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Methods in isolation and characterization of bovine monocytes and macrophages

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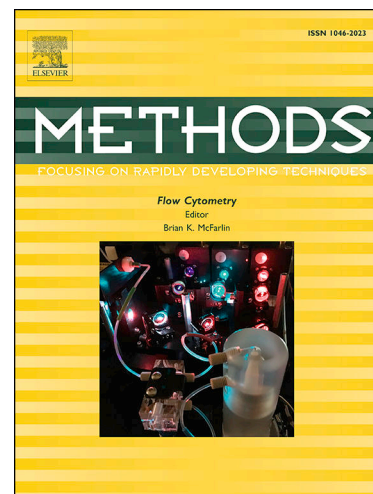
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1 Methods in isolation and characterization of bovine monocytes and macrophages

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14

15 Abstract

16

17 Monocytes and macrophages belong to the mononuclear phagocyte system and play important roles
18 in both physiological and pathological processes. The cells belonging to the monocyte/macrophage
19 system are structurally and functionally heterogeneous. Several subsets of monocytes have been
20 previously identified in mammalian blood, generating different subpopulations of macrophages in
21 tissues. Although their distribution and phenotype are similar to their human counterpart, bovine
22 monocytes and macrophages feature differences in both functions and purification procedures. The
23 specific roles that monocytes and macrophages fulfil in several important diseases of bovine species,
24 including among the others tuberculosis and paratuberculosis, brucellosis or the disease related to
25 peripartum, remain still partially elusive. The purpose of this review is to discuss the current
26 knowledge of bovine monocytes and macrophages. We will describe methods for their purification
27 and characterization of their major functions, including chemotaxis, phagocytosis and killing,
28 oxidative burst, apoptosis and necrosis. An overview of the flow cytometry and morphological
29 procedures, including cytology, histology and immunohistochemistry, that are currently utilized to
30 describe monocyte and macrophage main populations and functions is presented as well.

31

32

33 Keywords:

34 monocytes; macrophages; innate immunity; bovine

1. Introduction: monocytes/macrophages and their main functions.

35
36
37 Monocytes are myeloid cells produced by the bone marrow that play pivotal roles in the immune
38 response against infections and injuries. Monocytes and their corresponding tissue derivatives,
39 including macrophages and subpopulations of dendritic cells, are involved in almost all phases of the
40 inflammation and bridge innate and specific immune responses [1]. In the bone marrow,
41 hematopoietic stem cells differentiate to precursor cells. Pre-monocytic cells produce peripheral
42 blood monocytes that transform into resident macrophages upon tissue entry [2]. Resident
43 macrophages are among the first cell populations to sense a tissue pathogen or injury and generate
44 the initial wave of cytokines and chemokines, alerting the immune system to incoming pathogens or
45 tissue damage, skewing immune defences and activating and recruiting additional immune cells at
46 the site of inflammation [2]. Functions of macrophages vary according to their location and type of
47 activation. Alveolar macrophages are located in the lungs, and provide cellular defence against
48 inhaled antigens, clearing the air spaces of infectious, toxic, or allergic particles that have evaded the
49 mechanical defences of the respiratory tract [3,4]. In the brain, macrophages are identified as resident
50 microglial cells and protect the Central Nervous System (CNS). Microglial macrophages play a
51 pivotal role in synaptic pruning during development [5]. In a healthy status, microglia facilitate
52 learning and memory and scavenge cellular or other debris. After CNS infections and injury, activated
53 microglia fulfil the functions of resident macrophages during inflammation, recognizing pathogens
54 or injured cells, activating phagocytic, antigen-presenting and cytokine/chemokine secretion
55 functions. Activated microglia also contribute to the resolution of inflammation via switching to anti-
56 inflammatory cytokine patterns, and promote intercellular matrix synthesis and angiogenesis.[6]. In
57 the CNS, beside microglia, macrophages are also found in perivascular, meningeal and choroid plexus
58 spaces, participating to the blood-brain barrier by modulating the entrance and the phenotype of
59 immune cells during inflammation [7]. In lymph nodes, macrophages are classified as spinal or
60 subcapsular sinusoidal, their main role being that of capturing antigens and presenting them to B cells
61 [8]. The intestine provides one of the largest pool of macrophages in the body. Resident macrophages
62 act as sentinels for pathogen recognition and elimination, maintaining the intestinal homeostasis by
63 shaping host-microbiota symbiosis and regulating gut inflammation, promoting the crosstalking with
64 T cells [9,10]. The splenic red pulp is a large tissue compartment containing macrophage lines able
65 to perform several and diverse functions. The main role of splenic red pulp macrophages is to engulf
66 senescent and altered erythrocytes, break down haemoglobin, recovering iron to the bone marrow,
67 thus contributing to iron homeostasis [11] and to monitor, detect and phagocytize bloodborne
68 pathogens. Other equally important functions of splenic red pulp macrophages include presenting

