytotoxic effects of FTY720 are not a consideration because the in vivo blood concentration of FTY720 needed to cause lymphopenia is much lower than the concentration needed to induce apoptosis of peripheral blood lymphocytes.[22,23] A possible explanation for the increase in the number of dead feline neutrophils in the high-concentration treatments at 48 hours after incubation, but not 24 hours after incubation, may have been the short life span of peripheral neutrophils. In cats, neutrophils stay in the circulation for 5.5 to 7.6 hours after leaving the bone marrow and passing into the peripheral blood and subsequently survive in the tissues for 1 to 4 days.[24,25]

Inhibition of the production of neutrophils should not be a mechanism for FTY720–induced neutrophil depletion in cats. Depletion was evident within 4 hours after initiation of FTY720 treatment. Cats have a 3- to 5-day supply of neutrophils stored in the bone marrow.[26] Thus, assuming the production of neutrophils was inhibited immediately after FTY720 administration, neutropenia should not have been evident for at least 3 days. Therefore, we believe that depletion of peripheral blood neutrophils in cats may be primarily attributable to segregation of neutrophils in some area or areas of the body.

Use of FTY720 can reduce ischemia-reperfusion injury in rats via inhibition of T-cell infiltration into grafts.[27-29] Lymphocytes are depleted by FTY720 in the peripheral blood. Fewer lymphocytes penetrating into a transplanted graft results in a reduction in cytokine stimulation and the inflammatory response. In addition, FTY720 decreases vascular leakage and inflammation by enhancing endothelial cell barriers in mice.[30] The unique FTY720–induced depletion of peripheral neutrophils may provide more protection from ischemia-reperfusion injury in cats with a transplanted kidney because neutrophils play a critical role in the acute phase of reperfusion injury attributable to warm or cold ischemia. However, the migration ability of feline neutrophils is significantly increased by FTY720 without inhibition of ROS production in vitro. This phenomenon may imply that infiltration and damage by neutrophils that are able to reach the allograft will be promoted in FTY720–treated cats after transplanta-

tion. All of these effects of FTY720 on lymphocytes and neutrophils make it difficult to predict the potential protection that FTY720 could provide for ischemia-reperfusion injury in cats.

To our knowledge, the study reported here is the first evaluation of the effects of FTY720 on functions of feline neutrophils in vitro. Additional studies are required to delineate the effects and mechanisms of FTY720 in vivo before it can be used clinically in cats. If FTY720 proves to be a safe and effective immunosuppressive agent for cats, it could be combined with a low dose of CsA (lower than the dose of CsA currently used), which may reduce the prevalence of adverse events, such as diabetes and lymphoma.

a. FTY720-P was provided by Dr. V. Brinkmann, Auto Immunity and Transplantation, Novartis Institutes for Biomedical Research, Basel, Switzerland.
b. N-ethyl-maleinacidamide, Sigma Chemical Co, St Louis, Mo.
c. Phorbol myristate acetate, Sigma Chemical Co, St Louis, Mo.
d. Dihydroethidium, Invitrogen, Carlsbad, Calif.
e. 5-mL polystyrene round-bottom tube, BD Falcon, Franklin Lakes, NJ.
f. Fluorescein isothiocyanate–labeled *Staphylococcus aureus*, Invitrogen, Carlsbad, Calif.
g. CD11b, Clone CA16.3E10, Serotec, Raleigh, NC.
h. Ficoll–Paque plus, Amersham Biosciences, Piscataway, NJ.
i. Calcein-AM, Invitrogen, Carlsbad, Calif.
j. Chemo Tx, Neuro Probe, Gaithersburg, Md.
k. Zymosan, Sigma Chemical Co, St Louis, Mo.
l. Spectra Max, Gemini XS, Molecular Devices, Sunnyvale, Calif.
m. Propidium iodide, 0.5 mg/mL, Roche Applied Science, Indianapolis, Ind.

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