Expression of the receptor for IgM (FcμR) by bovine neutrophils

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ABSTRACT

Bovine neutrophils possess a particular set of receptors for immunoglobulins. They have been shown to express a distinctive receptor for IgG_2, and it has long been known that they interact poorly with IgG_1 but that they can use IgM antibodies as opsonins. We show that the binding of labeled IgM was inhibited by unlabeled IgM but not by IgA, suggesting that bovine neutrophils express a specific IgM receptor. The binding of non-aggregated IgM is strong at 4 °C, but shedding occurs at 37 °C. We designed anti-peptide antibodies based on the sequence of the FcµR, the newly described receptor for IgM. These antibodies bound to bovine neutrophils at 4 °C. At 37 °C, labeling was lost, but the loss was inhibited by pretreatment with cytochalasin D, indicating internalization of the receptor after cross-linking by antibodies. Neutrophils that had internalized the receptor were no longer able to bind IgM. Eosinophils showed a low level of FcµR expression. FcµR expression by neutrophils was not increased by stimulation with Toll-like receptor agonists or the complement anaphylatoxin C5a, and decreased by TNF-α. Exposure of neutrophils to IFN-γ for 18 h increased FcµR expression without augmenting the binding of IgG_1 or IgG_2. We confirmed that bovine neutrophils can use IgM to phagocytose and kill bacteria without the help of Complement. Neutrophils that have migrated into the lumen of inflamed lactating mammary glands expressed the FcµR. These results indicate that bovine neutrophils express an IgM receptor, the FcµR, which is functional to contribute to the opsonophagocytosis of bacteria at inflammatory sites. Expression of the FcµR by neutrophils gives IgM a particular importance for the immune defense in the bovine species.

1. Introduction

Neutrophil are effective antimicrobial cells, in particular through phagocytosis and killing of bacteria (Burn et al., 2021). Recognition of bacteria is facilitated by antibodies that bind to bacterial surface components by the antigen-binding fragment (Fab) and present their Fc domain to receptors expressed by the neutrophils. This opsonophagocytosis phenomenon is of major importance for the defense of the host against microbes. There is a wide variety of receptors to match the different isotypes and sub-isotypes of immunoglobulins (Lu et al., 2018; Wang and Jönsson, 2019). The Fc domain variants have unique structural features that condition their interaction with the receptor. The IgG_2 immunoglobulin isotype of ruminants has a very short hinge, which results in close apposition of the Fab to Fc domains (Clarkson et al., 1993). This likely prevents the interaction of IgG_2 with the classical FcγRs (CD16, CD32, CD64) due to steric hindrance. This is mirrored by the occurrence of a FcγR peculiar to ruminants, the Fcγ2R, which

interacts with IgG_2 at the interface of the Cγ2 and Cγ3 domains (Kacskovics, 2004; Zhang et al., 1995). Another peculiarity of dairy ruminant neutrophils is their capacity to use IgM as a complement-independent opsonin: IgM-dependent phagocytosis of bacteria (*E. coli* and *S. aureus*) by bovine neutrophils occurs in the absence of complement activation (Barrio et al., 2003; Williams and Hill, 1982). It has been shown that bovine neutrophils are able to bind IgM, either by rosette formation with IgM-coated erythrocytes (Grewal et al., 1978) or by flow cytometry with non-aggregated IgM (Rainard et al., 2000; Worku et al., 1994).

Apart from cattle, binding of IgM to neutrophils is a controversial issue. Aggregated and unaggregated human IgM did not bind to human neutrophils (Lawrence et al., 1975). Aggregated but not non-aggregated rabbit IgM have been shown to bind to rabbit neutrophils (Furriel et al., 1992). Identifying an IgM receptor has long been an elusive quest, culminating in the discovery of a specific receptor expressed on lymphocytes (Kubagawa et al., 2009). Initial studies indicated that the

cellular distribution in mice is restricted to the B cell lineage, in humans in B and T cell populations (Kubagawa et al., 2009). The receptor, which binds exclusively IgM with high affinity, may not be expressed by human or murine phagocytic cells (Honjo et al., 2013; Kubagawa et al., 2009), although there is a controversy on this subject (Kubli et al., 2019, 2022; Lang et al., 2013a, 2013b; Skopnik et al., 2022).

We hypothesized that the IgM specific receptor (FcµR) is responsible for the ability of bovine neutrophils to use IgM as opsonins. The *Fcmr* gene is located adjacent to genes encoding the other IgM receptors, the polymeric Ig receptor (PIGR) and the IgA/IgM receptor FcαµR, on homologous chromosomes as in other mammals, but not necessarily in the same order (Akula and Hellman, 2017). The availability of the cow genome database (Elsik et al., 2016) allowed us to test the hypothesis that the binding of IgM by bovine neutrophils was due to this newly described Fcµ receptor. We developed antibodies to the bovine FcµR to investigate its expression by bovine neutrophils and its contribution to the phagocytosis of IgM-opsonized particles.

2. Material and methods

2.1. Preparation of anti-bovine FcµR antibodies

Two synthetic peptides corresponding to residues 66–81 of bosFCMR (NCBI reference sequence XP_002693941.2), KYVREEFKHRVTLEQC (peptide 1), and residues 171–187 with an added cysteine at the N terminus, C-TTPAQRIKSPRAHQASPN (peptide 2) were coupled to keyhole limpet hemocyanin and used to immunize rabbits (Eurogentec). The obtained rabbit antibodies were purified from the antisera by affinity chromatography using the corresponding peptides coupled to EAH-Sepharose 4B gels (Cytiva) with maleimidobenzoyl-N-hydroxysuccinimidyl ester as described (Rainard et al., 2008). The reactivity of antibodies to the peptides was tested by indirect ELISA using the synthetic peptides as antigens. The binding of affinity-purified antibodies was revealed with a Donkey F(ab')2 anti-rabbit– RPE prior to flow cytometry analysis.

2.2. Blood sampling and preparation of bovine granulocytes

Jugular venous blood was collected in 10-mL K_3EDTA vacutainers (Becton Dickinson) from animals of the INRAE UE-PAO herd of Holstein cows. Samplings were performed with the approval of the "Val de Loire" Ethics Committee for Animal Experimentation and were registered to the French National Committee for Animal Experimentation under N°2016040410531486. Animal care was compliant with all applicable provisions established by the European directive 2010/63/UE.

Granulocytes were purified from the erythrocyte-containing layer of centrifuged blood after lysis of the red blood cells. In cattle, granulocytes can be isolated by simple centrifugation of blood because of their high density which separates them from the buffy coat and allows them to segregate with red blood cells (Carlson and Kaneko, 1973; Rambault et al., 2021). Vacutainer tubes were centrifuged at 1400 *g* for 20 min at 20 °C. Plasma and buffy coat were discarded and 2.0 mL of the red cell fraction containing the granulocytes were transferred to a 50-mL polypropylene centrifuge tube containing 5 mL of lysis buffer (ACK Lysing buffer, Gibco) and incubated with mild agitation for 5 min at room temperature. Then, 20 mL of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS^{2-}) were added, and the cells were centrifuged at 200 *g* for 6 min. When necessary, a second lysis step was performed by adding 1 mL of lysis buffer and incubation for 2 min before addition of 20 mL of DPBS^{2-} supplemented with 1 mg/mL bovine serum albumin (BSA; low endotoxin for cell culture, Sigma). After centrifugation, cells were resuspended in cold DPBS with calcium and magnesium and BSA (DPBS-A) and adjusted to 5×10^6/mL for cytometric analyses. When required, cells were incubated for 30 min at 37 °C without agitation to remove cytophilic immunoglobulins (Hsu and Mayo, 1973; Watson, 1976). The cells were then washed twice in cold DPBS-A. To replace cytophilic immunoglobulins, granulocytes were incubated with purified IgM or IgG at 4 °C for 30 min and washed twice in cold DPBS-A. For functional assays (receptor internalization, phagocytosis), the cells were resuspended in RPMI 1640 supplemented with 1 mg/mL BSA and 20 mM HEPES.

To investigate the expression of FcµR by migrated neutrophils, cells were isolated from the milk of 4 cows, from 7 quarters with moderate to high cell counts (300 000 to 5 500 000 cells/mL). Milk was cooled on ice, half diluted with DPBS^{2-} with 1 mg/mL BSA and centrifuged (1300 *g*) for 20 min at 4 °C. Cells were resuspended with 20 mL DPBS-A, pelleted by centrifugation (300 *g*, 8 min, 4 °C), and adjusted to 10^7 cell/mL in buffer containing 0.01% sodium azide before flow cytometry analysis.