69 antigens to T lymphocytes of the splenic periarteriolar sheaths [12]. In the liver, finally, resident
70 macrophages are named Kupffer cells. In humans, Kupffer cells account for a large pool of tissue
71 macrophages, and their main role is to remove toxins and pathogens [13].

72 Although the main role of macrophages is related to the protection against pathogens, they contribute
73 to the resolution of inflammation by at least three ways: a) removing the pathologic stimulus by
74 phagocytosis; b) producing anti-inflammatory cytokines, including for example TNF- α soluble
75 receptor of IL1-R antagonist and c) removing leukocytes and necrotic debris in the inflammatory site.
76 Macrophages carry out also tissue-repairing function and actively participate in the development of
77 many autoimmune diseases development [1].

78 Peripheral blood (circulating) monocytes are cells with a consistent morphology (Fig. 1), and for
79 many years they were regarded as a single functional population identified by the surface expression
80 of the lipopolysaccharide receptor (CD14). However, the development of flow cytometry and multi-
81 colour immunofluorescence, have allowed more detailed differentiation of monocytes, based on the
82 additional expression of CD16 (Fc γ IIIR), the low-affinity receptor for the Fc regions of IgG [14].

83 According to CD14 and CD16 expression over the surface of the cells, at least two subpopulations of
84 human monocytes: (CD14⁺⁺(bright)CD16⁻ and CD14⁺(dim)CD16⁺) have been identified. Further
85 studies introduced, according with CD16 intensity of expression, other monocyte subpopulations,
86 namely CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ [15]. Given their CD14 and CD16 membrane surface
87 asset, circulating human blood monocytes can be divided into three different subpopulations [16]:

- 88 a) Classical monocytes (cM): “CD14⁺⁺ CD16⁻” cells providing approximately 90% of human
89 blood monocytes.
- 90 b) Intermediate monocytes (intM): “CD14⁺⁺ CD16⁺ cells” (approximately 10% of human blood
91 monocytes)
- 92 c) Non-classical monocytes (ncM): “CD14⁺ CD16⁺⁺” cells (in humans, a population with
93 variable percentage).

94 Similar to humans, three monocyte subsets have been identified in bovine peripheral blood as well
95 [17,18]. The heterogeneity and the functional differences between the three populations of bovine
96 monocytes have been recently and extensively reviewed [19]. The authors compared bovine
97 monocytes with their analogue human population, focusing on sub-type distribution and their main
98 inflammatory functions, including adhesion and chemotaxis. Indeed, bovine monocyte specific
99 functions (Table 1) have been studied in a relatively limited number of papers [18–20] and only
100 partially resemble those of human counterparts.

101
102**Table 1**

The inflammatory activity of bovine monocytes subsets				
Sub-type of monocytes	Relative percentage	Phagocytic capability ¹	ROS production ²	Inflammasome activation
Classical monocytes (cM) CD14 ⁺⁺ CD16 ⁻	89%	+++	++	++
Intermediate monocytes (intM) CD14 ⁺⁺ CD16 ⁻	5-10%	++	+++	+++
Non-classical monocytes (ncM) CD14 ⁺ CD16 ⁺⁺	5/10%	+	+	+

103

¹ Phagocytic capability is expressed as percentage of FITC-positive cells within viable monocytes [18]: +++ = 80%, ++ = 56%, + = 40%

² ROS production is expressed in Mean Fluorescence intensity (MFI). +++ = 30 x 10³, ++ = 50 x 10³, + = 5 x 10³.

