Biologicals xxx (2013) 1–9

Contents lists available at ScienceDirect

Biologicals

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Porcine granulocyte-colony stimulating factor (G-CSF) delivered via replication-defective adenovirus induces a sustained increase in circulating peripheral blood neutrophils

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ARTICLE INFO

Article history:
Received 23 January 2013
Received in revised form
8 May 2013
Accepted 2 July 2013

Keywords:
Neutrophils
G-CSF
Porcine
Neutrophilia
Adenovirus gene delivery
Alternatives to antibiotics

ABSTRACT

The use of immunomodulators is a promising area for biotherapeutic, prophylactic, and metaphylactic use to prevent and combat infectious disease. Cytokines, including granulocyte-colony stimulating factor (G-CSF), have been investigated for potential value as biotherapeutic proteins. G-CSF enhances the production and release of neutrophils from bone marrow and is already licensed for use in humans. A limitation of cytokines as immunomodulators is their short half-life which may limit their usefulness as a one-time injectable in production-animal medicine. Here we report that administration of recombinant G-CSF induced a transient neutrophilia in pigs; however, delivery of porcine G-CSF encoded in a replication-defective adenovirus (Ad5) vector significantly increased the neutrophilia pharmacodynamics effect. Pigs given one injection of the Ad5-G-CSF had a neutrophilia that peaked between days 3 −11 post-treatment and neutrophil counts remained elevated for more than 2 weeks. Neutrophils from Ad5-G-CSF treated pigs were fully functional based on their ability to release neutrophil extracellular traps and oxidative metabolism after *in vitro* stimulation. Since acceptable alternatives to the use of antibiotics in food-animal production need to be explored, we provide evidence for G-CSF as a possible candidate for agents in which neutrophils can provide protection.

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1. Introduction

Porcine respiratory disease complex (PRDC) is a multifactorial disease of swine caused by interactions of numerous viruses, bacteria, and adverse environmental conditions. Critical periods of peak disease incidence include the neonatal period, weaning and transportation. Commingling stress is typically associated with both weaning and transportation to next stage production sites. While the stressful event may be short-lived, the effect of the stress on the host immune response may be longer lasting. Although primary respiratory infectious agents can cause serious disease, self-limiting disease is more common with uncomplicated infections of most of these agents. It is when these primary infections become complicated with secondary bacteria that more serious and chronic respiratory disease results and the most burdensome economic losses are incurred. Many primary pathogens act on the host immune system to enable secondary invaders by lowering the local innate mucosal, and sometimes the systemic, defense mechanisms of the host [1]. Secondary bacterial infections are typically managed with prophylactic, metaphylactic or therapeutic antibiotic intervention for both clinical and subclinical bacterial diseases in swine. Today, however, much emphasis is being placed on identifying safe alternatives to antibiotics.

One possible alternative includes biotherapeutic proteins engineered for pharmaceutical use. One advantage of biotherapeutic proteins is they often provide a better safety margin than synthetic small molecules because they are metabolized by the same pathways as the natural protein in the body. Biotherapeutic immune modulators can be given to prevent or lessen disease symptoms caused by various pathogens (viral and bacterial). Therefore, a desired goal for such a biotherapeutic compound is to provide the desired effect on host immunity, and thus sustain or bolster immunity through a period of immune dysfunction that the host is experiencing as a consequence of normal physiological development or stress.

Cytokines are one class of compounds that have been investigated for potential value as biotherapeutic proteins. Administration

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1045-1056/$36.00 Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.
http://dx.doi.org/10.1016/j.biologicals.2013.07.001

of recombinant cytokines to modulate immunity in immune compromised hosts has long been thought to prevent bacterial infections [2]. One possible biotherapeutic cytokine alternative to antibiotic use is the immunomodulator granulocyte-colony stimulating factor (G-CSF). Colony stimulatory factors are cytokines that cause the bone marrow to produce leukocytes, which in turn, fight infectious disease. G-CSF controls the production, differentiation and function of granulocytes from bone marrow [3,4]. Human recombinant G-CSF (filgrastim) is available in a few FDA-approved forms with an indicated use to decrease the incidence of infection, as manifested by febrile neutropenia, in patients with non-myeloid malignancies receiving myelosuppressive anti-cancer drugs [5,6]. In cattle G-CSF has proven to be safe to administer and efficacious in reducing disease severity and duration following experimental pathogen challenge, including protection pneumonia caused by *Mannheimia haemolytica*, a bacterial infection that is associated with shipping stress in cattle [7–11].

We hypothesize that prophylactic or metaphylactic application of G-CSF to pigs, especially during typical times of stress and pathogen exposure (weaning, transportation) would be beneficial to the overall health of the pig population. However, a limiting factor to date for successful deployment of biotherapeutic proteins in livestock species is their short half-life and need for repeated injections. Therefore, we sought to develop a method to provide an extended duration of neutrophilia with a single injection. To date, administration of a recombinant cytokine such as G-CSF has been limited to subcutaneous injection of the purified or pegylated versions of the recombinant protein, which elicit neutrophilia for approximately 5–7 days. For use in livestock, this however, may not be of sufficient duration to be of value in animals experiencing immune dysfunction, which may persist beyond 7 days. Here we tested use of a replication-defective vector to deliver porcine G-CSF cDNA to enable the host to transiently increase its own production of G-CSF. Our report includes development and testing in pigs of a replication-defective human adenovirus 5 vector (Ad5) containing selected constructs of porcine G-CSF, including alternative gene sequences in an attempt to extend the physiological half-life of G-CSF [12–14]. We demonstrate an advantage of the Ad5 vector to obtain, in a single-dose, a sustained neutrophilia superior to that of the FDA-approved pegfilgrastim (Neulasta®) in pigs.

