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Generation and characterization of monoclonal antibodies to equine CD16

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Abstract

The low-affinity Fc receptor CD16 plays a central role in the inflammatory and innate immune responses of many species, but has not yet been investigated in the horse. Using the predicted extracellular region of equine CD16 expressed as a recombinant fusion protein with equine IL-4 (hIL-4/CD16), we generated a panel of mouse monoclonal antibodies (mAbs) that recognize equine CD16. Nine mAbs were chosen for characterization based upon recognition of CD16, but not IL-4, in ELISA. All nine mAbs recognized full-length, cell-surface CD16 expressed as a GFP fusion protein by CHO cells, but not the closely related Fc receptor CD32 expressed in the same system. In flow cytometric analysis with equine peripheral leukocytes, the mAbs labeled cells in the granulocyte, monocyte, and lymphocyte populations in a pattern consistent with other species. Monocytes that were strongly labeled with CD16 mAb 9G5 were also positive for the LPS receptor CD14. Cytospins made with peripheral leukocytes were immunohistochemically labeled and showed mAb recognition of primarily mononuclear cells. ELISA revealed that the nine mAbs can be grouped into three patterns of epitope recognition. These new antibodies will serve as useful tools in the investigation of equine immune responses and inflammatory processes.

Keywords

FcγRIII; FCGR3; Horse; Cell surface receptor; Leukocyte

1. Introduction

The recognition of immune complexes is a key initiating event in the establishment of innate immune responses and inflammation. A primary mediator of this process is the membrane glycoprotein CD16 (FcγRIII), a low-affinity receptor for the Fc region of aggregated IgG. Together with CD64 (FcγRI) and CD32 (FcγRII), it forms a family of three closely related Fcγ receptors (Ravetch and Kinet, 1991). CD16 is an activating receptor; its effector function is initiated when the extracellular region, comprised of two C2-type

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immunoglobulin-like domains, binds to the Fc regions of IgG in immune complexes (Lanier et al., 1989; Simmons and Seed, 1988; Trinchieri and Valiante, 1993). Depending on the cell type, activation can lead to phagocytosis, antibody-dependent cytotoxicity (ADCC), release of inflammatory mediators, or degranulation. A soluble form of CD16 in human plasma has also been shown to bind complement receptors, resulting in the production of inflammatory cytokines (Galon et al., 1996). Mice deficient in CD16 demonstrate impaired NK cell, monocyte, and mast cell effector functions, as well as reduced inflammation-induced pathology (Hazenbos et al., 1996).

In the human, CD16 exists as two isoforms encoded by closely related genes: a transmembrane form (IIIa) and glycosylphosphatidylinositol (GPI)-linked form (IIIb). The extracellular regions of the two proteins are nearly identical and differ by only six amino acids (Ravetch and Perussia, 1989). The transmembrane form is expressed on NK cells, monocytes, macrophages, mast cells, and small T-cell subsets, while the GPI-linked form is expressed exclusively on neutrophils (Ravetch and Kinet, 1991). In non-primate species that demonstrate CD16 expression, no glypiated forms have been identified, and all CD16⁺ cell populations are thought to express the transmembrane form (Collins et al., 1997; Halloran et al., 1994; Hughes, 1996; Nishimura et al., 2000).

Gene expression of an equine CD16 ortholog has recently been demonstrated in peripheral and endometrial cup lymphocytes (Noronha et al., *in press*). CD16 molecules of other livestock species have been successfully identified at the protein level with commercially available cross reactive antibodies (Boysen et al., 2008; Elhmouzi-Younes et al., 2010). However, this has not been the case in the horse. Ibrahim and Steinbach (2007) reported that the anti-human CD16 mAbs 5D2 and MEM-154 failed to react with horse cells in a manner consistent with the expected pattern for CD16. We obtained similar results using the anti-human CD16 mAb 3G8 (data not shown). Researchers studying equine immunology and inflammation have therefore been limited by an inability to study this fundamental immunoreceptor. Here, we describe the generation of a recombinant equine CD16 protein, and the subsequent development of a panel of CD16-specific monoclonal antibodies that can be used in multiple immunological applications to address this knowledge gap.