³ IL1 β production (pg/mL) after ATP and LPS stimulation. +++ 4000 pg/mL, ++1000 pg/mL, + negligible

109

110

Differences between macrophage activation patterns between cattle and other species also exist. For example, stimulating bovine monocyte-derived macrophages with pro-inflammatory signals do not produce the same reactions as compared to human and murine macrophages [19,21].

In humans and rodents, several other macrophage categories have been reviewed [22], the most important of which are listed in Table 2. The presence of most of these sub-groups in bovine peripheral blood has yet to be demonstrated.

116

Table 2

118 Principal macrophage subcategories and phenotypes in human and rodents

Macrophages sub-group	Type of response	Activity	Reference
M1	Pro-inflammatory,	Facilitate immunity to remove foreign pathogens and tumour cells, mediate tissue damage induced by ROS, impair wound healing and tissue regeneration	[22,23]
M2	Anti-inflammatory		[24]
	M2a	Tissue repair and wound healing	[25,26]
	M2b	Stress oxidative, efferocytosis	[27]
	M2c	Pro-fibrotic activity phagocytosis of apoptotic cells	
	M2d	Tumour-associated macrophages, angiogenesis and cancer metastasis	[28]
M3	Pro-inflammatory	Switch between M2 to M1 response, anti-tumour effect	[29,30]
M4	Pro-inflammatory	Predominant in atherosclerotic lesions	[31]

		low phagocytosis capability, high tissue repair capability	
M17	Pro-inflammatory	Increased phagocytosis in the presence of oxidized LDL	[21,32]
Mreg	Anti-inflammatory	Produce high levels of nitric oxide, CD86 and MHC Class II, Intense activation and proliferation, of IL-10 producing T cells	[33]
Mox	No clear pro- or anti-inflammatory activity	Weak phagocytosis, proatherogenic Development in response to oxidative stress damage	[34,35]

119
120 The transcriptomic data also revealed differential gene transcription between the three major
121 monocyte subclasses of genes involved in immune defences, providing the evidence that monocytes
122 subsets express different transcriptomes [36]. Indeed, the functions related to CD16⁺ have to be
123 revised and probably attributed between intermediate and non-classical subsets [37,38]. A sequential
124 development between the two subsets was suggested [39], based on the concept that macrophages
125 adopt *in vivo* several functional phenotypes, depending on continuous changes of the tissues'
126 microenvironments. Given this background, M1/M2 and other subgroups are therefore not definite
127 subsets but may represent the extremes of a continuum of different functional states [40].

128 129 2. Purification of bovine monocytes from blood: sorting or adherence

130
131 Normal blood values for monocytes in cows range from 25 to 840 cells/ μ L (average 400 cells/ μ L)
132 and changes with age [41]. Purification of bovine monocytes starts from the collection of peripheral
133 blood from the jugular or coccygeal veins, using a sterile Vacutainer™ blood collection vial
134 containing EDTA (Becton Dickinson, USA). When a large amount of blood is needed (e.g. more than
135 200 μ L) the use of blood bags containing citrate phosphate dextrose (Terumo, Japan) is preferred as
136 a less stressful procedure for the animal. Blood collection must be as fast as possible for welfare
137 issues and because the production of glucocorticoids associated with prolonged containment can
138 profoundly affect *in vitro* monocyte activities. Although published several years ago, the paper by
139 Godderies and co-workers [42] still provides a very good background for monocyte purification from
140 bovine blood, specifically for the techniques related to adherence to glass or plastic wares. In general,
141 methods to purify monocytes can be divided into two groups: those related to the adhesive capability
142 of monocytes and those related to monocyte sorting by magnetic-activated cell sorting (MACS,
143 Miltenyi Biotec, Germany).
144 The purification procedure based on the adhesiveness to tissues has been previously described by the
145 team of Godderies and coworkers [42], whereas the procedure for the monocyte sorting by MACS