2. Materials and methods

2.1. Production and mutagenesis of porcine G-CSF cDNA

Porcine G-CSF cDNA was obtained by RT-PCR of mRNA extracted from peripheral blood mononuclear cells (PBMC) collected from two pigs. The PBMC were isolated using sodium heparin cell separation tubes according to manufacturer's recommendations (BD Vacutainer® CPT™, Becton, Dickinson and Company, Franklin Lakes, NJ). PBMC were cultured (10^7 per 200 μL) in RPMI 1640 with 10% fetal bovine serum (heat-inactivated) and gentamicin at 37 °C for 96 h with concanavalin A (10 μg/mL) and then phorbol myristate acetate (2.5 μg/mL) and ionomycin (25 μg/mL) were added, and cells cultured an additional 4 h before processing for RNA extraction following manufacturer's instructions (RNeasy Mini Kit, QIAGEN, Valencia, CA). Following reverse transcription, primers designed from the porcine G-CSF sequence in GenBank (Accession U68482) were used to amplify a full-length cDNA. Each primer began with 8 random nucleotides (5′-ACTATTAC-3′), followed by either a Kpn I (5′-GGTACC-3′) or Hind III (5′-AAGCTT-3′) restriction endonuclease recognition/cut site in the forward and reverse primers respectively. In the forward primer, a Kozak sequence (5′-GCCGCCACCATGGGC-3′) was introduced after the KpnI site immediately before the start codon. The forward primer was 48 nt in length and the reverse primer was 34 nt in length.

Based on a rational design for increased half-life and potency of human G-CSF [13], the orthologous and conserved amino acids in porcine G-CSF were identified and dual point mutations were introduced into the porcine wild-type G-CSF encoding cDNA construct by PCR primer site-directed mutagenesis per manufacturer's instructions (QuikChange IIXL Site-directed Mutagenesis Kit, Stratagene, La Jolla, CA). Two synthetic oligonucleotide primers, were designed to each introduce a G to C mutation at bp 980 and 989 based on the porcine sequence (GenBank Accession U68482) both of which would result in amino acid conversions from an aspartic acid to a histidine at positions 130 and 133 (mutations indicated in bold font; forward primer: 5′-ACATACTGCAGCTG **C**ATGTCACC**C**ACTTAGCCACCAAC-3′, and the respective antisense primer: 5′-GTTGGTGGCTAAGT**G**GGTGACAT**G**CAGCTGCAGTATGT-3′. Mutations and full-length sequence fidelity were confirmed by sequencing. Fig. 1 is a diagram of wild-type and mutated porcine G-CSF amino acid sequences.

2.2. Generation of replication-defective adenoviruses containing wild-type & mutated G-CSF and IL-18

Adenoviral expression vectors were constructed using the AdEasy™ XL System (Stratagene, La Jolla, CA) as described in the AdEASY™ vector system manual (v1.4 Q BIOgene, Carlsbad, CA). In brief, cDNA constructs were directionally cloned into a transfer vector (pShuttle-CMV), introducing a promoter from the immediate early (IE) region of the cytomegalovirus (CMV) and a simian virus 40 polyadenylation signal. pShuttle-CMV-G-CSF constructs were then transformed into One Shot® Top10® electrocompetent cells (Invitrogen, Grand Island, NY) for DNA preparations.

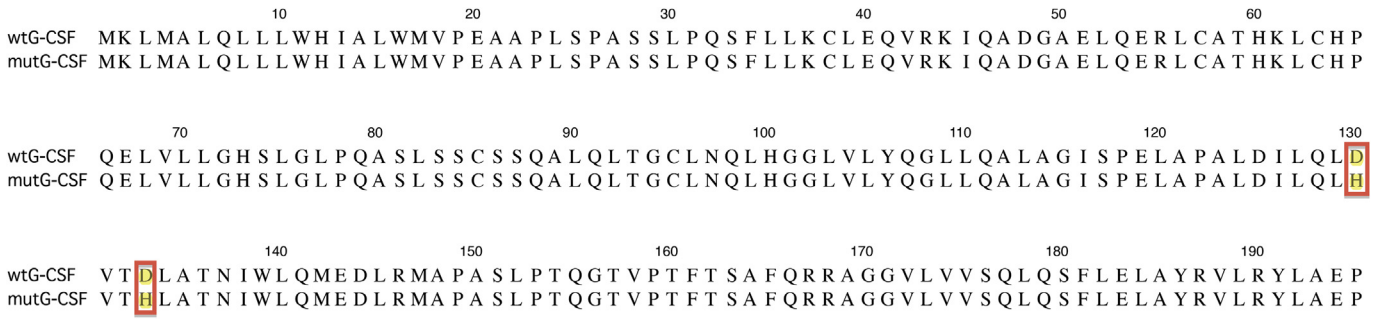

Fig. 1. Amino acid alignment of wild-type and mutated porcine G-CSF. Based on rational design for increased half-life and potency of human G-CSF, the conserved amino acids in porcine G-CSF were identified and dual point mutations introduced as described in materials and methods. The end result was a switch from aspartic acid at positions 130 and 133 to histidine (boxed text).

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Transformed cells were selected by antibiotic resistance and resistant colonies were further screened for retention of correct insertion by restriction enzyme digestion and DNA gel electrophoresis. Preparations of positive isolate colonies were subsequently grown in 5 mL LB with kanamycin, DNA was purified, and the resulting plasmids linearized by restriction endonuclease digestion prior to transformation into $RecA^+$ BJ5183-AD-1 electroporation competent cells, pre-transformed with the repli cation-defective human adenovirus-5 vector plasmid pAdEasy-1 (huAd5ΔE1ΔE3). Positive recombinants were selected and prepared for transformation into XL10-Gold™ ultra-competent cells for large DNA preparations. Selected colonies were prepared using an SNAP Midiprep Kit (Invitrogen, Grand Island, NY). DNA from the SNAP preparations was lipotransfected into HEK-293 cells per manufacturer's protocol for Lipofectamine 2000 (Invitrogen, Grand Island, NY). Isolated, well-developed plaques were collected per manufacturer's instructions. Several rpHuAd5 vector clones were selected for each G-CSF construct and sequenced to confirm correct insertion, Kozak sequence, and desired porcine G-CSF sequence.

A replication-defective human adenoviral expression vector containing porcine IL-18 was constructed as above using a cDNA amplified from a plasmid containing porcine IL-18 (kindly provided by Dr. Michael P. Murtaugh, University of Minnesota, GenBank accession number U68701). In brief, cDNA constructs were directionally cloned into a transfer vector (pShuttle-CMV) as above but using the following primers incorporating a Bgl II cut site (italics) and a Kozak sequence and start codon (in bold) and a Xho I cut site (in italics) and stop codon (in bold) in the reverse primer using the following primers:

Forward: 5′ATCATATTAGATCTGCC**GCCGCCATGG**CTGCTGAACCG3′.