2. Materials and Methods

2.1. Recombinant IL-4/CD16 (rIL-4/CD16)

Equine *CD16* was amplified from horse PBMC cDNA using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). A multispecies comparison of the amplified gene product and analysis of predicted protein structural motifs are described in detail elsewhere (Noronha et al., *in press*). The amplification product was gel purified/extracted, cloned into pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and sequenced on an Applied Biosystems Automated 3730 DNA Analyzer at the Cornell Life Sciences Center; sequence IDs and PCR primers listed in Table 1. Sequences were analyzed using the DNASTar software suite. The amplified sequence was identical to a region of equine chromosome 5 that is syntenic to the FcγR region of human chromosome 1. No evidence of a second soluble form of *CD16* was found in the genome. The extracellular domain (bases 52-579 of the coding sequence) was predicted by performing Clustal W alignments with validated *CD16* sequences of other species, and was directionally cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) 3' to the coding sequence of equine *IL-4* as previously described (Wagner et al., *under review*). CHO K-1 cells were transfected with the linearized vector using the Geneporter2 system (Genlantis, San Diego, CA). Stable transfectants were selectively cultured in G418 (Invitrogen), cloned by limiting dilution, and screened for IL-4 production by flow cytometry and ELISA as previously described (Wagner et al., *under review*). rIL-4/CD16 was purified from serum-free supernatant by fast protein liquid chromatography using an

anti-IL-4 affinity column as previously described (Wagner et al., *under review*). A fraction of the purified fusion protein was resolved by SDS-PAGE on a 10% non-reducing polyacrylamide gel to determine molecular weight.

2.2. Immunization and splenic fusion

Immunization was performed as previously described (Wagner et al., 2003). Briefly, female BALB/c mice were immunized i.p. on days 0, 14, 21, 28, 29 and 30 with α IL-4/CD16 in PBS and Gerbu Adjuvant MM (Gerbu Biotechnik, Gaiberg, FRG). Animal response was measured by monitoring serum titers to equine IL-4 using ELISA. On day 31, spleen cells were fused to SP2/0 myeloma cells as previously described (Appleton et al., 1989). Nascent hybridomas were plated into 96 well tissue culture plates and supernatants from all wells were screened for reactivity to α IL-4/CD16 and α IL-4/IgG (Wagner et al., 2005) using ELISA, and for cell surface labeling of equine PBMC using flow cytometry. Antibodies which labeled PBMC and detected α IL-4/CD16 but not α IL-4/IgG were selected for further study. Cultures were cloned by performing three rounds of limiting dilution, measuring sensitivity and specificity of secreted immunoglobulin by ELISA and flow cytometry as above after each round. Mouse immunoglobulin isotypes of secreted antibodies were determined by ELISA (Sigma, St. Louis, MO). Antibodies were purified by fast protein liquid chromatography using a protein G affinity column (GE Healthcare, Piscataway, NJ). Proteins were quantified using a Bradford assay (Bio-rad, Hercules, CA). Selected antibodies were biotinylated using Sulfo-NHS-Biotin (Thermo Fisher Scientific, Waltham, MA).

2.3. Antibody screening and ELISA

Cell culture supernatants were screened for mAbs to α IL-4/CD16 by ELISA as described previously (Wagner et al., *under review*) and against α IL-4/IgG1 (Wagner et al., 2005) to confirm their specificity to CD16. Isotype controls were mAbs recognizing canine parvovirus (CPV, IgG1) or H3N8 (IgG2b). For the epitope ELISA, plates were coated with 1 μ g purified mAbs, washed, then followed with α IL-4/CD16 fusion protein. Following washing, 1 μ g of biotinylated antibodies were then added, followed by streptavidin-HRP. Reactions were developed and analyzed as previously described (Wagner et al., 2006). For mAb 2A2, an additional ELISA was performed where plate-bound fusion protein was incubated with unlabeled antibody prior to adding the biotinylated antibody.