146 technique has been first described by Ceciliani et al. [43]. Figure 2 presents the morphology of
 147 cultured monocytes following MACS purification technique after 1, 2 and 4 days of culture. The first
 148 steps of purification are similar for both methods. Blood is collected into tubes (or bags) containing
 149 anticoagulant and processed within 1 hour of collection. Blood is centrifuged at $1260 \times g$ for 30 min
 150 at RT to obtain the buffy coat. Mononuclear cells are recovered after dilution of the buffy coat 1:2.5
 151 (v/v) in PBS/2mM EDTA, and 30 ml of this solution is overlaid on 15 ml Ficoll 1077 (Amersham
 152 Biosciences) and centrifuged for 30 min at $1500 \times g$ at 4°C . Mononuclear cells (i.e. monocytes and
 153 lymphocytes) are collected from the interface, also called mononuclear ring. The cells are
 154 resuspended in 50 ml PBS/2 mM EDTA and centrifuged for 7 min at $400 \times g$ at 4°C to remove
 155 platelets. To avoid non-specific activation of monocytes, it is pivotal to remove as many platelets as
 156 possible from the mononuclear cell fraction by performing second centrifugation in a 4% to 20%
 157 sucrose gradient layered over Ficoll-Paque PLUS (Sigma-Aldrich, USA). This procedure will
 158 effectively remove any platelet contamination [45,46]. Alternatively, consecutive centrifugations at
 159 low speed ($400 \times g$ for bovine monocytes), considerably reduces the number of platelets, that remains
 160 in the supernatant [43]. Table 3 summarizes the two protocols.

161

162 **Table 3.**

163 Purification methods for bovine monocytes *

Methods	Protocol
Adherence	A. Polystyrene surfaces
	1. Isolated PBMCs are cultured in 75 cm^2 polystyrene culture flasks and incubated for 2 h at 38°C .
	2. No-adherent cells are removed by pipetting out the supernatant and rinsing the flask with warm medium (38°C).
	3. Adherent cells (monocytes) are detached by incubating them with Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} , containing a mixture of 0.5 mg/mL Trypsin + 0.6 mM EDTA for 10 min at 23°C .
	4. Cells are pelleted and washed (10 min $180 \times g$, 4°C) with unsupplemented RPMI 1640 medium, and finally resuspended in the culture medium.
	5. After detaching monocytes from polystyrene flasks, to avoid monocytes loss by the binding to polystyrene, all procedures should be carried out at 4°C in polycarbonate conical tubes.
	B. Plasma coated gelatin surfaces
	1. PBMCs are cultured in 75 cm^2 plasma coated gelatin (2%) culture flasks and incubated for 1 h at 38°C .
	2. No-adherent cells are aspirated, and the flask rinsed with culture medium (38°C).
	3. Adherent cells are detached by incubating the cells with HBSS without Ca^{2+} and Mg^{2+} , containing 10 mM EDTA for 5 min at 23°C .
4. Flasks are gently shaken, and cells recover with pipette and pelleted.	
5. Cells are washed and resuspended in culture medium at 4°C .	

- Sorting
1. Monocytes are isolated from PBMCs with magnetic-activated cellular sorting technique, using a monocyte-specific monoclonal antibody (CD14⁺).
 2. PBMCs are incubated with anti-human CD14 labelled superparamagnetic beads (Miltenyi-Biotech) for 15 min at 4°C.
 3. Cells are separated through magnetic separation using Macs column (Miltenyi-Biotech), by first eluting the cells not bound to the column (CD14⁻) and then CD14⁺ cells by removing the column from the magnet.
 4. The purity of sorted cells is evaluated by flow cytometry.

164

165 The aforementioned techniques have both advantages and disadvantages that must be considered
 166 when choosing the adequate isolation method according to each experimental requirement. MACS
 167 technique is based on the immunomagnetic separation of the cells, which allows the specific isolation
 168 of a subset of monocytes of interest (e.g., CD14⁺ fraction) with higher purity (> 98%) [43] than with
 169 the adherence based method [44]. Such specific marker separation is not possible when working on
 170 the isolation of monocytes by adherence from PMBC, where the authors often report the cells only
 171 as adherent (monocytes) and non-adherent (lymphocytes) [42]. Despite its advantages, this immune-
 172 based method also presents some disadvantages such as higher costs and the unavailability of
 173 antibodies used for the separation of monocytes of other species than humans. This issue can be
 174 addressed by purifying cells with the adherence-based method. Finally, the latter method is also
 175 cheaper and simpler, as no complex equipment is needed. The main disadvantage of the adherence
 176 method is the potential presence of other cellular population, such as eosinophils. When blood is
 177 collected from animals with a high concentration of eosinophils, the first step of purification, e.g.
 178 centrifugation over Ficoll layer, cannot entirely remove eosinophils, which in turn can contaminate
 179 monocyte population.