Reverse: 5′ATACTCATCTCGAG**CTA**GTTCTTGTTTTGAACAGTGAACA TTATAG3′.

All subsequent steps for recombination into the Ad5 vector were as before and a 579-bp insert was sequenced to confirm proper orientation of the IL-18 cDNA and Kozak sequence. *In vitro* expression of IL-18 in infected AD-HEK-293 cells was confirmed by western blot using supernatants from the cell culture and a goat anti-porcine IL-18/IL-1F4 biotinylated affinity purified polyclonal (BAF588, R&D Systems, Minneapolis, MN).

Low passage replication deficient adenovirus isolates expressing wild-type G-CSF, mutated G-CSF, IL-18 or without an inserted gene (Ad5-empty) were propagated in specialized AD-HEK-293 cells genetically altered to support replication. For use as inocula, adenovirus isolates were purified and concentrated by double CsCl density gradients per manufacturer's instructions (AdEasy™ vector system manual, v1.4 Q BIOgene, Carlsbad, CA). In short, forty-T150 cell culture flasks (Corning, Corning, NY) were inoculated per virus. Samples were collected when CPE had reached 100%. Media was consolidated and spun at 300 rcf for 10 min to obtain a single cell pellet, discarding the supernatant. Pellets were freeze/thawed three times to lyse cells and release the virus. Discontinuous gradients (1.4–1.2 CsCl sp gr) were prepared in 50-mL ultracentrifuge tubes. Samples were overlaid on top of discontinuous gradient and centrifuged at 100,000 rcf for 90 min. Concentrated adenovirus was collected by aspiration of the adenoviral band and immediately overlaid on previously prepared continuous gradients (1.4–1.2 CsCl sp gr) and spun at 100,000 rcf for 20–24 h. Resulting concentrated adenoviruses were desalted by dialysis in 10 mM Tris (pH 8.0), 2 mM $MgCl_2$, 5% sucrose buffer. Purified adenoviral isolates were titered by tissue culture infectious dose 50% ($TCID_{50}$/mL) as described in the AdEasy™ vector system manual. Adenoviral inoculums were diluted to the titer described in each figure legend in sterile dialysis buffer shortly before administration.

2.3. Animal studies

Piglets were received to the facility as three-week-old weaned piglets from a high-health status herd or farrowed on-site from sows received from high-health status herds. The study was completed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal experiments were approved by the USDA-National Animal Disease Center's Institutional Animal Care and Use Committee (protocol #2320) and all efforts were made to minimize stress and suffering. Prophylactic antibiotics were given to some of the pigs but no vaccinations were administered. Blood samples were collected by venipuncture at the indicated days relative to treatment. At the end of each experiment pigs were humanely euthanized with a lethal dose of pentobarbital (Fatal-Plus®, Vortech Pharmaceuticals, Dearborn, MI). Pegfilgrastim (Neulasta®), which is dispensed as 0.6 mL in a single-dose in a syringe at 6 mg/mL, was prescribed from a local pharmacy. Contents of the syringe were collected into a 15 mL conical tube, diluted 1:10 in PBS for a working stock of 100 μg per 0.1 mL. This working stock was taken to the barn where pigs were weighed, and each dose mixed from the working stock just prior to subcutaneous injection (1 mL final volume). The dose administered (μg/kg) is indicated in each figure.

2.4. Whole blood cell differential

Blood was collected by venipuncture into EDTA-treated vacutainer tubes and single-tube whole blood staining technique utilized. All primary antibodies were specific to porcine antigens and two different staining cocktails were used, which is indicated in each figure legend. The first included CD45 (74-9-31A, VMRD) and a granulocyte marker (PG68A, VMRD). Fifty microliters of each antibody, diluted to 10 μg/mL, was added to 50 μL of whole blood. The second cocktail included a pan-lymphocyte antibody (PG106A, VMRD), pan-granulocyte marker (6D10) and anti-CD14 (CAM36A, VMRD). Fifty microliters of a cocktail with each antibody at 10 μg/mL, 6 μg/mL and 10 μg/mL, respectively was added to 50 μL of whole blood. Fluorochrome-conjugated secondary antibodies specific to mouse immunoglobulin included anti-IgM-FITC, anti-IgG1-PE (both from Southern Biotechnology) and anti-IgG2a-PE-Cy5.5 (Invitrogen). Fifty microliters of the antibody cocktail at 12 μg/mL, 3 μg/mL and 6 μL/mL respectively, was used. For enumeration, 50 μL of beads (Spherotech, 10^6 beads/mL) was added and ultimately 1 mL of FacsLyse (BD Biosciences) was added to lyse red blood cells and fix leukocytes. Data was acquired using a FacsScan with CellQuest data acquisition and analysis software.

2.5. ELISA assays

Blood was collected into vacutainer serum separator tubes and processed according to manufacturer's recommendations (BD, Franklin Lakes, NJ). A multiplex ELISA specific for porcine TNF-α, IL-1β, IFN-γ and IL-6 was performed according to manufacturer's recommendations (Aushon Biosystems, Billerica, MA). An ELISA for porcine IL-8 was used according to manufacturer's recommendations (R&D Systems, Minneapolis, MN). An ELISA designed for detection of human G-CSF was used according to manufacturer's recommendations (Ray Biotech, Inc., Norcross, GA).

2.6. Neutrophil functional assays

2.6.1. Neutrophil isolation

Three to five mL of EDTA-treated whole blood was mixed with 100 mL of cold lysing solution (10.6 mM Na_2HPO_4, 2.7 mM

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NaH_2PO_4) by gentle inversion for 1 min. Isotonicity was restored by adding 50 mL 3X cold restoring solution (10.6 mM Na_2HPO_4, 2.7 mM NaH_2PO_4, 462 mM NaCl). Samples were centrifuged at 1165 $\times g$ for 20 min to pellet leukocytes. The supernatant was discarded and the pellet subjected to a second round of lysing as described above. The final pellet was resuspended in 10 mL phosphate-buffered solution (PBS) which was then underlayed with 10 mL of 45% Percoll (Sp Gr = 1.058), followed by 10 mL 70% Percoll (Sp Gr = 1.088) and 81% Percoll (Sp Gr = 1.105). Tubes were centrifuged at 400 $\times g$ for 40 min at room temperature. The neutrophil fraction (between 70% and 81%) was aseptically removed using a sterile cannula and syringe. Collected cells were washed once with PBS and resuspended in 1 mL PBS. Cell counts were then performed using Beckman Coulter Z2 Cell Counter and adjusted to 5×10^6 cell/mL. Purity was confirmed by flow cytometry after staining with a pan-granulocyte marker (PG68A) as described above. Purity was greater than 95%.