2.3. Flow cytometry analysis

Single color flow cytometric analyses were carried out on blood granulocytes or two-color analyses on milk cells. Granulocytes in DPBS-A were kept on ice and labeled with primary antibodies for 30 min. After two washes in DPBS-A (400 *g* at 4 °C for 3 min), they were labeled with the corresponding fluorescent-conjugated secondary antibodies (Table 1). After labeling, milk cells were washed in DPBS without BSA and dead cells were identified by incubation for 30 min on ice using the Fixable Viability Dye eFluor®450 (eBioscience). This step was not systematically performed with blood granulocytes as they were more than 90% viable. Cells were kept on ice prior to flow cytometric analysis. Labeled cells were examined using a LSR Fortessa™ X-20 (Becton-Dickinson) flow cytometer and data were analyzed with the Kaluza software (Beckman Coulter). Granulocytes were gated based on their scattering (FSC^{medium}, SSC^{high}), the side scatter clearly distinguishing them from mononuclear cells. Eosinophils were differentiated from neutrophils based on their strong autofluorescence in the AF488 (Supplementary Fig. 1A) or BV421 channel. Milk cells were gated on live, single cells. The cells were labeled with the MM20A (anti-granulocytes, Kingfisher) and anti-CD14 (Tük4-SBV790) antibodies, or alternatively with rabbit antibodies to ovalbumin or anti-peptide 1 (Table 1). Phenotypes were expressed as the geometric mean fluorescence intensity (MFI) and/or % positivity. A minimum of 50 000 events were acquired.

2.4. Purification of immunoglobulins and binding to granulocytes

Immunoglobulins were prepared from a pool of serum from 10 cows from the UE-PAO dairy herd. The serum was filtered (0.2 µm) and dialyzed against 5 mM NaHPO$_4$/Na2PO$_4$ pH 5.5 at 4 °C for 24 h. The precipitated euglobulins were collected by centrifugation (17 000 *g* for 30 min), washed with pure water and solubilized with DPBS. The euglobulins were passed over a Hi-Trap Protein G column (Cytiva) connected to an FPLC Biologic chromatography system (Bio-Rad) to remove most of IgG. The fall-through fraction was passed over a size exclusion chromatography HPLC column (60 cm × 7.5 mm, TSK G4000PW, TosoHaas) with a GE Äkta Purifier 10 FPLC system (Supplementary Fig. 2A). The first peak, which contains the IgM, was collected, put in a dialysis bag (molecular cut-off 6000), and concentrated with polyethylene glycol (MW 25 000). The concentration (mg/mL) of the IgM was evaluated by absorbance (Nanodrop, ThermoScientific) A_{280}/1.2. The purity of IgM was controlled by SDS-PAGE. The purified IgM did not migrate into the gel under non reducing conditions and bands at 75 kDa and 25 kDa corresponding respectively to the IgM heavy and light chains apparent MW were visible under reducing (mercaptoethanol 2% (v/v)) conditions. No 50 kDa band indicative of IgG contamination was visible (Supplementary Fig. 2B). The absence of IgG in the IgM preparation was also controlled by indirect ELISA. Wells were coated with the purified IgM at 5, 2.5, 1, 0.5 and 0.25 µg/mL in PBS and after blocking and washing steps, revealed with different bovine isotypes-specific antibodies conjugated to HRP (IgA, IgG1, IgG2 and IgM, BioRad). No OD signal (at 405 nm) above the control (no Ig) was obtained with anti-isotype-HRP other than anti-IgM (Supplementary Fig. 2C).

Table 1
Primary and secondary antibodies used for flow cytometry analyses.

Antibody	Target	Reactivity	Clone & isotype	Dye	Supplier
Primary antibodies	IgM	Bovine	Rabbit IgG	FITC	Bio-Rad
	Ovalbumin	Chicken	Rabbit IgG		In-house
	Anti-peptide 1	Bovine	Rabbit IgG		In-house
	Anti-peptide 2	Bovine	Rabbit IgG		In-house
	Granulocytes	Bovine	MM20A Mouse IgG1		KingfisherBiotec
	CD14	Human x Bovine	Tük4	SBV790	Bio-Rad
Secondary antibodies	IgG(H + L)	Human	F(ab')2 fragment	Alexa Fluor 488	Jackson ImmunoResearch
	IgG(H + L)	Rabbit	Donkey F(ab')2 fragment	Phycoerythrin	Jackson ImmunoResearch
	IgG(H + L)	Rabbit	Goat F(ab')2 fragment	Alexa Fluor 488	Molecular Probes
	IgG(H + L)	Bovine	Goat F(ab')2 fragment	Alexa Fluor 594	Jackson ImmunoResearch
	IgG(H + L)	Mouse	F(ab')2 fragment	Alexa Fluor 546	Bio-Rad
Anti-human FcµR	FcµR	Human	HM7 Mouse IgG2b	Allophycocyanin	BioLegend
	FcµR	Human x mouse	992338 Mouse IgG2b		R&D Systems
	FcµR	Human	Rabbit IgG		Sigma (HPA003910)
	FcµR	HUman	Rabbit IgG		Abnova (H00009214)
Fixable viability dye	Dead cells			eFluor®450	eBioscience

Purification of IgG_1 and IgG_2 from blood serum was based on their different affinity for staphylococcal protein A (Lawman et al., 1985). The IgG trapped on a Hi-Trap Protein G column (5 mL, Cytiva) were eluted with 0.1 M glycine-HCl buffer pH 2.7 and equilibrated in 0.2 M sodium acetate buffer pH 8.0. The IgG_1 and IgG_2 were separated by affinity chromatography on Protein A Sepharose. The IgG fraction was applied to a HiTrap protein A HP column equilibrated with 0.2 M sodium acetate buffer (pH = 8.0): the fall-through fraction contains the IgG_1 and the fraction eluted with a 0.2 M citrate buffer pH 3.5 contains the IgG_2. Between these 2 steps, the column was washed with 0.2 M sodium acetate at pH 6.5. Concentrations (mg/mL) of purified IgG_1 and IgG_2 were determined by $A_{280}/1.4$. Purity was checked by ELISA by coating wells with the purified immunoglobulins at 10, 5, 2.5, 1, 0.5 and 0.25 µg/mL in PBS and using bovine isotypes-specific monoclonal antibodies conjugated to HRP. The results indicated good separation of the two IgG subisotypes. Serum human IgA were from Sigma-Aldrich.

To investigate the binding of immunoglobulins to granulocytes various concentrations of IgG_1, IgG_2, IgM, and IgA immunoglobulins were incubated with granulocytes for 30 min on ice, washed twice with ice-cold media and labeled with F(ab')2 fragment anti-bovine IgG (H + L)-Alexa Fluor 594 prior to flow cytometry analysis. This antibody identifies all isotypes of bovine Ig by recognizing the light chain.

2.5. Binding of anti-FcµR antibodies to granulocytes and internalization

Granulocytes (5 x 10^5 cells) were incubated with various concentrations of anti-peptide 1 or anti-peptide 2 antibodies for 30 min at 4 °C in a total volume of 100 µL. To test receptor internalization, the cells were then incubated for 30 min at 37 °C. Binding of antibodies to peptide 1 was revealed with Donkey F(ab')$_2$ anti-rabbit– PE (Table 1). When indicated, the granulocytes were treated with 5 µg/mL cytochalasin D to prevent endocytosis (Ogle et al., 1988) during the incubation with the anti-peptide 1 antibodies. A portion of the cells was maintained at 4 °C, another at 37 °C for 30 min before labeling with the secondary antibody at 4 °C. In parallel, the binding of IgM to granulocytes was measured to test whether internalization of FcµR coincided with the presence of the receptor at the surface of the cells.

2.6. Stimulation of bovine neutrophils

To investigate whether the expression of surface FcµR varies according to the activation status, neutrophils (5 x 10^5 cells) were exposed to various stimulating agents before flow cytometric analysis. Synthetic ligands of Toll-like receptors (TLR) known to be active on bovine neutrophils (Conejeros et al., 2015) were used: FSL-1 (TLR2/6), Pam3CSK4 (TLR2/1), both from InvivoGen. The complement-derived chemoattractant C5a was purified from bovine plasma as described (Nemali et al., 2008; Rainard et al., 1998). Recombinant bovine TNF-α and IFN-γ were from Bio-Rad. Granulocytes were incubated for 30 min with FSL-1 (10, 100, or 1000 ng/mL) or Pam3CSK4 (1 or 10 µg/mL), 15 min with C5a (10 nM), or 1 h with TNF-α (10 or 100 ng/mL), and the expression of the FcµR was measured by flow cytometry. To investigate the modulation of FcµR expression by IFN-γ, granulocytes were incubated with various concentrations (10, 30, or 100 ng/mL) of bovine IFN-γ for 18 h at 37 °C before flow cytometry analysis.