180 Once purified, population purity is assessed through flow-cytometric techniques.

181 Briefly, a total of 100 µL of cell suspensions are labelled with FITC-anti-CD14 antibody (clone
 182 TÜK4) and incubated in the dark for 15 minutes. Cells are washed twice and resuspended in PBS for
 183 flow cytometry. Fig. 3 shows representative flow cytometry dot plots of CD14⁺ cells before and after
 184 MACS purification. The recovery of cells from either method yields an amount of homogenous
 185 monocyte population ranging from 8 to 10 millions of cells, starting from an initial blood volume of
 186 200 mL.

187 Flow cytometric analysis shows that after MACS sorting, an enrichment of CD14⁺ monocytes
 188 population > of 95% is obtained.

189

190 3. Monocyte apoptosis, pyroptosis and necrosis (necroptosis)

191

192 In multicellular organisms, a balance between cell proliferation and cell death is maintained to assure
193 homeostasis [47]. Cells that are useless or superfluous, aged, damaged, and/or potentially harmful are
194 selectively eliminated using genetically encoded mechanisms [48]. Up to now several types of cell
195 death have been described: apoptosis, necrosis, both accidental and programmed (necroptosis),
196 pyroptosis, cell death associated with autophagy, ferroptosis, netosis, among others [49]. However,
197 apoptosis and necrosis are the best-characterized cell death ways so far. A comprehensive list of
198 different cell death mechanisms has been recently published by the Nomenclature Committee on Cell
199 Death [48].

200 Apoptosis is an ATP-dependent type of programmed cell death, generally characterized by distinctive
201 morphological and biochemical changes. The morphological features of apoptosis include reduction
202 of cellular volume, condensation of the chromatin, nucleus fragmentation, plasma membrane
203 blebbing, maintenance of plasma membrane integrity until late stages and formation of apoptotic
204 bodies followed by dead cells phagocytosis [50]. Cells undergoing apoptosis also show biochemical
205 changes such as externalization of phosphatidylserine on the outer plasma membrane [51], the release
206 of mitochondrial intermembrane space proteins (Cytochrome C, Smac/DIABLO) [52], DNA
207 fragmentation and caspase-dependent activation [53]. Several of these features were exploited to
208 measure apoptosis rate. Apoptosis is generally not associated with inflammation, due to the absence
209 of cells' contents release, resulting in cell clearance from the body with minimal damage to the
210 surrounding tissues [54].

211 Understanding the monocyte cell death mechanism(s) is important, as the way of cell death (necrosis
212 vs. apoptosis vs. pyroptosis) can influence the pro- and anti-inflammatory responses [55] and
213 therefore interfere with the proper resolution of inflammation, or the clearance of the inflammatory
214 focus from the pathogens, or cancer development [56]. Moreover, apoptosis rate is critical for normal
215 cell turnover and proper development and functioning of the immune system. Indeed, apoptosis
216 provides a way to control the activity of blood monocytes, by increasing or reducing their abundance
217 in the inflammatory environment.

218 In contrast, necrosis is generally described as an “accidental or uncontrolled” way of cell death caused
219 by extreme physical or chemical stress [57]. The rapid cytoplasmic granulation/vacuolization, cellular
220 and organelles' swelling in necrotic cells, results in plasma membrane rupture, organelle breakdown,
221 intracellular content spillage, inflammation and surrounding tissue damage [47,54]. Nevertheless, it
222 is now known that necrosis can also occur as a caspase-independent form of regulated cell death, also
223 known as necroptosis, following many defined steps and signalling events, just like apoptosis [57].