2.6.2. Neutrophil extracellular trap (NET) formation

The NET formation assay was performed as previously described with some modifications [15]. One mL of isolated neutrophils were resuspended in RPMI 1640 media at 5×10^6 cells/mL and then 20 μL of heat-inactivated fetal bovine sera was added to each sample. Cells (100 μL per well) were seeded into 96-well flat-bottom, non-tissue culture treated plates (BD 351172) in triplicate for each animal for each stimulant. Cells were treated with each of the following in 100 μL − phorbol myristate acid (PMA, 10 ng/mL) and ionomycin (1 μM), opsonized zymosan, or media alone. Opsonized zymosan was prepared as described previously [16], with the exception of omitting phenol red from all solutions. Plates were centrifuged at 500 $\times g$ for 5 min and then incubated for 30 min at 37 °C in 5% CO_2. After the incubation, plates were washed with PBS and 50 μL of Sytox Orange (5 μg/mL) added to each well. Plates were incubated at room temperature for 10 min and then washed with PBS. Plates were read on an M5 SpectraMax spectrophotometer (Molecular Devices) using excitation of 530 nm, emission of 570 nm and cut-off 550 nm. The stimulation index was calculated by dividing the average of fluorescence of triplicate stimulated wells by the average of fluorescence of non-stimulated wells for each respective animal. Neutrophils isolated from five animals per treatment (Ad5-empty, Ad5-mutG-CSF and Ad5-wtG-CSF) were used and each stimulant was run in triplicate.

2.6.3. Neutrophil oxidative metabolism assay

The assay was performed using components of the FLMPO 100-3 kit according to manufacturer's recommendations (Cell Technology). In this assay, a non-fluorescent detection reagent is oxidized by myeloperoxidase (MPO) in the phagolysosome in the presence of hydrogen peroxide (produced by the activated neutrophil) to produce a fluorescent analog. Briefly, 50 μL of diluted detection reagent was seeded into each well of a 96-well optical bottom plate. Fifty microliters of 5×10^6 cells/mL were added in duplicate for each animal for each stimulant. Cells were treated with opsonized zymosan to activate phagocytosis as described for the NET assay. Plates were immediately placed into the spectrophotometer, which maintained a temperature of 37 °C. A reading was performed every minute for 30 min using an excitation of 550 nm and emission at 596 nm. Area under the curve analysis was performed for each well, duplicate well averages calculated and data expressed using a stimulation index that was calculated by dividing the first saturation point of stimulated wells by the corresponding non-stimulated wells.

2.7. Data analysis

Stimulation index results were analyzed using analysis of variance (ANOVA) with a P-value ≤ 0.05 considered significant and a Tukey post-test performed for pair-wise comparisons (GraphPad Prism, GraphPad Software, La Jolla, CA). Log_{10} transformed neutrophils counts for each day relative to G-CSF delivery were analyzed using a mixed linear model for repeated measures (Proc Mixed, SAS 9.2 for Windows, SAS Institute, Cary, NC, USA). Linear combinations of the least squares means estimates for neutrophil counts were used in a priori contrasts after testing for a significant ($P < 0.05$) effect of the G-CSF delivery on neutrophil counts. Comparisons were made between groups at each time-point using a 5% level of significance ($P < 0.05$) to assess statistical differences.

3. Results

3.1. Pegylated recombinant human G-CSF induces a rapid, but transient, neutrophilia in pigs

To determine if recombinant human G-CSF would induce a dose-dependent increase in circulating neutrophils in pigs, four different doses of pegfilgrastim were administered to groups of 2 or 5 pigs. Within one day of injection, circulating neutrophil numbers exceeded 6×10^4 per μL of blood in pigs given 100 μg/kg and 50 μg/kg of the drug (Fig. 2A). The number of circulating neutrophils decreased rapidly thereafter, and by day 7 following administration, 1 of 2 pigs in each group had returned to baseline levels (approximately 8×10^3 per μL). However, the other pig in both groups still had approximately 2×10^4 neutrophils per μL of blood (Fig. 2A). The effects of pegfilgrastim were dose-dependent, and when given at the 5 μg/kg dose, the number of circulating neutrophils averaged 3.1×10^4 cells per μL on day 1 and returned to baseline by day 5 following administration (Fig. 2B).

3.1.1. Replication-defective adenovirus 5 (Ad5) encoding mutated porcine G-CSF induces neutrophilia in a dose-dependent manner

Replication-defective adenovirus can be used as a vector to deliver a gene of interest for expression in a variety of mammalian species. In order to evaluate the ability of Ad5 to serve as a vector for porcine G-CSF to induce a neutrophilia, and determine if this response was dose-dependent, groups of pigs ($n = 2$) were administered Ad5-mutG-CSF at varying doses and bled daily to enumerate circulating neutrophils (Fig. 3). Circulating neutrophil numbers peaked at day 3 regardless of the dose; however, there was a significant dose effect. On day 3, there were approximately 4.9×10^4 neutrophils per μL in the group given 10^{11} $TCID_{50}$, and still on day 7, approximately 3.5×10^4 neutrophils per μL. The lowest Ad5-mutG-CSF dose of 10^9 $TCID_{50}$ did not cause an overwhelming increase in circulating neutrophils, as values on days 2 through 7 were not increased over values collected on day −3. In this particular study pigs were euthanized on day 7 following administration of the Ad5-mutG-CSF constructs so resolution of the neutrophilia could not be assessed.