2.4. GFP fusion protein expression and flow cytometric analysis

Full-length sequences (minus termination codons) of equine *CD16*, *CD32*, and *IL-4* genes were PCR amplified using *Pfu* DNA polymerase from equine PBMC cDNA and directionally cloned into the multiple cloning region of the pEGFPN1 vector 5' to the *EGFP* gene (PCR primers listed in Table 1). CHO-K1 cells were transfected with the vectors using the Geneporter2 system and assayed for protein expression 48 hours post-transfection. Successful expression of GFP was confirmed by fluorescence microscopy and indicated correct reading frame cloning of the fusion protein, as GFP sequence was downstream of the protein of interest. Cells were detached with trypsin and used either fresh or fixed with 2% PFA for 20 minutes. Cells were labeled in FACS buffer [PBS, 0.5% BSA, 0.02% NaN₃ with (fixed) or without (fresh) 0.5% saponin] with mAb supernatants, IgG1 and IgG2b isotype controls, or anti IL-4 mAb 13G7 (Wagner et al., 2006) followed by DyLight649-conjugated goat-anti-mouse IgG (H+L) F(ab')₂ (Jackson ImmunoResearch, West Grove, PA). Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and with FlowJo Software (Tree Star, Inc., Ashland, OR).

2.5 Leukocyte flow cytometry and immunohistochemistry

Leukocytes were isolated from horses of the Cornell Equine Genetics Center (animal details in Table S1). Erythrocytes were removed from heparinized blood samples using isotonic lysis (0.2% NaCl followed by 1.6% NaCl). Leukocytes were washed and assayed for viability using trypan blue exclusion and phase contrast microscopy. One million fresh cells were labeled with mAb 9G5 and/or a monoclonal antibody recognizing equine CD14 (Kabithe et al., 2010), or an isotype control mAb recognizing canine parvovirus (CPV, IgG1) or H3N8 (IgG2b), and analyzed by flow cytometry. Dead cells were excluded following staining for viability with propidium iodide. Five hundred thousand leukocytes were adhered to a glass slide with a Cytospin centrifuge, fixed in acetone, and labeled with mAbs as previously described (de Mestre et al., 2010).

3. Results

3.1. Expression of α IL-4/CD16 and selection of mAbs to equine CD16

To generate monoclonal antibodies to equine CD16, a system was employed that uses a recombinant secreted fusion protein made with equine IL-4 and the protein of interest (Wagner et al., *under review*). To create the fusion protein, the predicted extracellular domain of CD16 was PCR-amplified from equine PBMC cDNA and inserted into a mammalian expression vector downstream of equine IL-4 (Fig. 1A). CHO-K1 cells were stably transfected with this construct and the secreted fusion protein was purified from the culture medium by fast protein liquid chromatography using an equine IL-4 affinity column. The size of the recovered protein was consistent with the 36.7 kDa predicted molecular weight of α IL-4/CD16 (Fig. 1B). The extracellular domain of equine CD16 contains two predicted N-linked glycosylation sites (N-X-S/T-X), therefore the three bands observed on SDS-PAGE likely represent differentially glycosylated species of the fusion protein (Fig. 1B, arrowheads). The fusion protein was used to immunize mice, the spleens of which were recovered to generate hybridomas. Media samples from the hybridomas were screened for the ability to recognize the α IL-4/CD16 fusion protein in ELISA (Fig. 1C). Specificity of this recognition was determined by testing the media samples against a α IL-4/IgG1 fusion protein (Fig. 1C). Antibodies that recognized the CD16 fusion protein, but not α IL-4/IgG1, were considered CD16 specific. MAb generated by nine hybridoma clones that met these initial screening criteria were selected for further characterization.

3.2. MAbs recognize full-length equine CD16

To test the ability of the nine mAbs to recognize full-length CD16 expressed on the cell surface the full coding sequence (minus the stop codon) was cloned into the pEGFPN-1 mammalian expression vector upstream of the *EGFP* gene (Fig. 2A). This allowed membrane expression of CD16 with minimal alterations to surface protein conformation, as eGFP would be expressed on the cytosolic side of the cell membrane. CHO-K1 cells were transfected with the construct and GFP expression was verified by fluorescence microscopy. The cells were labeled with the nine mAbs and analyzed by flow cytometry, where they demonstrated robust recognition of the full-length CD16 protein (Fig. 2B). Because CD16 is one of three closely related Fc receptors known to have significant sequence similarity in other species, we also tested the specificity of the selected mAbs to CD32, the Fc γ R with the most predicted similarity to CD16. Equine *CD32* was similarly cloned into pEGFPN-1 and expressed in CHO cells; none of the nine antibodies recognized the CD32 construct in flow cytometric analysis (Fig. 2B). MAb labeling was performed on fresh cells, so that only native, cell surface protein could be recognized (data not shown) as well as fixed, permeabilized cells, so that control cells producing soluble IL-4-GFP could be included to confirm the lack of mAb reactivity to IL-4 seen in ELISA (Fig. 2B). All mAbs demonstrated the ability to recognize membrane-expressed CD16 in a specific manner.