224 Necrosis is the term currently used for nonapoptotic death and has been characterized as passive,
225 accidental cell death resulting from environmental perturbations with the uncontrolled release of

226 inflammatory cellular contents. The presence of necrosis provides evidence that a cell has died, but
227 not necessarily how the death occurred. The necroptosis is defined as the caspase-independent,
228 regulated cell death (RCD). In the recent years, research studies concerning caspase-independent,
229 non-apoptotic forms of programmed cell death (PCD) have enabled the identification of backup
230 mechanisms to allow the cells to undergo suicide or RCD under conditions where the caspase
231 machinery is defective or inhibited [58].

232 Another type of regulated necrosis so far described and measured in monocytes and macrophages is
233 pyroptosis. Pyroptosis occurs in response to microbial products or other intracellular perturbations,
234 which cause the production of proinflammatory cytokines like IL-1 β , leading to cell swelling and
235 death [57]. Being initially thought to occur only in macrophages and leukocytes during inflammatory
236 conditions, pyroptosis has now been observed in several other cell types, including fibroblasts and
237 endothelial cells [59]. Pyroptosis is generally characterized morphologically by membrane pore
238 formation, cell swelling, membrane disruption and DNA damage, whereas biochemically by an
239 inflammatory caspase activation (non-apoptotic caspase-1), that mediates cytokine maturation
240 [57,60]. The identification of distinctive and specific markers in all of these three types of cell death
241 has long been used for their *in vitro* and *in vivo* discrimination in many cell types including monocytes
242 and macrophages.

243 244 3.1. *In vitro* assays measuring monocytes/macrophages apoptosis

245
246 *In vitro* apoptosis can be determined through several methods: by characterizing cell morphology
247 through microscopy or by measuring biochemical cell death features, like caspase activation, that is
248 activated uniquely during the apoptosis [47]. Methods relying on flow cytometry have also been
249 widely applied in human and murine research to distinguish apoptosis from necrosis [61].

250 Specifically, the measurement of the activity of the two main effectors enzymes in apoptosis, namely
251 caspase-3 and -7, has been already reported and validated in bovine monocytes (CD14⁺) [20,32]. In
252 these studies, the activation of the two enzymes was measured through fluorometry, using a detection
253 assay that contains aa pro-fluorescent substrate the rhodamine 110, bis-(N-CBZ-L-aspartyl-L-gluta-
254 myl-L-valyl-L-aspartic acid amide - Z-DEVD-R110) and a cell lysis buffer that supports optimal
255 caspase activity. The assay is adapted to small well formats, such as 96 or 384-well plates. Depending
256 on the selected format, between 5x10⁴ cells for 384-well up to 1x10⁵ cells for 96-well plates are seeded
257 in final volumes of 25 μ L to 50 μ L, respectively [43,63]. In a second step, an equal volume of Apo-
258 ONETM Homogeneous Caspase 3/7 Reagent (Promega, USA), containing the pro-fluorescent
259 substrate and previously diluted 1:100 in the reaction buffer, is added to each well. Plates are then

260 incubated in a plate shaker at 300 rpm for 1 h at room temperature. According to the manufacturer's
261 instructions, incubation times should be optimized for each model as minimal apoptotic induction
262 and low cell numbers may require extended incubation periods. Finally, upon cleavage of the DEVD
263 peptides by caspase-3 and -7 activities, the fluorescent compound rhodamine 110 is released and
264 fluorescence intensity can be measured using a fluorescence plate reader (excitation at 485 nm,
265 emission at 530 nm).