3.1.2. Ad5-mutG-CSF construct affects the course of neutrophilia in pigs

During the recombination event that incorporates the gene of interest from the transfer vector into the adenovirus vector, recombination may occur at different sites of the pAdEasy-1 plasmid. During the development of the Ad5-mutG-CSF, several clones were selected and propagated. Eventual sequencing of four selected clones showed that for two of the clones recombination occurred through the origin (S15-3HA and S12-4IA) and for the other two clones recombination occurred through the left

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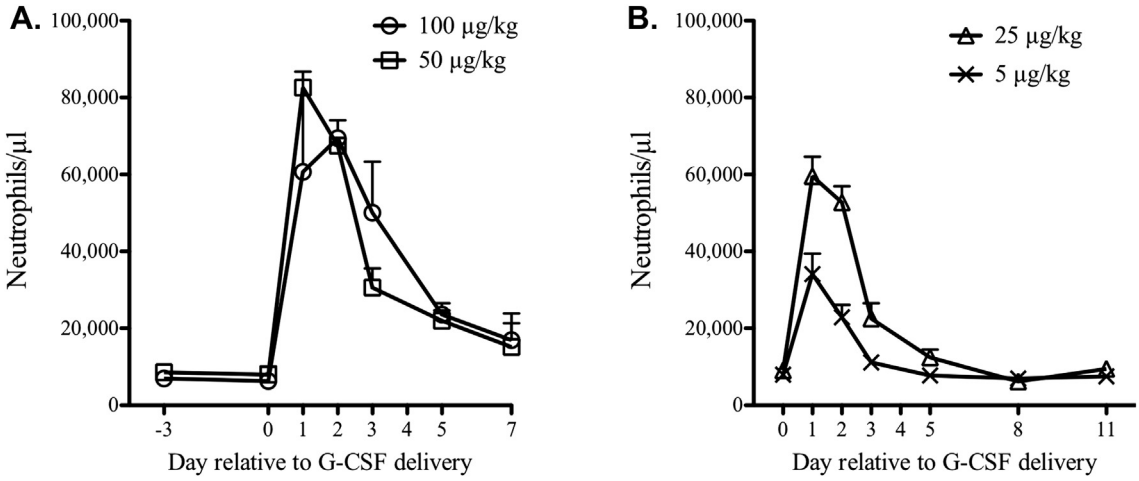

Fig. 2. The number of circulating neutrophils following a single-dose of Neulasta® (recombinant human G-CSF). Two separate experiments were performed in which A) 2 pigs per group were administered 100 ug/kg or 50 ug/kg or B) 5 pigs per group were administered 25 ug/kg or 5 ug/kg of Neulasta by subcutaneous injection. Blood samples were taken at the noted day relative to treatment for neutrophil enumeration by flow cytometry as described in materials and methods. The PG68A anti-porcine neutrophil antibody was used for this analysis. Data is reported as the mean + SEM for each group.

homologous region (S12-4HB and S15-3AA) and sequences were identical for each respective recombination event (data not shown). All four of the recombinant plasmids were propagated in 293 cells and 1 mL per pig of 10^{10} $TCID_{50}$/mL was administered to evaluate the subsequent increase in circulating neutrophils (Fig. 4). Within a day of administration, increased neutrophil numbers were the same across groups. However, by day 3 and later, there were differences in the numbers of neutrophils in circulation between the groups. A biphasic response was appreciated with all constructs, but most apparent after administration of the S12-4HB and S15-3HA constructs. For the S12-4HB construct, circulating neutrophil numbers were actually highest during the second wave, averaging more than 70,000 neutrophils per µL of blood. The second peak for all constructs decreased significantly between days 11 and 14, with a steady decrease through the final sampling 24 days following initial administration. The increased neutrophil response was specific to administration of Ad5-G-CSF, as pigs that received the Ad5-empty or Ad5-IL-18 did not display an increase in circulating neutrophils throughout the course of the study (Fig. 4).

3.1.3. *Wild-type G-CSF encoded in Ad5 vector induces more neutrophils into circulation compared to mutated G-CSF*

Previous work on human G-CSF suggested that changing two amino acids to histidine would increase half-life and potency of the protein; thus, similar changes were made in porcine G-CSF to evaluate the effects of these changes compared to wild-type G-CSF. Interestingly, making the changes in porcine G-CSF and delivering the gene via the replication-defective Ad5 vector did not increase protein potency. Instead, the Ad5 vector encoding wild-type G-CSF induced greater numbers of neutrophils into circulation than the vector encoding mutated G-CSF (Fig. 5). The biphasic response appreciated with different Ad5-mutG-CSF constructs was even

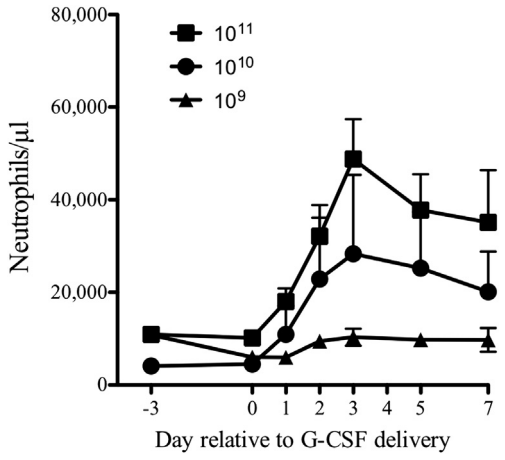

Fig. 3. The number of circulating neutrophils following administration of a replication-defective adenovirus encoding mutated porcine G-CSF (S15-HA) at various doses. Pigs received a single intramuscular injection of Ad5-mutG-CSF of 10^{11}, 10^{10} or 10^{9} tissue culture infectious dose 50 ($TCID_{50}$) and blood samples taken at the noted day relative to treatment for neutrophil enumeration by flow cytometry as described in materials and methods. The PG68A anti-porcine neutrophil antibody was used for this analysis. Data is reported as the mean + SEM for two pigs in each group.

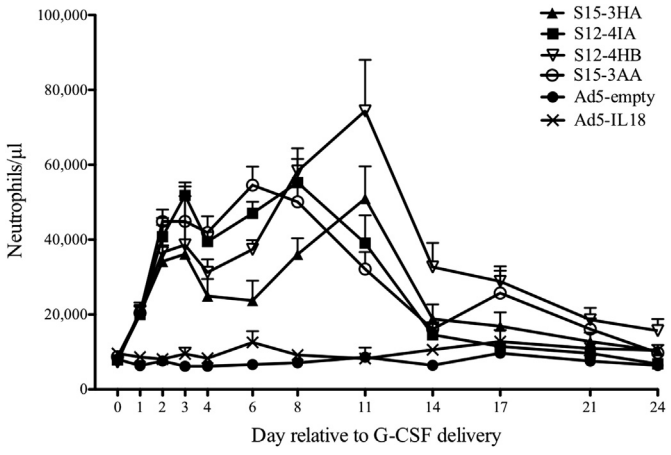

Fig. 4. The number of circulating neutrophils following administration of different replication-defective adenovirus 5 (Ad5) constructs encoding mutated porcine G-CSF. Pigs received a single intramuscular injection of the noted Ad5-mutG-CSF construct at 10^{10} tissue culture infectious dose 50 ($TCID_{50}$) per pig. Pigs received the same Ad5 construct without an inserted gene (Ad5-empty) or with the porcine IL-18 gene (Ad5-IL18) as controls. Blood samples were collected at the noted day relative to treatment for neutrophil enumeration by flow cytometry as described in materials and methods. The PG68A anti-porcine neutrophil antibody was used for this analysis. Data is reported as the mean + SEM for five pigs in each group.