2.7. Phagocytosis assays

Escherichia coli P4, originally isolated from the milk of a cow with mastitis (Bramley, 1976), was chosen as the phagocytosis assay target because it has been extensively used in many in vivo, ex vivo, and in vitro studies of phagocytosis in relation to bovine mastitis (Bannerman et al., 2004; Herry et al., 2017; Williams and Hill, 1982). Ingestion of bacteria by granulocytes was measured by cytometric analysis using fluorescent bacteria. Bacteria (*E. coli* P4) were transformed with the DsRed-carrying plasmid pDL278_P23-DsRed-Express2 (pDL278_P23-DsRed-Express2 was a gift from Robert Shields; Addgene plasmid # 121505; http://n2t.net/addgene:121505; RRID:Addgene_121505) (Shields et al., 2019). For the assay, transformants (P4-DsRed) were grown overnight in brain-heart infusion broth in the presence of 50 µg/mL spectinomycin. Live bacteria were adjusted to 5×10^7 CFU/mL and opsonized for 15 min at 37 °C with 5% normal bovine serum (NBS, a pool of serum from 10 healthy cows, used as a positive control for opsonization) or with purified immunoglobulins at concentrations equivalent to about 5% NBS: 0.5 mg/mL for IgG_1 or IgG_2, 0.15 mg/mL for IgM. Then, C5a was added (10 nM final concentration) along with granulocytes to obtain a bacteria/cell ratio of 10 (5×10^6 bacteria for 5×10^5 cells in a total volume of 500 µL per tube). Tubes were incubated for 45 min at 37 °C under end-over-end agitation. After incubation, the tubes were put on ice, and the content analyzed by flow cytometry. To check the internalization of bacteria, neutrophils were isolated after phagocytosis and sorted based on DsRed fluorescence and side scatter into two phagocytic populations with a MoFlo Astrios^{EQ} cells sorter (Beckman Coulter). To visualize the interaction of neutrophils with bacteria, smears of sorted cells were prepared by centrifugation (Cytospin, Shandon Instruments), stained with May--Grünwald and Giemsa using the RAL kit (RAL Diagnostics) and examined microscopically.

For the phagocytic killing assay, bacteria were grown overnight in

Brain Heart Infusion broth (BHI) at 37 °C without agitation, washed twice in HBSS, suspended in HBSS supplemented with BSA (1 mg/mL). The bacterial suspension (*E. coli* P4) was adjusted to 5×10^7 cfu/mL, and 50 μL were distributed in centrifuge tubes (1.5 mL Eppendorf) along with 50 μL immunoglobulin preparations to reach a final concentration of 0.5 mg/mL IgG_1 or IgG_2, or 0.15 mg/mL IgM. A positive control (one tube with 5% NBS) and a negative control (one tube without opsonins) were included. Tubes were incubated for 15 min at 37 °C (opsonization step) before addition of 100 μL of either C5a (400 ng/mL), LPS (500 ng/mL), or RPMI-AH (unstimulated control). Addition of 200 μL of granulocyte suspension (5×10^6/mL) followed, providing a bacteria/cell ration of 2.5/1, and the volume was brought to 500 μL by addition of 100 μL of RPMI-AH. The tubes were incubated for 45 min at 37 °C with slow end-over-end rotation (20 rpm). At the end of the incubation period, 100 μL of the phagocytic mixture were transferred to tubes containing PBS +0.2% Triton X100. After vigorous homogenization, serial 1/10 dilution were performed, and 100 μL of the dilutions were spread on TSA agar. Colony-forming units (cfu) were enumerated after overnight culture at 37 °C.

2.8. Statistical analysis

Prism 7 (GraphPad) was used to perform statistical analysis. Data were analyzed using a Wilcoxon matched-pairs signed rank test or Mann-Whitney test, when indicated. Data with $P < 0.05$ (two-tailed P values) were considered significant. Each experiment has at least two biological replicates unless stated.

3. Results

3.1. Binding of bovine IgG1, IgG2, IgM, and human IgA to bovine granulocytes

As a preliminary step, we checked the binding of immunoglobulin isotypes to bovine neutrophils with purified bovine immunoglobulin fractions. Neutrophils can bind antibodies non-specifically through their receptors for the Fc part of Ig, so that it is common to use Fc blocking procedures (immunoglobulin or antibodies to FcRs) to prevent unspecific labeling. However, when studying the ability of neutrophils to bind immunoglobulins, this is not possible. We could distinguish the binding of IgG_2 to neutrophils by flow cytometry with a sheep anti-IgG_2-FITC, but this secondary antibody gave a strong signal, likely due to its recognition by Fc receptors, presumably the Fcγ2R (Fig. 1A). Consequently, we used as secondary antibody an $F(ab')_2$ fragment goat anti-bovine IgG(H + L)-AF594 to measure the binding of IgG_1, IgG_2, or IgM to granulocytes. This secondary antibody gave a dose-response labeling of neutrophils to increased concentrations of bovine IgG (Fig. 1B). None of the neutrophils bound IgG_1 but 100 % bound IgG_2 (Fig. 1C). Bovine neutrophils have been reported to bind human IgA (Zhang et al., 1995). We used an $F(ab')_2$ fragment anti-human IgG (H + L)-AF488 secondary antibody to check the binding of human IgA to bovine neutrophils, as this antibody identifies all isotypes of human Ig by recognizing the light chain. The labeling of bovine neutrophils increased with increased concentrations of human IgA (Fig. 1D).

In the absence of Fc blocking agent, the binding of IgM could not be visualized with a sheep anti-bovine IgM-FITC, due to high non-specific binding (Fig. 1E). By contrast, binding of IgM was obvious when biotinylated IgM were revealed with streptavidin-PerCP or when IgM were labeled with PerCP-Cy5.5 (Fig. 1E).

The presence of a noticeable amount of eosinophils in the blood of several cows allowed us to analyze the binding of immunoglobulins to these cells. Bovine eosinophils did not bind IgG, readily bound IgA, and bound lightly IgM in comparison to neutrophils (Fig. 1F).

Next, we showed that the binding of labeled IgM was inhibited by unlabeled IgM but not by IgA, indicating that bovine neutrophils express a specific IgM receptor (Fig. 1G). We hypothesized that the receptor was

the identified FcμR and tested this possibility by developing specific antibodies.

3.2. Antibodies to bovine FcμR bind to bovine granulocytes

Several antibodies to human or mouse FcμR were tested for binding to bovine neutrophils (Table 1). None of them labeled bovine granulocytes when analyzed by flow cytometry (results not shown). Based on the amino acid sequence of the bovine FcμR we selected two peptide sequences to induce antibodies susceptible to bind to the extracellular part of the receptor. The obtained rabbit antibodies were purified from the antisera by affinity chromatography on peptide-coupled chromatography gel columns. As control for unspecific labeling, we used affinity-purified antibodies from rabbits immunized with ovalbumin. These antibodies hardly bound to bovine neutrophils (Supplementary Fig. 1B). This allowed us to use affinity-purified rabbit antibodies to FcμR peptides to label bovine neutrophils with a $(Fab')_2$ fragment secondary antibody conjugated to phycoerythrin. Antibodies to peptide 1 bound to all neutrophils (Fig. 2A), antibodies to peptide 2 also labeled neutrophils but with less intensity (Fig. 2B). The binding of peptide 1 antibodies to eosinophils was weak (Fig. 2C) and almost undetectable for peptide 2 antibodies (not shown).

Pre-incubation of neutrophils with IgM did not inhibit the binding of peptide 1 antibodies to bovine neutrophils (Fig. 2D). Pre-incubation of neutrophils with antibodies to peptide 1 and peptide 2 did not reduce the binding of IgM (Fig. 2E). From that, we can conclude that the binding sites of the antibodies were outside the IgM-FcμR interaction site.

3.3. Internalization of the FcμR suppresses the binding of IgM

We observed that after fixation of IgM at 4 °C, incubation of neutrophils at 37 °C led to the loss of labeling by the anti-bovine IgG(H + L), and therefore the disappearance of surface IgM (Fig. 3A). This could be due to the release of IgM or their internalization. We also noted that incubation of neutrophils at 37 °C after incubation at 4 °C with anti-peptide1 caused the loss of labeling, and therefore the disappearance of FcμR from the surface of the cells (Fig. 3A). To distinguish release from internalization, we inhibited actin polymerization with cytochalasin D. Neutrophils treated with the inhibitor retained the labeling with anti-peptide 1 after incubation at 37 °C, supporting the internalization hypothesis (Fig. 3B). We suppose that incubation at 37 °C enabled the engagement of the FcμR by cross-linking anti-peptide 1 with $F(ab')2$ anti-rabbit Ig– PE to induce internalization of the receptor. Finally, we tested the binding of IgM after internalization of the receptor recognized by anti-peptide 1. It appeared that neutrophils which no longer bound anti-peptide 1 were unable to bind IgM (Fig. 3C). Altogether, these results indicate that antibodies to peptide 1 induce the internalization of the FcμR when neutrophils are incubated at physiological temperature, and that the receptor is indispensable for IgM binding. These results also indicate that the molecule recognized by anti-peptide 1 antibodies is indeed the only IgM receptor expressed by bovine neutrophils.