266 Caspase activity can also be measured through colourimetric assays. For example, a caspase
267 colourimetric protease assay (Aportaget; Invitrogen, Canada) kit has been used to determine the
268 activity of caspases-3, -6, and -9 in monocytes infected with *Mycobacterium bovis* [64]. This assay
269 can be carried out by lysing a pellet of infected monocytes containing 5×10^6 cells with 50 μL of
270 chilled cell lysis buffer provided by the kit and incubated for 10 min, before centrifuging them at
271 $10,000 \times g$. The supernatant containing the cytosolic fraction is transferred to a fresh tube and the
272 protein concentration is quantified. After having adjusted the cytosolic fraction concentration (200
273 $\mu\text{g}/\text{mL}$) in the cell lysis buffer provided in the kit, 50 μL of it are transferred to 96-well round-plates
274 and 50 μL of 2 \times kit reaction buffer, containing 10 mM of dithiothreitol (DTT) and 200 μM the
275 respective substrate for the caspases, is added in a volume of 5 μL . Finally, samples are incubated at
276 37°C for 2 h in the dark and then read at 405 nm with a 490-nm reference filter in a microplate reader.
277 Bovine monocyte-derived macrophages apoptosis has also been detected through fluorometric analysis
278 of caspase-3 activity [63] and relative quantification of histone-complexed DNA fragments, that are
279 often out of the cytoplasm of cells undergoing apoptosis [65], using Cell Death Detection
280 ELISAPLUS kit (Roche, Switzerland) [66]. Because histone-complexed DNA release is not an
281 exclusive phenomenon of apoptosis but also can occur in necrotic cells, using complementary assays
282 is preferred [67].

283

284 3.2. *In vitro* assays measuring monocytes necrosis

285

286 **The permeabilization** of cells' plasma membranes and its consequent release of cellular content are
287 some of the hallmarks of necrosis. The release of lactate dehydrogenase (LDH), an ubiquitous
288 cytosolic enzyme, into the supernatant after membrane integrity loss has long been measured to detect
289 necrosis in multiple cell types by spectrophotometry, including bovine monocyte-derived
290 macrophages [63,66,68]. Specifically, in the study of Denis and coworkers [66], a commercially
291 available kit (CytoTox 96[®], Promega, USA) was used to measure the LDH released to the
292 supernatants by a 30-minute coupled enzymatic assay that leads to the conversion of a tetrazolium
293 salt (iodonitro-tetrazolium violet; INT) into a red formazan product, which is proportional to the

294 amount of LDH present and thus to the number of damaged cells. Besides INT, the kit reagents also
295 contain the substrates lactate, NAD⁺ and diaphorase to complete the reaction, as the first two are
296 needed to produced reduced nicotinamide adenine dinucleotide (NADH) that is later used to convert
297 INT into the coloured product in the presence of diaphorase [68]. According to the manufacturer
298 instructions, cells are first seeded in 96- or 384-well plates and treated with the desired experimental
299 design. Afterwards, the supernatants are transferred to a fresh plate (50 µL for a 96-well and 12.5 µL
300 for a 384-well plate) and an equal volume of CytoTox 96[®] Reagent is added. Plates are protected
301 from light and incubated for 30 min at room temperature. Finally, 50 or 12.5 µL of Stop Solution are
302 added and absorbance measured 490nm or 492nm [66]. Other techniques based on differential uptake
303 of DNA binding dyes, like propidium iodide (PI), that do not enter living cells, have also been used
304 for determination of bovine necrotic neutrophils [69], blood monocytes and macrophages (CD14⁺)
305 isolated from the mammary gland through flow cytometry [70].

306

307 *3.3. In vitro assays measuring monocytes pyroptosis*

308

309 The discrimination between necrosis and pyroptosis can be a challenging task as many of the
310 hallmarks for regulated necrosis such as plasma membrane rupture, cytoplasmic
311 granulation/vacuolization and cellular/organelle swelling are also shared by pyroptosis [71]. The
312 measurement of LDH release has also been implemented for pyroptosis determination of human and
313 murine bone marrow-derived macrophages, as it is a simple and cost-effective technique [62,72].
314 DiPeso and coworkers monitored the cells' LDH secretion, as previously described [59,73], after
315 reacting with a pyroptosis inducer called RodTox that activates the NAIP2/NLRC4 inflammasome,
316 leading to caspase-1 activation and pyroptosis. Additionally, a more specific way to measure
317 pyroptotic death of individual cells *in vitro* can be carried out through the measurement of caspase-1
318 activation and inflammasome formation (NLRP3) by both fluorescence or confocal microscopy [74].
319 Cells are labelled with the caspase-1 activity probe FAM-YVAD-FMK that specifically binds to the
320 caspase-1 family proteases, and with antibodies against inflammasome components such as the
321 adaptor protein ASC that recruits pro-caspase-1. For that, murine marrow-derived macrophages are
322 first seeded in 96-well plates and stimulated with 100 ng/mL of LPS for 3 h at 37°C to prime Toll-
323 Like Receptor 4 (TLR4), the first step needed for NLRP3 activation. Afterwards, the medium is
324 removed, and cells are stimulated with fresh DMEM medium-containing 5 µM nigericin to induce
325 the second step for the activation of the NLRP3 inflammasome, and 5 mM glycine to reduce the cell
326 lysis caused during caspase-1 activation. Cells are then incubated for 60 min at 37°C and 5% CO₂,
327 and during the last 45 min incubated as well with 30x of the FAM-YVAD-FMK probe, which is