3.3. MAbs recognize native CD16 on equine leukocytes

Next, the ability of the antibody panel to recognize native CD16 on intact horse cells was tested. Total equine leukocytes were used so that the granulocyte, monocyte, and lymphocyte populations could be investigated. First, the labeling patterns of the nine mAbs were compared on samples from one animal. The mean fluorescence intensity (MFI), an indicator of relative antigen density, varied between the three cell populations in a pattern that was similar for all mAbs (Fig. 3A, exceptions noted in Table 3). Granulocytes were dimly labeled, most lymphocytes and monocytes were labeled with moderate intensity, and a small population of cells in the monocyte/large lymphocyte size range labeled very intensely, indicating the highest levels of expression. The percent of total leukocytes labeled varied between 1.5 and 7.6 (median=3.0%) and the observed patterns were similar to Fig. 3A for all mAbs.

In order to further investigate the leukocyte populations labeled by these mAbs, cells were isolated from seven additional horses, labeled with representative mAb 9G5, and analyzed by flow cytometry. The lymphocyte, granulocyte, and monocyte/large lymphocyte populations were gated based upon morphology (Fig. 3B) and analyzed for percent labeled within each group (Fig. 3C). Individual animal variability of roughly 3- to 4-fold was observed for each cell population. The number of labeled granulocytes varied from 1.2% to 8.3% (median=3.1%); monocytes and large lymphocytes varied from 4.6% to 13.9% (median=8.4%); and lymphocytes varied from 1.1%-3% (median=2.1%).

In order to visualize the labeled cells, leukocytes were adhered to glass slides and labeled with the mAbs by immunohistochemistry (Fig. 3D). No positively stained granulocytes were observed, possibly due to a low receptor density as evidenced by the low MFI observed in the flow cytometry experiments. Among the labeled cells, the most intensely stained (9G5^{hi}) had large lymphocyte and monocyte morphologies, while smaller lymphocytes and some monocytes were moderately or dimly stained (9G5^{lo}). These observations are consistent with the MFI values of these populations observed in Fig. 3A.

The 9G5^{hi} monocyte/large lymphocyte population was further investigated using 2-color flow cytometry. In humans, cows, and sheep, a substantial fraction of CD16⁺ monocytes are positive for the LPS receptor CD14 (Elhmouzi-Younes et al., 2010; Ziegler-Heitbrock, 2007). To determine whether this is also the case in the horse, leukocytes from 6 horses were labeled with mAb 9G5 and a monoclonal antibody to CD14. Cells in the monocyte/large lymphocyte population were gated and analyzed as above. In all horses, the 9G5^{hi} cells in this population were consistently CD14⁺ (Fig. 3E).

3.4. MAbs recognize three different epitopes

Finally, the nine mAbs were assayed by ELISA in order to determine if they recognized different epitopes of the antigen. *h*IL-4/CD16 protein was captured using each of the mAbs. Biotinylated mAbs were then applied to determine if the remaining free sites on the fusion protein could be bound, indicating recognition of distinct epitopes (Table 2). Based on this assay, the mAbs were assigned to one of three groups of epitope recognition. A summary of the anti-equine CD16 mAbs is shown in Table 3. One mAb, 2A2, appeared to recognize a repeating epitope based upon its ability to bind *h*IL-4/CD16 already captured by 2A2. To test this, we incubated the captured fusion protein with increasing concentrations of unlabeled 2A2 to saturate remaining epitopes prior to probing with biotinylated 2A2. Binding of the biotinylated antibody was reduced in a dose-dependent manner, indicating that the mAb bound multiple sites on the protein (data not shown).