3.4. Modulation of FcμR expression by stimulation of bovine neutrophils

Neutrophils can be primed or stimulated by a variety of stimuli. To investigate whether FcμR expression could be modulated by neutrophil stimulation, we submitted isolated granulocytes to agonists of the Toll-like receptors TLR2-TLR6 (FSL-1) or TLR1-TLR2 (Pam3CSK4), C5a and TNF-α. None of these agents increased the surface expression of FcμR (Fig. 4A). Pre-incubation with TNF-α reduced FcμR expression (geometric MFI; $P = 0.03$, Mann-Whitney test) (Fig. 4A). We tested whether IFN-γ could modulate the FcμR expression. We also investigated whether IFN-γ induced the expression of the high affinity receptor for IgG, the FcγR1 (CD64), which has been shown to bind bovine IgG_1 (Yan et al., 2000). As a prolonged incubation with IFN-γ is necessary to induce the
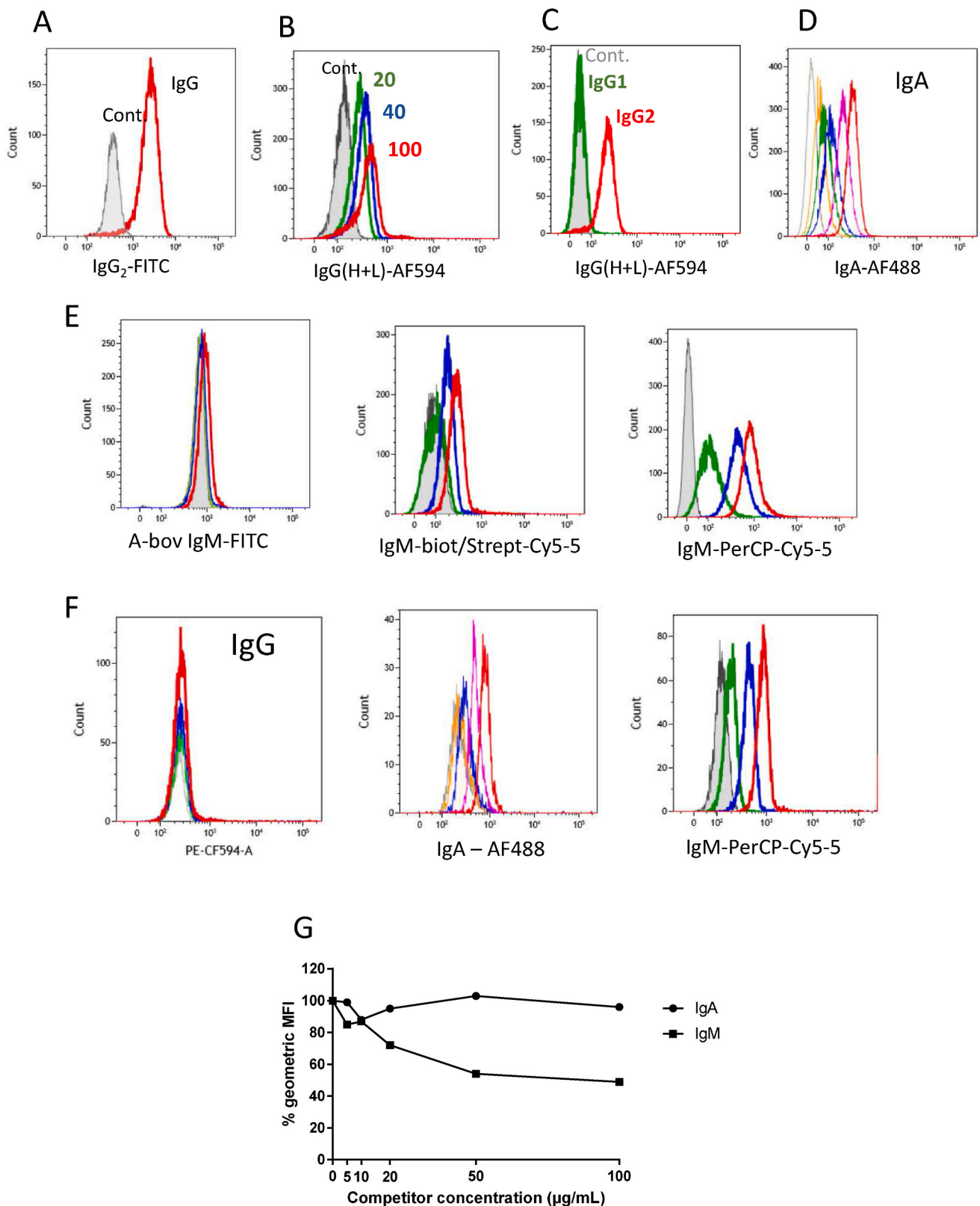
Fig. 1. Binding of immunoglobulins to bovine granulocytes. (A) Binding of bovine IgG$_2$ to neutrophils revealed with sheep anti-bovine IgG$_2$-FITC. Grey trace RPMI-AH followed by secondary antibody, red trace IgG followed by secondary antibody. (B) Binding of increased concentrations of IgG (0, 20, 40, 100 µg/mL) revealed with Goat F(ab')$_2$ fragment anti-bovine IgG (H + L)-AF594. (C) Binding of 100 µg/mL IgG$_1$ (green trace) or IgG$_2$ (red trace) to blood neutrophils. Result from a representative experiment out of four, showing no binding of IgG$_1$ and binding of IgG$_2$ to all neutrophils. (D) Binding of increasing concentrations (0, 5, 10, 20, 50, 100 µg/mL) of human IgA to neutrophils. (E) Binding of IgM (0, grey trace; 1, green trace; 10, blue trace; 40, red trace, µg/mL) to neutrophils evaluated with either sheep anti-IgM-FITC, biotinylated IgM and streptavidin-PerCP, or IgM-PerCP-Cy5.5. (F) Binding of immunoglobulins (IgG, IgA, and IgM) to eosinophils (same concentrations as in 1E). (G) Inhibition of the binding of 2 µg/mL IgM (labeled with PerCP-Cy5-5) by pre-incubation with increased concentrations of unlabeled IgM but not with IgA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
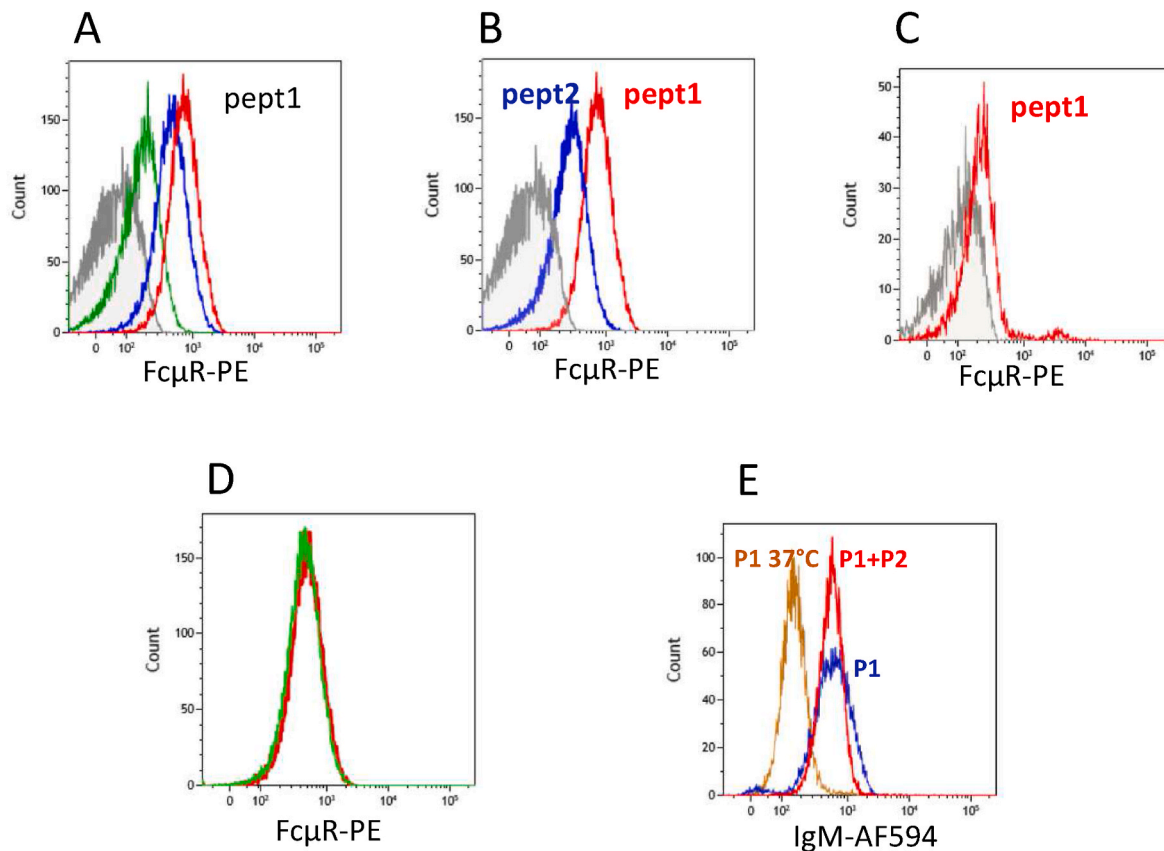
Fig. 2. Binding of antibodies to bovine FcμR peptides 1 and 2 to bovine granulocytes. (A) Overlay histogram showing the binding of increasing concentrations of anti-peptide 1 (pept1) of FcμR to neutrophils: 5 μg/mL (green trace), 10 μg/mL (blue trace), and 20 μg/mL (red trace). Grey histogram: secondary antibody only. (B) Overlay histogram showing the comparison of the binding of anti-peptide 1 (20 μg/mL, red trace) or anti-peptide 2 (20 μg/ML, blue trace) to neutrophils. (C) Binding of anti-peptide 1 (20 μg/mL, red trace) to eosinophils. (D) No inhibition of anti-peptide 1 (10 μg/mL) binding to neutrophils (red trace) by pre-incubation of neutrophils with 40 μg/mL IgM (green trace). (E) Pre-incubation of neutrophils with a mix of anti-peptide 1 and anti-peptide 2 did not impair the binding of IgM to neutrophils. Granulocytes were incubated with either anti-peptide 1 (P1, blue trace) alone or a mixture of anti-peptide 1 (20 μg/mL) and anti-peptide 2 (40 μg/mL) (P1+P2, red trace), washed, incubated with IgM (40 μg/mL), washed, labeled with anti-bovine IgG-AF594 and analyzed by flow cytometry, all at 4 °C. The binding of IgM was also measured after incubation with anti-peptide1 at 4 °C, followed by incubation at 37 °C for 30 min before incubation with IgM (P1 37 °C, brown trace), showing the loss of IgM binding. Results representative of at least three cows from two different experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