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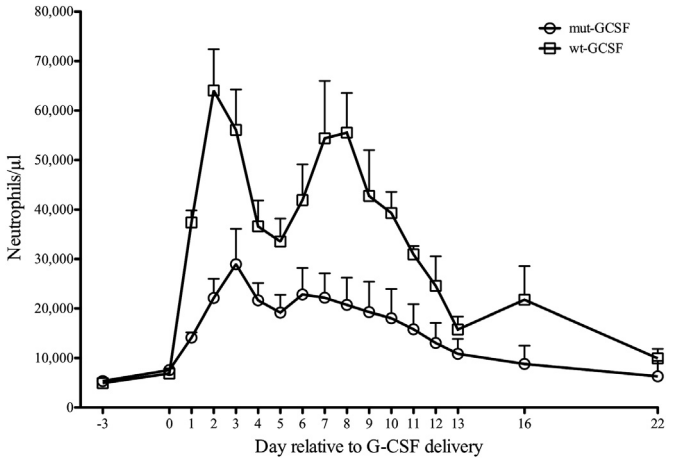

Fig. 5. The number of circulating neutrophils following administration of replication-defective adenovirus 5 (Ad5) encoding mutated porcine G-CSF (mut-G-CSF, S15-3AA) or wild-type G-CSF (wt-G-CSF). Pigs received a single intramuscular injection of the noted Ad5-construct at 10^{10} tissue culture infectious dose 50 ($TCID_{50}$) per pig. Blood samples were collected at the noted day relative to treatment for neutrophil enumeration by flow cytometry as described in materials and methods. The PG68A anti-porcine neutrophil antibody was used for this analysis. Data is reported as the mean + SEM for five pigs in each group.

more apparent with administration of the Ad5-wtG-CSF construct, with a second peak of increased circulating neutrophils on days 7 and 8 following Ad5-wtG-CSF administration. On day 17 following administration of the Ad5-wtG-CSF circulating neutrophil numbers were still significantly elevated, but by day 24 had returned to baseline.

3.1.4. Ad5-G-CSF administration increases the number of other circulating leukocyte populations

Given the magnitude of increased neutrophils in circulation following Ad5-G-CSF administration, regardless of using mutated or wild-type G-CSF, we sought to evaluate the numbers of circulating monocytes and lymphocytes in response to Ad5-mutG-CSF and Ad5-wtG-CSF as well. Results indicate that not only are circulating neutrophil numbers increased, but also circulating monocyte numbers are significantly increased following Ad5-G-CSF administration (Fig. 6). The effect was more pronounced after Ad5-wtG-CSF administration compared to Ad5-mutG-CSF; however, the response was sustained for at least 2 weeks in both groups. While neutrophil numbers continued to increase between days 3 and 5 following Ad5-wtG-CSF treatment, monocyte numbers appeared to peak on day 3 and steadily declined to baseline thereafter. Lymphocyte numbers also increased following administration of Ad5-wtG-CSF, but there was no significant increase between Ad5-empty and Ad5-mutG-CSF groups.

3.1.5. Neutrophils elicited into circulation following administration of Ad5-G-CSF are functional

While a large number of neutrophils were elicited into circulation following administration of Ad5-G-CSF, their functional capacity was unknown. To confirm that these neutrophils were functional, two different assays were performed. Neutrophils were isolated from groups of pigs 5 days after administration of Ad5-mutG-CSF, Ad5-wtG-CSF, or Ad5-empty and their ability to form neutrophil extracellular traps (NETs) and undergo a phagocytosis-associated oxidative burst following stimulation were assessed. Our results indicate that the functional capacity of circulating neutrophils elicited following Ad5-G-CSF treatment is not significantly different than neutrophils isolated from Ad5-empty treated