4. Discussion

We determined that the nine mAbs characterized here specifically recognize CD16 in multiple assays. In addition to the specificity demonstrated by ELISA and recognition of transfected cells, the consistent pattern observed in flow cytometric analysis of leukocytes indicates recognition of the same cell surface molecule among the numerous immunoreceptors on the leukocyte surface. Some variation in the percentage of labeled leukocytes was observed between mAbs. This may be due to factors such as differences in affinity for antigen, variability in antibody concentration within the hybridoma culture media samples, or subtle differences in epitope recognition due to differential glycosylation of leukocyte populations. Some variation was also observed when leukocytes isolated from different horses were labeled. This difference among individuals is not unexpected. We have previously observed that cell surface expression of immunoreceptors can vary markedly among individual horses of mixed genetic backgrounds housed in an outdoor environment (de Mestre et al., 2010). Furthermore, expression of CD16 is known to be up regulated during inflammation in other species (Fingerle et al., 1993; Strauss-Ayali et al., 2007; Ziegler-Heitbrock, 2007). As the sampled horses are exposed to standard environmental insults, it is reasonable that individual animals may be in different states of normal, sub-clinical inflammation, therefore contributing to variability in CD16 expression.

Comparison of leukocytes among livestock species shows some variations in CD16 expression patterns. In the pig, CD16 is expressed on 13% lymphocytes, 70% monocytes, and ~95% neutrophils (Dato et al., 1992). CD16 expression on sheep PBMC is reported at a median 9.1% (7.5-12.6) and 10.2% (8.1-11) for cows, in a pattern similar to Fig. 3A (Elhrouzi-Younes et al., 2010). To compare our data in context, the percent of labeled equine PBMC was calculated by gating only the mAb 9G5-labelled monocyte and lymphocyte populations, yielding a median 3.7% (1.1-8.1). One explanation for this difference among livestock species may be an age-related difference in CD16 expression. The animals used for the cow and sheep studies were juveniles, while the horses used here were adults over 5 years of age. The difference may also be species-related, which is not surprising as CD16 expression levels vary significantly even among closely related non-human primates (Rogers et al., 2006). Notably, however, the pattern of equine CD14⁺CD16⁺ monocytes that we observed here was strikingly similar to that seen in humans (Fingerle et al., 1993). This is particularly interesting considering that mice do not have a monocyte population with this phenotype (Sunderkotter et al., 2004). Therefore, the horse may serve as a useful model species for the study of this human cell type.

5. Conclusion

The antibodies described here, summarized in Table 3, demonstrate recognition of recombinant CD16 in ELISA and full-length CD16 in flow cytometry. This recognition appears to be specific for CD16 and does not occur with other structurally-similar immunoreceptors. These antibodies label equine leukocyte populations in a pattern that is consistent with CD16 expression observed in other species. We therefore conclude that this panel of monoclonal antibodies recognizes the equine CD16 antigen in ELISA, flow cytometry, and immunohistochemistry, and will expand our ability to study horse health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Construction and expression of *rIL-4/CD16*, and utilization to identify anti-CD16 hybridomas. (A) The predicted extracellular domain of equine *CD16* was directionally inserted 3' to equine *IL-4* in a pcDNA3.1 expression vector (*CMV*, Human cytomegalovirus immediate-early promoter; *pA*, Bovine Growth Hormone polyadenylation signal). (B) Soluble *rIL-4/CD16* was expressed in CHO cells, affinity purified, and resolved by SDS-PAGE on a 10% gel under non-reducing conditions (lane 2). The size of the purified protein was consistent with the predicted molecular weight of 36.7 kDa. Arrowheads denote multiple glycosylation forms; lane 1 shows a molecular weight marker. (C) Culture media from hybridomas were tested by ELISA for the ability to recognize *rIL-4/CD16* and *rIL-4/IgG1*; negative mAb control, anti-CPV.

Figure 2.