expression of CD64 on human neutrophils (Hoffmeyer et al., 1997; Klebanoff et al., 1992), we incubated bovine neutrophils with recombinant bovine INF-γ overnight at 37 °C. Incubation with IFN-γ consistently augmented the binding of anti-peptide 1 (Fig. 4B), with a mean ratio (geometric MFI) with/without 10 ng/mL IFN-γ of 1.67 (3 cows), with/without 30 ng/mL of 1.75 (3 cows), and with/without 100 ng/mL of 1.77 (5 cows, 2 experiments, P = 0.03, Wilcoxon test). Even at the highest concentration tested, IFN-γ did not induce the binding of IgG$_1$ to neutrophils, which indicates that the treatment did not induce the expression of FcγRI (CD64), the high affinity receptor for bovine IgG$_1$ and IgG$_3$ but not IgG$_2$ (Noble et al., 2023) (Fig. 4C). Treatment with IFN-γ also did not augment the binding of IgG$_2$ (Fig. 4C).

3.5. Bovine FcμR enables neutrophils to ingest and kill bacteria opsonized by IgM

We then checked that IgM antibodies allow neutrophils to ingest and kill bacteria opsonized with only IgM, and compared this activity with that of IgG$_1$ and IgG$_2$. Ingestion (phagocytosis) was evaluated with fluorescent *Escherichia coli* bacteria (P4-DsRed) by flow cytometry. The ability of purified IgM, IgG$_1$ and IgG$_2$ immunoglobulins to bind to *E. coli* P4 was also evaluated by flow cytometry. There were antibodies to *E. coli* P4 in all isotypes, however IgG$_1$ contained the most antibodies, followed by IgM, while the IgG$_2$ preparation was much less active

(Supplementary Fig. 2). We used IgG$_1$ and IgG$_2$ at 0.5 mg/mL and IgM at 0.15 mg/mL to opsonize the bacteria, which corresponds to isotype concentrations in approximately 5% of serum, the concentration of the serum pool (NBS) which served as a positive reference for the comparison of opsonizing activities. With 5% NBS most neutrophils ingested bacteria and became fluorescent (Fig. 5A). Neutrophils segregated in two gates, one of single cells (gate C) and the other of aggregated cells (gate B) based on SSC (Fig. 5A). The two categories were cell sorted, cytocentrifuged, stained, and examined under the microscope. Cells from gate C were mainly scattered and most had ingested a few bacteria, whereas those from gate B were in clusters and had ingested many bacteria (Fig. 5B). To calculate the phagocytic index (% fluorescent cells x MFI), we chose to use only the granulocytes that did not aggregate during the phagocytic incubation, that is the cells from gate A (Fig. 5A). Clearly, IgM had an opsonizing activity much higher than that of IgG$_1$ and IgG$_2$, comparable to that of serum (Fig. 5C and D). This hierarchy was respected in the data from aggregated cells present in gate B (not shown).

To evaluate the capacity of IgM to promote killing of bacteria by neutrophils, *E. coli* P4 opsonized under the same conditions as for phagocytosis were incubated with granulocytes in suspension, and the survivors were enumerated by culture on TSA plates after lysis of the cells (Fig. 6). In the absence of stimulating agents, the IgG$_1$ preparation, despite its content in antibodies to P4, did not induce a decrease in
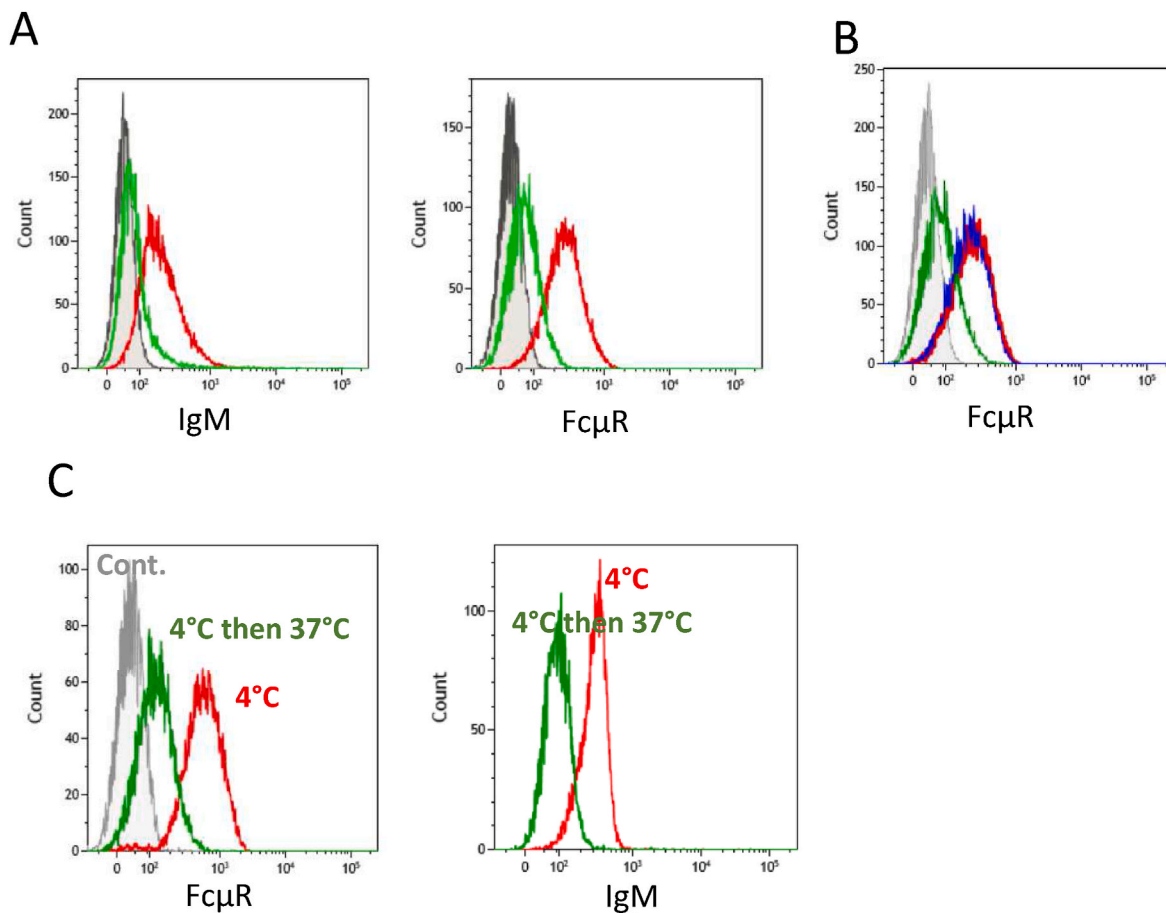
Fig. 3. Loss of surface labeling for IgM and FcμR with incubation at 37 °C, its inhibition with cytochalasin D and relation between expression of FcμR and IgM binding. (A) Internalization of FcμR and IgM. Granulocyte were incubated with either IgM (40 μg/mL) or anti-peptide 1 (20 μg/mL) and incubated for 30 min at 4 °C. After washing, cells were incubated for 30 min at either 4 °C (red trace) or 37 °C (green trace), washed, and labeled with secondary antibody. (B) Cytochalasin D inhibits the internalization of FcμR. Granulocytes were incubated with anti-peptide 1 (20 μg/mL) for 30 min at 4 °C. After washing, they were incubated for 30 min à 37 °C with (blue trace) or without (green trace) cytochalasin D (5 μg/mL, final concentration). Cells maintained at 4 °C without cytochalasin D (red trace) served as labeling positive control. Representative of two experiments. (C) Internalization of FcμR impairs the binding of IgM. Granulocytes were incubated with anti-peptide1 (20 μg/mL) at 4 °C for 30 min, washed, and either maintained at 4 °C (red trace) or incubated at 37 °C for 30 min (green trace). Then they were either incubated with anti-rabbit-RPE to show FcμR surface expression or with IgM (40 μg/mL) for 30 min at 4 °C, washed, and labeled with anti-bovine IgG(H + L)-AF594 to show IgM binding. Control (Cont.): incubation with anti-OVA instead of anti-peptide 1. Representative of three cows (78% reduction in FcμR MFI, 94% reduction in IgM MFI). Eosinophils were excluded from the analyses based on their autofluorescence in the BV421 channel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

bacteria viability, contrary to IgG$_2$ and IgM preparations that enabled neutrophils to kill bacteria. Stimulation with LPS increased killing efficiency mainly for IgG$_2$. Extemporaneous activation of neutrophils with C5a increased the killing in the presence of IgG$_1$, but not more than in the absence of immunoglobulins (BSA + C5a). The strongest effect of C5a was with IgM, bringing about killing as efficient as with 5% NBS.