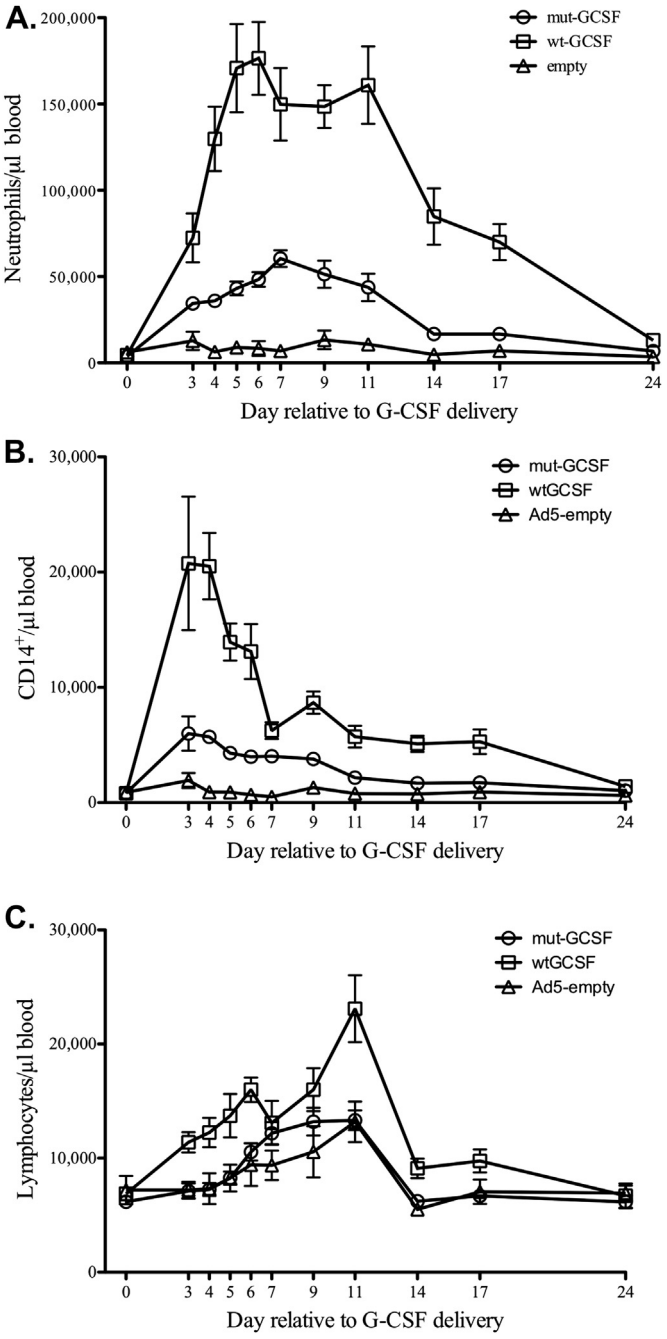

Fig. 6. The number of circulating neutrophils, monocytes, and lymphocytes following administration of replication-defective adenovirus 5 (Ad5) encoding mutated porcine G-CSF (mut-G-CSF, S15-3AA) or wild-type G-CSF (wt-G-CSF). Pigs received a single intramuscular injection of the noted Ad5-construct at 10^{10} tissue culture infectious dose 50 ($TCID_{50}$) per pig. Pigs received the same Ad5 construct without an inserted gene (Ad5-empty) as a control. Blood samples were collected at the noted day relative to treatment for enumeration of each population by flow cytometry as described in materials and methods. The following antibody clones were used for determining the designated population (neutrophils, 6D10; $CD14^+$ monocytes, CAM36A; lymphocytes, PG106A) Data is reported as the mean + SEM for ten pigs in Ad5-mutG-CSF group and 5 pigs each in Ad5-wtG-CSF and Ad5-empty groups.

pigs (Fig. 7). Specifically, oxidative metabolism following exposure to opsonized zymosan was the same regardless of whether neutrophils were isolated from Ad5-G-CSF treated pigs or Ad5-empty treated pigs. In addition, NET release was similar regardless of Ad5-G-CSF treatment.

Please cite this article in press as: Loving CL, et al., Porcine granulocyte-colony stimulating factor (G-CSF) delivered via replication-defective adenovirus induces a sustained increase in circulating peripheral blood neutrophils, Biologicals (2013), http://dx.doi.org/10.1016/j.biologicals.2013.07.001

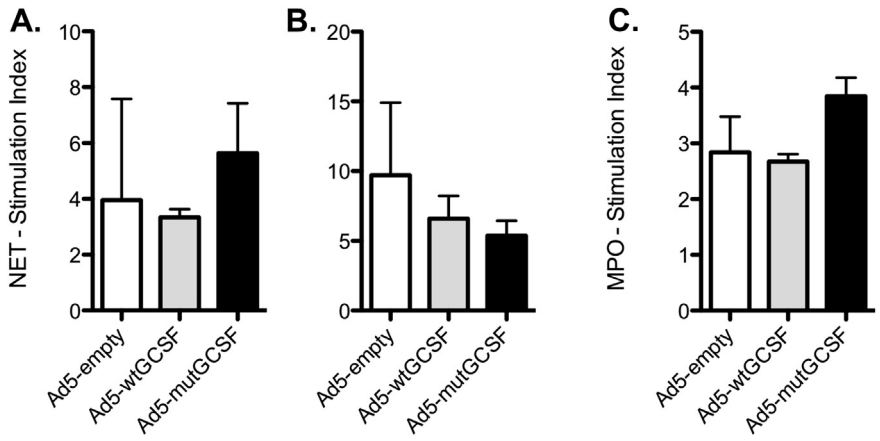

Fig. 7. Functional assessment of neutrophils elicited into the periphery following administration of replication-defective adenovirus (Ad5) encoding mutated porcine G-CSF (mut-G-CSF, S15-3AA), wild-type G-CSF (wt-G-CSF) or empty vector (Ad5-empty). Pigs received a single intramuscular injection of the noted Ad5-construct at 10^{10} tissue culture infectious dose 50 ($TCID_{50}$) per pig and neutrophils isolated from peripheral blood 5 days later to assess neutrophil extracellular trap (NET) formation following stimulation with A) opsonized zymosan or B) PMA/ionomycin and c) oxidative burst following stimulation with opsonized zymosan. Assays were performed as described in materials and methods. Data is reported as the mean ± SEM for 5 pigs in each treatment group.

4. Discussion

Neutrophils play an important role in antimicrobial defense as well as innate and adaptive immunity, with a noted role in protection from extracellular pathogens (reviewed in Ref. [17]). As increased emphasis is placed on finding alternatives to antibiotics in livestock species, one obvious option is identifying methods to boost an animal's own immune system, particularly by using proteins that would be metabolized through normal pathways in the body. Herein, we showed that a single, intramuscular injection of porcine G-CSF encoded in a replication-defective human adenovirus 5 (Ad5) resulted in a neutrophilia that lasted more than 2 weeks. The neutrophils elicited into circulation were able to produce NETs and exhibit MPO activity following *in vitro* stimulation, indicating their full functional capacity. Thus, we provide a method to boost innate immunity as a potential alternative to antibiotic therapy for further investigation in prevention of subclinical or clinical disease in pigs.

The biological activity of G-CSF has been extensively reviewed [18,19]. While G-CSF can mediate effects on a variety of cells in the body, the main target of G-CSF activity is neutrophils. Exogenous G-CSF acts in the bone marrow to increase the number of neutrophil precursors as well as stimulate the rate of maturation and release into circulation [19] and has been shown to increase the number of monocytes in circulation [20]. Following administration of pegfilgrastim to pigs, an increase in circulating neutrophils was appreciated, though this response was dose dependent and not sustained much past a week at the highest dose (Fig. 2). Given the intended use in pigs, which would be to enhance innate immunity at times of immunosuppression associated with normal physiologic changes (i.e. weaning), this rapid, but relatively transient increase in circulating neutrophils would likely be suboptimal for prevention of clinical disease over a 2- to 3-week period. Instead, the neutrophilia that lasted for at least 3 weeks when porcine G-CSF gene was encoded in the adenovirus vector is more optimal because it likely provides a longer duration of protection by the innate immune system. Initially, in an attempt to maximize the response to G-CSF in the pig, mutations were introduced into the porcine G-CSF gene based on a rational design approach shown to increase ligand-binding and protein half-life of human G-CSF [13]. However, this strategy did not correlate to responses in the pig, as wild-type porcine G-CSF elicited substantially more neutrophils into circulation than the mutated version (Figs. 5 and 6). Another factor that affected the number of neutrophils elicited into circulation was the Ad5 construct used in the pig (Fig. 4); however, the reason for this is not completely clear given that the gene sequence was the same for all constructs (data not shown). While the back-titer of each was at the expected 10^{10} $TCID_{50}$/mL slight differences undetectable in the titering assay may have significant effects *in vivo*.