Generation of full-length CD16-, CD32-, and IL4-GFP expression constructs and flow cytometric analysis showing recognition by anti-equine-CD16 mAbs. (A) Full length *CD16*, *CD32*, and *IL-4* coding sequences (minus stop codons) were directionally inserted into the pEGFP-N1 mammalian expression vector 5' to *EGFP* (CMV, Human cytomegalovirus immediate-early promoter; *pA*, SV40 polyadenylation signal). (B) CHO cells transfected with fusion constructs were labeled with anti-equine-CD16 mAbs and a fluorescently-labeled anti-mouse immunoglobulin secondary antibody (goat-anti-mouse-IgG (H+L)-647) and analyzed by flow cytometry. MAb 9G5 is shown as a representative mAb. Anti-equine-IL-4 mAb (13G7) and sera taken from the mouse spleen donor prior to immunization (*pre-immunization*) and at spleen collection (*post-immunization*) were used as controls.

Figure 3.

Recognition of horse peripheral blood leukocytes by anti-CD16 mAb 9G5. (A) Isolated leukocytes were labeled with IgG1 control mAb (anti-CPV, left) or clone 9G5 (right) and analyzed by flow cytometry. (B) Dot plot showing morphology of positive staining cells (black) in relation to total cells (grey) and gating strategy for leukocyte populations (G, granulocytes; M/LL, monocytes/large lymphocytes; L, lymphocytes). (C) Graph depicting percentages of 9G5-labeled cell populations for eight horses. (D) Immunohistochemical labeling of peripheral leukocytes with mAb 9G5 (1000X) showing a representative brightly-stained lymphocyte (top) and dimly-stained monocytic cell (bottom). (E) Leukocytes were labeled with mAb 9G5 in conjunction with a mAb recognizing equine CD14. Dot plots show M/LL populations as indicated in B.

Table 1

Sequences of genes and primers used.

Gene	Accession #	Primers (5'-3')
<i>CD16</i>	JN795139	<i>IL-4 fusion construct</i> F-GGCGGATCCTGGCACACGAGCTGAAGATC R-GGCAAGCTTTCAACCTTTAACAATGACGTTTCATAGCC
		<i>GFP fusion construct</i> F-GGCGCTAGCATGTGGCAGATGCTATCACCAACGG R-GGCGGTACCTCAGAGCCCCGGCTCCATGTG
<i>IL-4</i>	GU139701	<i>GFP fusion construct</i> F-GGCGCTAGCATGGGTCTCACCTACCAACTGATTCCAG R-GGCGGTACCTCACACTTGGAGTATTTCTTTTCATGATCGTCTTTAGC
<i>CD32</i>	JN795138	<i>GFP fusion construct</i> F-GGCGCTAGC ATGACTATGGAGATCCTGATGTTTCCAAATGTACATC R-GGCGGTACCTCTTGTTCAGGGCTCTGGCTCCA

Table 2
ELISA and Epitope Recognition Group Assignments

		2. Biotinylated Antibody										Group Assignment
		1A2	2A2	3B8	3E8	7G8	8B7	8D1	9H4	9G5		
1. Capture Antibody	1A2										A	
	2A2		*								C	
	3B8										A	
	3E8										B	
	7G8										A	
	8B7										B	
	8D1										B	
	9H4										B	
	9G5										A	

Black: Reduced binding by biotinylated antibody; White: No reduction in binding;

* based on additional assays, mAb 2A2 binds a repeating epitope on CD16

Table 3

Summary of αCD16 mAbs

Epitope Group	mAb	Isotype	Application			
			ELISA	FACS ¹	IHC ⁴	
A	1A2	IgG1	+	++	++	
	3B8	IgG1	+	++	+	
	7G8	IgG1	+	+	+ ⁵	
	9G5	IgG1	+	++	++ ⁵	
B	3E8	IgG2b	+	+ ²	+++ ⁵	
	8B7	IgG1	+	+	+	
	8D1	IgG1	+	+ ³	+	
	9H4	IgG1	+	+	+	
C	2A2	IgG1	+	++	++	

¹Labels both fresh and PFA-fixed cells;
²Labels additional lymphocyte population (common IgG2b artifact with fresh horse cells);
³Labels a small fraction of granulocytes more brightly;
⁴Frozen acetone-fixed cells (and tissues, data not shown);
⁵Also labels PFA-fixed cells (other mAbs not tested);