Since the FcμR is the only receptor for IgM on neutrophils, the results of the phagocytosis and killing assays show that the FcμR is functional and enables neutrophils to ingest and kill IgM-opsonized bacteria.

3.6. Neutrophils recruited into the lumen of the mammary gland express the FcμR

As phagocytosis by neutrophils in the mammary gland lumen is a primordial defense against infection by the most common mastitis pathogens (staphylococci and streptococci) (Paape et al., 2002), we investigated whether neutrophils migrated into the milk of infected mammary quarters expressed the FcμR. We isolated the cells from milk samples of inflamed quarters (>300 000 cells/mL, subclinical mastitis) by centrifugation. Most of the cells were neutrophils (76%–97%, median 89.6%), identified with MM20A antibody (Fig. 7A). All the neutrophils

expressed the FcμR, although with some variability among cows and quarters (Fig. 7B).

4. Discussion

Our working hypothesis was that the receptor identified as specific for IgM, the FcμR, is expressed by bovine neutrophils, which allows them to phagocytose targets opsonized with only IgM. Although we have known for a long time that bovine neutrophils are capable of phagocytizing bacteria simply opsonized with IgM, we did not know which receptor could be responsible for this activity. Indeed, the major opsonins in serum, colostrum and milk for mastitis pathogens such as *Staphylococcus aureus* and *E. coli* are in the IgM isotype (Miller et al., 1988; Seto et al., 1976; Williams and Hill, 1982). Three receptors interact with IgM: the polymeric Ig receptor (PIGR), the FcαR which binds both IgA and IgM, and the FcμR which binds exclusively IgM (Akula and Hellman, 2017). The PIGR has not been reported to be expressed by neutrophils, as well as the FcαR. Moreover, although serum IgA binds to bovine neutrophils, we showed that IgA does not compete with the binding of IgM to neutrophils (Fig. 1G). By elimination, we can infer that the FcμR is the only receptor that could account for the interaction of IgM with
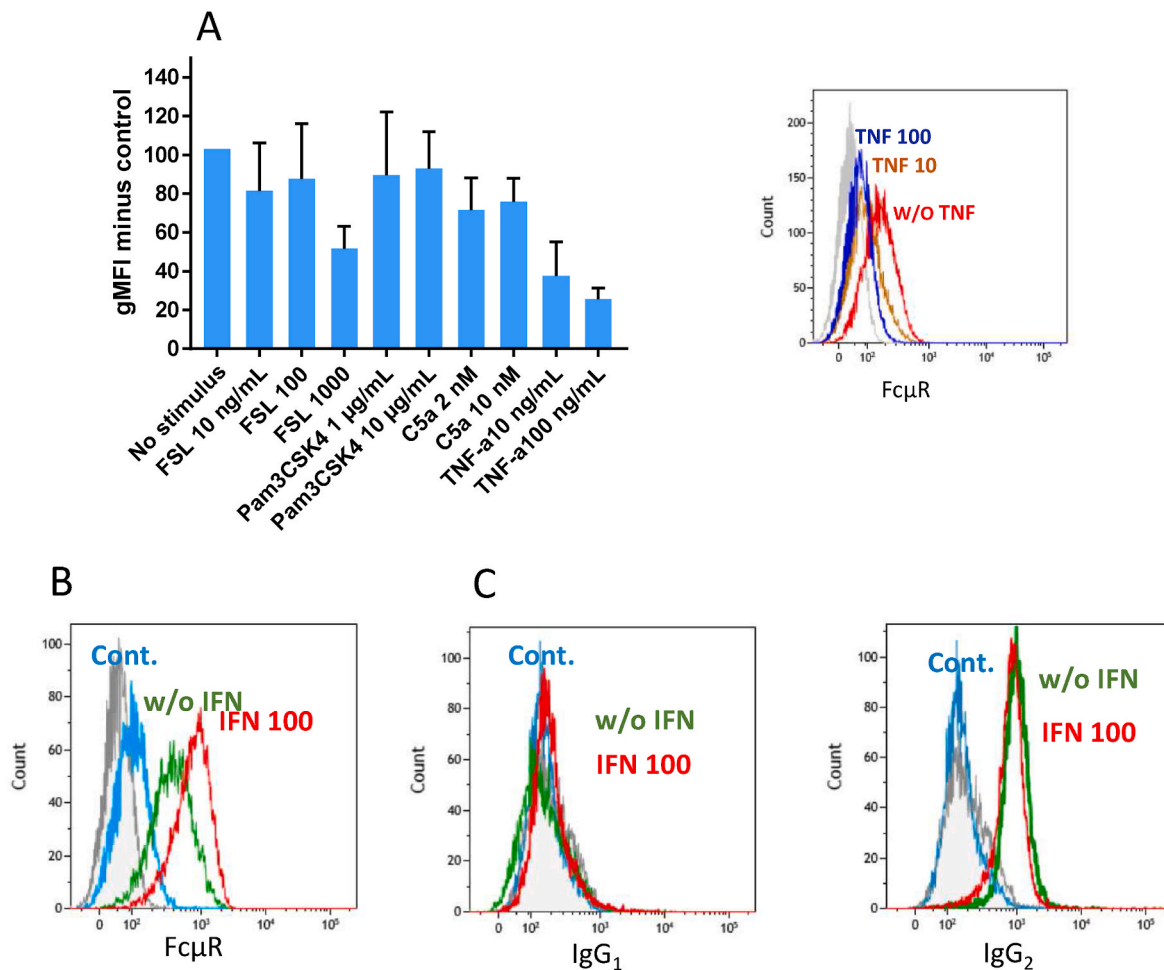
Fig. 4. Modulation of the expression of the FcμR by stimulation of bovine neutrophils. (A) Effect of neutrophil agonists on the expression of the FcμR. Granulocytes were incubated for 30 min with FSL-1 or Pam3CSK4, 15 min with C5a, or 1h with TNF-α, and the expression of the FcμR was measured by flow cytometry (binding of anti-peptide 1). Values are mean MFI (minus MFI by control without agonist) from 2 experiments with 2 cows. Result from a representative flow cytometry analysis without (red trace) or with TNF-α (10 μg/mL brown trace, 100 μg/mL blue trace) is shown. (B) Effect of IFN-γ on the expression of FcμR by neutrophils. Granulocytes were treated for 18 h with medium alone or 100 ng/mL recombinant bovine IFN-γ (red trace). Expression of FcμR was measured by binding of anti-pept1 by flow cytometry (histograms are representative from two experiments with 5 cows). Control: rabbit anti-ovalbumin antibodies with (blue trace) or without (grey trace) IFN-γ. (C) Effect of IFN-γ on the binding of immunoglobulins to neutrophils. Granulocytes were treated for 18 h with medium alone or 100 ng/mL recombinant bovine IFN-γ. Binding of IgG$_1$ or IgG$_2$ was measured by flow cytometry after incubation with 50 μg/mL IgG$_1$ or IgG$_2$. Control: medium alone with (blue trace) or without (grey trace) IFN-γ. The % of viable cells in the single cell window after overnight incubation was ≈90 % with medium alone and >95 % with IFN-γ as assessed with Fixable Viability Dye eFluor®450. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

bovine neutrophils. We sought out to investigate the expression of the newly described FcμR expressed by murine and human lymphocytes (Kubagawa et al., 2009; Shima et al., 2010). As commercially available antibodies to the human or murine FcμR did not bind to bovine neutrophils, we developed anti-peptide antibodies designed from the sequence of the bovine *Fcmr* gene. Two anti-peptide antibodies (anti-peptide 1 and anti-peptide 2) bound to bovine neutrophils as shown by flow cytometry analysis (Fig. 2). The anti-peptide 1, which was more efficient than the anti-peptide 2, labeled 100% of blood neutrophils and slightly labeled eosinophils. None of the antibodies inhibited the binding of IgM to neutrophils, and IgM binding did not interfere with the binding of the anti-peptides (Fig. 2D). Although these results did not provide us with direct evidence that the anti-peptides recognize FcμR, a set of indirect arguments support this assertion. The main observation is that the disappearance of surface FcμR labeling with anti-peptide 1 antibodies renders neutrophils incapable of binding IgM (Fig. 3). We suppose that the disappearance of labeling when cells were incubated at 37 °C likely resulted from the FcμR cross-linking by antibodies to peptide 1, which induced the internalization of the receptor. Another indirect argument comes from the binding behavior of the IgM and anti-peptide1 to