We were somewhat surprised by the biphasic response associated with Ad5-G-CSF administration and do not know the exact mechanism associated with this response. The biphasic nature of the number of circulating neutrophils was appreciated more following administration of the wild-type Ad5-G-CSF as opposed to delivery of the mutated gene (Figs. 5 and 6). However, the days in which blood was collected and analyzed may alter this interpretation, as samples were not always collected and analyzed every day through each independent study. Thus, the biphasic response may not be as obvious and it may shift a day or two in either direction, also affecting our ability to measure the zenith or nadir in circulating neutrophil numbers. Regardless, two distinct peaks in circulating neutrophil numbers were measured in several independent experiments (Figs. 4–6) following administration of both Ad5-G-CSF constructs (mutated and wild-type). It's possible that the initial peak was due to the release of relatively more mature neutrophils from the bone marrow and the subsequent peak the result of increased progenitor production and the eventual maturing and release of those cells into circulation.

Another surprising result was the sustained neutrophilia well past what was predicted based on the neutrophil response following pegfilgrastim administration. A possible explanation for this is increased stability of the G-CSF mRNA given the removal of the 3′-untranslated region of G-CSF, which encodes for two *cis*-acting mRNA destabilization elements [21]. Thus, the G-CSF mRNA may persist for some time, leading to a steady production and release of G-CSF from the injection site. Reagents are available to measure human G-CSF, and we were able to detect G-CSF protein in the sera of pigs treated with pegfilgrastim. However, in spite of the cross-species biological activity of human G-CSF in pigs, a human specific G-CSF ELISA was not able to detect G-CSF in sera from Ad5-G-CSF treated pigs, suggesting that the reagents do not cross-react with porcine G-CSF (data not shown). Thus, further work is needed to determine the kinetics of G-CSF mRNA degradation and protein expression associated with administration of Ad5-G-CSF in pigs.

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During the course of the study, the antibody used in the flow cytometric assay to detect circulating neutrophils was changed to accommodate measuring changes in circulating monocytes and lymphocytes numbers as well. As the primary effect of G-CSF is to increase neutrophil numbers, it may do so at the cost of other immune cell development. Thus, we wanted to evaluate neutrophil numbers as well as monocyte and lymphocyte numbers in circulation. With the exception of the final study (Fig. 6), the PG68A pan-granulocyte antibody was used to label neutrophils, which specifically labels mature neutrophils. The final study, in which lymphocytes and monocytes were also enumerated, used the 6D10 granulocyte marker, which can also label immature neutrophils. Thus, the large number of circulating neutrophils measured in Fig. 6 compared to neutrophil numbers in other figures may be explained by the broader reactivity of the 6D10 antibody. However, there were slight differences across experiments even when the same antibody was used, which may be explained by minor differences in the amount of Ad5 virus administered as previously discussed. Further work is ongoing to investigate the different cell populations elicited into circulation following Ad5-G-CSF administration, including immature cell populations.

Neutrophils are characterized by their ability to act as phagocytic cells as well as release lytic enzymes or produce reactive oxygen intermediates that have potent antimicrobial effects. Recombinant human G-CSF (filgrastim) has been used in humans for some time to treat neutropenia associated with chemotherapy. With purified recombinant G-CSF or filgrastim (sold under the name Neupogen®), single injections typically achieve a transient neutrophilia for about a day. Pegylated versions of G-CSF known as pegfilgrastim (sold under the names Neulasta® or Neulastim®), elicit a neutrophilia for approximately 5–7 days duration, and this approach to treating neutropenic individuals has been successful. We observed the same pharmacodynamic effect of increased neutrophil numbers in pigs treated with pegfilgrastim (Fig. 2). Host resistance to bacterial infection is enhanced by the production of G-CSF during the acute phase response [22] and administration of recombinant bovine G-CSF has been used to prevent mastitis [7–10] but was not effective as a treatment for chronic mastitis [23]. The body has mechanisms to control tissue damage associated with proteases and cytotoxic compounds released from neutrophils, but there is the possibility excessive neutrophils can cause immunopathology. However, we did not detect an increase in serum TNF-α, IL-1β, IFN-γ, IL-6 or IL-8 following Ad5-G-CSF delivery, indicating that administration did not induce a generalized inflammatory response (data not shown). Moreover, previous studies with recombinant G-CSF in pigs investigating possible immunopathology associated with endotoxin in the lung found no evidence of G-CSF adversely affecting physiological responses to lipopolysaccharide [24]. During the course of our work we did not observe any clinical symptoms as the pigs continued to eat normally and did not exhibit any vomiting or diarrhea. Research in other animal species indicates that antibodies to the adenovirus itself can be generated following administration; thus administration may be limited to a single-dose or additional adenoviruses of a different serotype also used. The adenovirus delivery system, encoding a foot-and-mouth disease virus gene, has been approved for use in cattle indicating it can safely be used in livestock species. While further studies are required to evaluate the efficacy of Ad5-G-CSF in prophylactic and/or metaphylactic prevention of disease in swine, as well as additional safety testing, our results provide an interesting method to increase the number of circulating neutrophils, which may serve as alternative to antibiotics used in the prevention of subclinical or clinical disease.

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Acknowledgments

We thank Gwen Nordholm, Zahra Olson, Sarah Anderson and Steve Kellner for excellent technical assistance and Bruce Pesch for assistance and expertise with flow cytometry. We thank Randy Atchison and John Lippolis for advice on neutrophil function assays. We thank Jason Huegal, Jason Crabtree, and Dalene Whitney for excellent animal care. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity employer.

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