neutrophils which corresponds to what is known about FcμR in other species. Several observations support that the binding of IgM to bovine neutrophils is carried out by the FcμR. The binding of nonaggregated IgM to neutrophils at 4 °C is consistent with the high affinity of the FcμR, which allows measurement of IgM binding capacity by maintaining the cells at 4 °C during the assay (Nakamura et al., 1993). The shedding of IgM when cells are incubated at 37 °C is also in conformity with the properties of FcμR (Kubagawa et al., 2014; Ohno et al., 1990). Incidentally, incubation at physiologic temperature induces shedding rather than internalization of IgM, because no IgM can be detected at the surface of neutrophils whereas FcμR is still expressed. On the contrary, internalization, not shedding, of FcμR is induced by antibodies to peptide 1, because pre-treatment of neutrophils with cytochalasin D, an inhibitor of actin polymerization, prevents the loss of surface expression of the receptor (Fig. 3B). The shedding and internalization of IgM-coated targets is also in accordance with what is known about IgM and bovine neutrophils. It has been reported that at least two washings in PBS at room temperature are necessary to wash off opsonic serum components that could remain on neutrophils isolated from blood (Williams and Bunch, 1981). The binding of anti-peptide 1 antibodies was not much
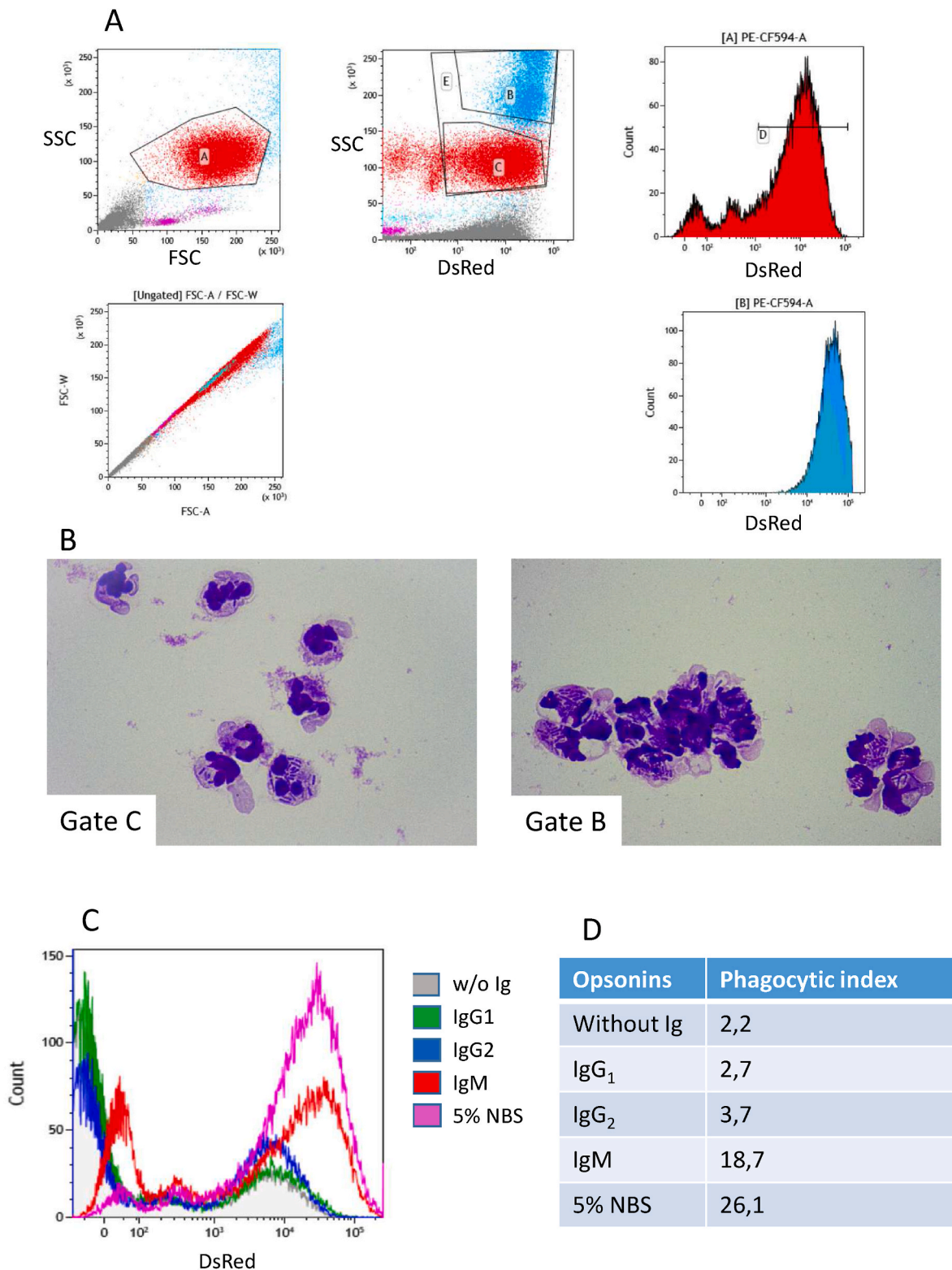
Fig. 5. Phagocytosis by blood neutrophils of fluorescent bacteria. Bacteria (*E. coli* P4-DsRed) opsonized with immunoglobulin isotypes purified from normal bovine serum (NBS) or purified immunoglobulins were incubated with granulocytes. Live bacteria were pre-incubated for 15 min at 37 °C with 0.5 mg/mL IgG$_1$ or IgG$_2$, or with 0.15 mg/mL IgM. Then, C5a was added (10 nM final concentration) along with granulocytes to obtain a MOI of 10. Vials were incubated for 45 min at 37 °C under end-over-end agitation. (A) After incubation, the vials were put on ice, and the content analyzed by flow cytometry. (B) Cells were sorted based on Side Scatter and the two sorted populations (in gate B and gate C) were cytocentrifuged, stained and examined under the microscope. Representative photograph (processed with Photoshop™ for contrast) are shown. (C) Overlay of the distribution of fluorescent cells after phagocytic incubation: in grey, without immunoglobulins, with IgG$_1$ (green trace), with IgG$_2$ (blue trace), with IgM (red trace), with 5% NBS (purple trace). (D) Relative phagocytic indexes were calculated (means from two experiments) as the product of % fluorescent cells x MFI relative to the value obtained without C5a and immunoglobulins (index 1.0): the product obtained with opsonins was divided by the product obtained without opsonins. DsRed fluorescence was measured in the AF594 channel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
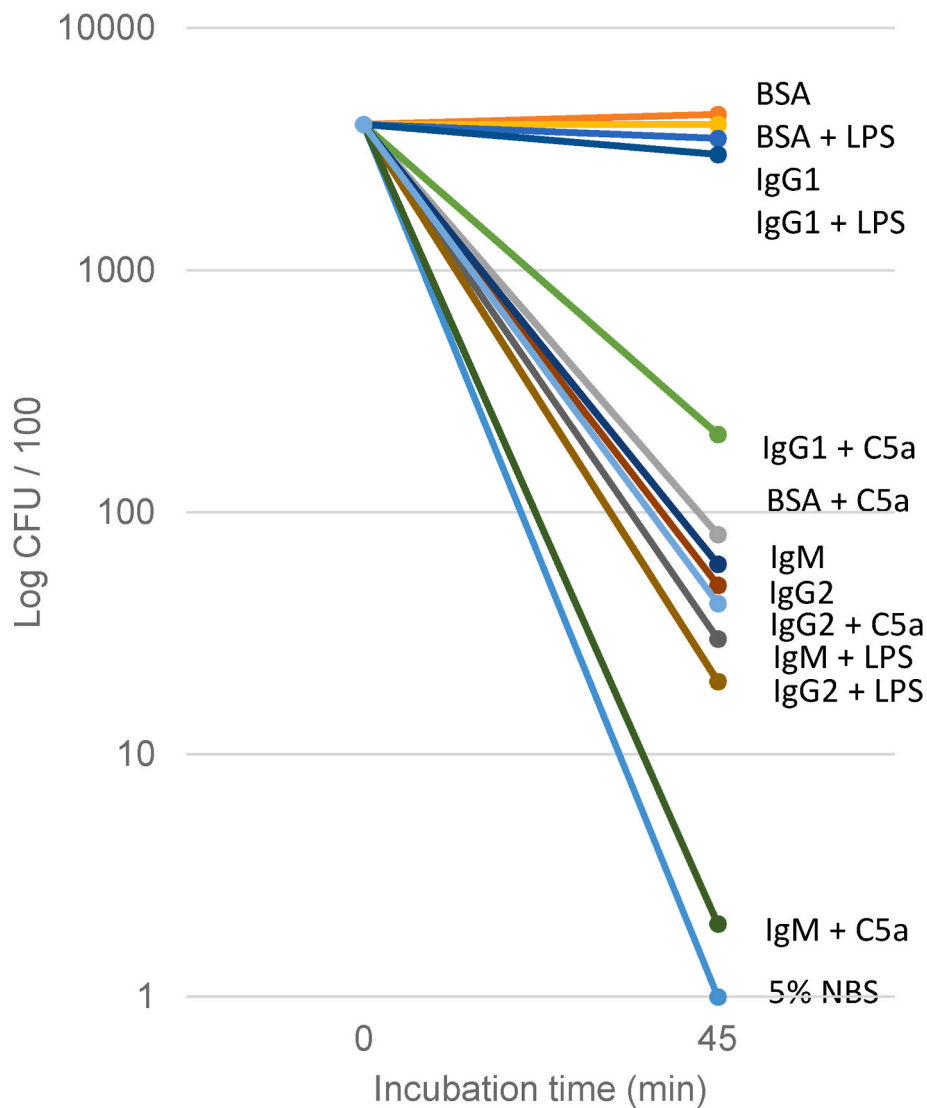
Fig. 6. Killing by neutrophils of bacteria (*E. coli* P4) opsonized with IgM, IgG$_1$, or IgG$_2$ preparations. Bacteria (*E. coli* P4) were suspended in RPMI1660 + BSA and HEPES, and pre-opsonized with either IgG1, IgG2, or IgM. Granulocytes were added with or without stimulating agents (C5a or LPS), and the CFU at the end of a 45 min incubation under agitation were enumerated. The numbers of CFU in the 100 dilution of cell lysate are indicated on a log scale at the beginning (T0) and at the end (T45) of incubation. Results are the means of two separate experiments.

affected by the stimulation of neutrophils with TLR agonists or with C5a, and we have little information in the literature on the binding of IgM to activated bovine neutrophils. However, the decrease in FcμR expression after priming with TNF-α is in accordance with the decrease in IgM binding by TNF-α-exposed neutrophils previously reported (Rainard et al., 2000).

Our results regarding the binding of immunoglobulin isotypes to neutrophils are also in agreement with previous knowledge. The binding of nonaggregated IgG$_2$, IgA, and IgM to bovine neutrophils at 4 °C has been previously documented (Howard et al., 1980; Noble et al., 2023; Zhang et al., 1994). Receptors for rabbit IgG were not found on bovine neutrophils (Howard et al., 1980), as shown in Fig. 1F. Unaggregated IgG$_1$, heat-aggregated IgG$_1$, or IgG$_1$-immune complexes do not bind to bovine neutrophils (Guidry et al., 1993; McGuire et al., 1979; Noble et al., 2023; Rainard et al., 2000). Indeed, we did not find binding of IgG$_1$ to neutrophils, and IgG$_1$-opsonized *E. coli* were not more ingested and killed by neutrophils than unopsonized bacteria (Figs. 5 and 6). As previously reported (Guidry et al., 1993; Howard, 1984; Barrio et al., 2003), bovine neutrophils ingested and killed IgG$_2$-opsonized bacteria. Interestingly, IgG$_2$ did not need a neutrophil stimulating agent to be

effective (Fig. 6). This is in agreement with the reported activating properties of purified IgG$_2$ on bovine neutrophils in the absence of plasma (Tao et al., 1995). The IgM preparation may also have some activating capacity, but this remains to be investigated. Whatever it may be, IgM antibodies were most effective with neutrophils stimulated with C5a. Under this condition, the IgM preparation was by far the most efficient opsonic isotype, rivalling with NBS (Figs. 5 and 6). This could partly result from the lower antibody activity against the assay microorganism (*E. coli* P4) in the IgG$_2$ than in the IgM serum fractions (Supplementary Fig. 2). This might be common to serum from non-vaccinated cows, as it has previously been reported that IgG$_2$ fractions of bovine serum did not opsonize efficiently *E. coli*, even at high concentration (5 mg/mL)(Williams and Hill, 1982). The observation of paucity of IgG$_2$ antibodies may extend to other bacteria such as *S. aureus* (Barrio et al., 2003). These observations suggest that natural or naturally acquired opsonic antibodies to these bacteria are mainly in the IgM isotype and not in the IgG$_2$ sub-isotype. Importantly, neutrophils that had migrated into the mammary gland lumen upon inflammation expressed a high level of FcμR (Fig. 7). This observation is in keeping with the increase in IgM binding to bovine neutrophils after in vivo
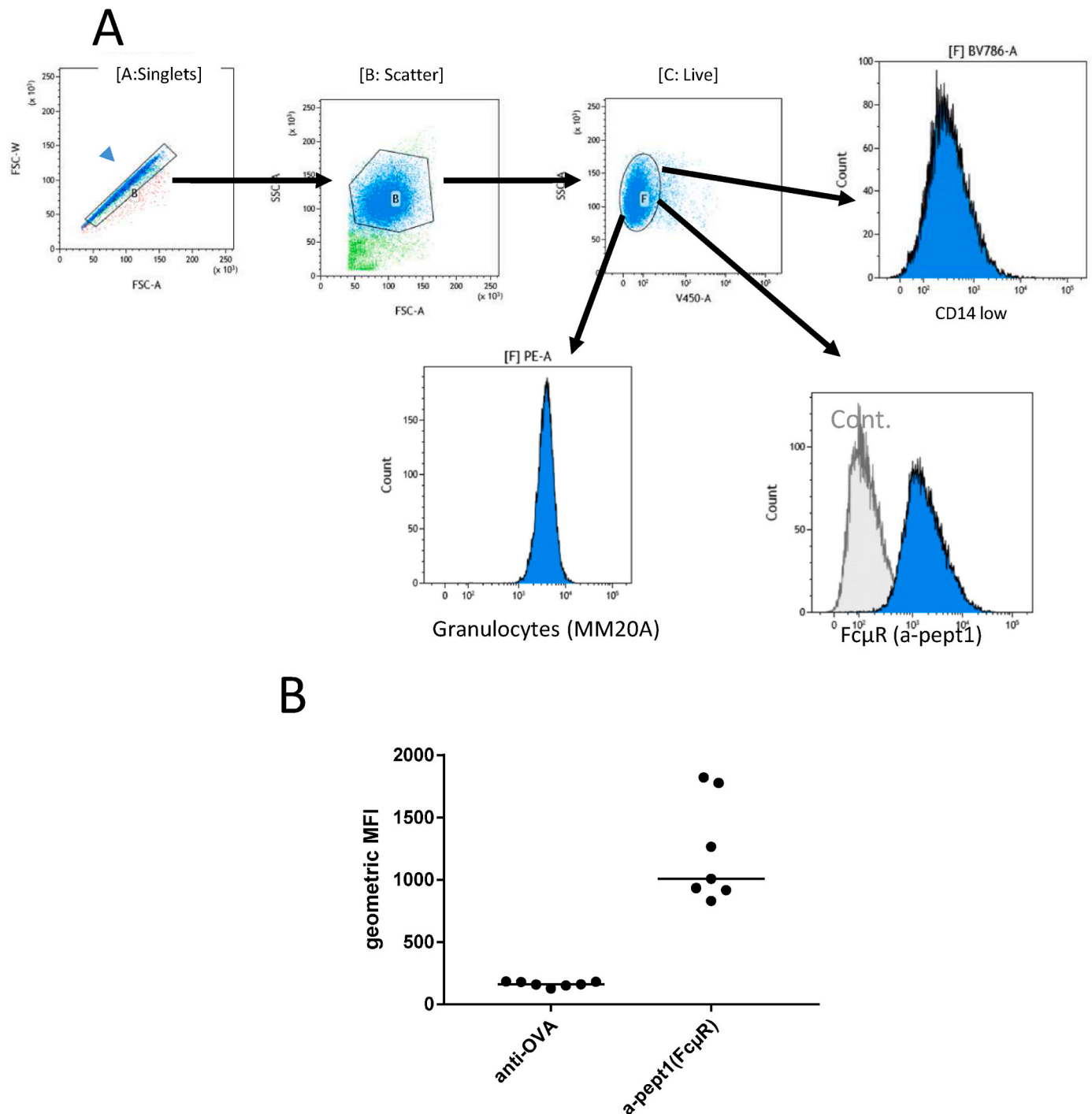
Fig. 7. Hierarchical gating to identify milk neutrophils bearing the FcµR and expression by migrated neutrophils. Milk cells were isolated by centrifugation from milk samples taken from inflamed glands (>300 000 cells/mL). (A) The cells were labeled with antibodies to CD14-SBV790, or to granulocytes (MM20A) and F(ab')$_2$ anti-mouse IgG AF546, or anti-peptide 1 and F(ab')$_2$ anti-rabbit-PE. (B) Cells from 7 quarters of four cows were analyzed. Results shown are the geometric MFI of live neutrophils (gate F) labeled with anti-peptide 1. Negative controls are geometric MFI of live neutrophils labeled with rabbit anti-ovalbumin antibodies. Bars indicate median values.

migration into the mammary gland (Worku et al., 1994).

The high expression of FcµR is a characteristic that distinguishes bovine neutrophils from human or murine neutrophils. Indeed, the expression of FcµR by human or murine neutrophils is a debated issue (Honjo et al., 2013; Lang et al., 2013b), but in any case the expression is weak at best, and human natural antibodies, including IgM, are usually not effective opsonins in the absence of Complement (Boero et al., 2021; Ferrante et al., 1993; Verbrugh et al., 1979). The other major immunoglobulin receptor of bovine neutrophils is the Fcγ2R, which is a characteristic of bovids (Kacskovics, 2004; Zhang et al., 1995), while the low expression of CD16 (FcγRIII) is a peculiarity of cattle since cows do not express this receptor (Noble et al., 2023) but goat, sheep and water buffalo do (Elnaggar et al., 2016, 2019). Expression by neutrophils of the FcµR that is highly effective for the phagocytosis and killing of bacteria, along with natural IgM antibodies that recognize a wide range of pathogens (Racine and Winslow, 2009; Ehrenstein and Notley, 2010)

endow this isotype with a prominent role in the defense of cattle against bacterial infections. This warrants further research on the bovine FcμR, such as its role in neutrophil activation, the existence of intracellular stores, and its expression by other bovine cell types.

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CRediT authorship contribution statement

Florence B. Gilbert: Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Pascal Rainard:** Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2024.105235.

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