328 prepared according to the manufacturer's instructions. After incubation, the medium is removed and
329 the cells are washed three times for 5 min with cold PBS. Finally, the cells are fixed and stained with
330 a fluorescent nucleic acid stain to label the nuclei and mounted for a correct visualization by confocal
331 microscopy, with an excitation/emission of 492/520nm for the FAM-YVAD-FMK probe. For the
332 antibody staining of the inflammasome component (ASC), after seeding, priming and exposing the
333 cells to nigericin, the cells are washed with cold PBS, fixed and permeabilized on ice and in dark for
334 30 min. Macrophages are washed with the washing buffer and the primary antibody anti-ASC (1:500)
335 is added and cells are incubated for 1 h on ice. The cells are again washed and the secondary antibody
336 (fluorescent dye conjugated goat anti-mouse; 1:500) is added for another 1 h on ice. During the last
337 5 min of this incubation, the dye to stain the cells' nuclei is also added. Finally, cells are washed and
338 prepared for confocal analysis with an excitation/emission for the secondary antibody of 555/580 nm.
339 According to Hartigh and Fink, to successfully determine pyroptotic cells the nuclear morphology
340 should be considered, as cells with active caspase-1 possess a rounded and condensed nuclei without
341 visible nucleoli, often present in cells without caspase-1 activation [74].
342 Even though the previously described techniques have been carried out mostly in human and murine
343 macrophage models, the same ones can be applied to bovine monocytes/macrophages' pyroptosis
344 determination, although they have been not tested so far.

345 346 3.4. Flow cytometric assays to evaluate apoptosis, necrosis (necroptosis) and pyroptosis

347
348 Determining the number of dead cells during a flow cytometry study is a prerequisite to obtain reliable
349 data, given that dead cells can bind in a non-specific way to many reagents. Therefore, removing dead
350 cells from flow cytometry data is a prerequisite and a critical step to ensure accurate results and
351 analysis [55]. A widely used flow cytometric assay to define cell viability is based on the reaction of
352 a fluorescent dye, such for example propidium iodide (PI), with cellular amines. This die cannot
353 penetrate live cell membranes: consequently, only cell surface proteins are available to react with the
354 dye, resulting in dim staining. The reactive dye can permeate the damaged membranes of dead cells
355 and stain both the interior and exteriors amines, resulting in more intense staining [72]. In normal
356 living cells, the phosphatidylserine (PS) is located at the side of the cytoplasmic surface of the cell
357 membrane. However, during early apoptosis, PS is translocated from the cytosolic side of the intact
358 plasma membrane to the extracellular surface. Early apoptotic cells cannot, therefore, be reliably
359 identified using approaches that are based on membrane permeability. Annexin V is a protein with
360 high affinity, specificity, and sensitivity for PS. Thus, the binding of annexin V to cells can be used

361 as a marker of early apoptosis [75] and provides the most commonly used approach for determining
362 apoptosis [55].

363 A typical experimental protocol to detect apoptosis is the following: 100 μL of cells suspension
364 (1×10^6 cells/mL) in 1X annexin-binding buffer are incubated with 5 μL of fluorescein isothiocyanate
365 (FITC) annexin V and 1 μL of PC5.5-PI (100 $\mu\text{g}/\text{mL}$) in the dark at room temperature for 15 min.
366 Finally, 400 μL of the annexin V binding buffer is added and the cells are analyzed by flow cytometry.
367 The binding of annexin V to PS is reversible, and so samples must be analyzed as soon as possible
368 and no later than 1 h after labelling. Fig. 4 illustrates the typical aspect of a dot plot flow cytometry
369 analysis using annexin V/PI double staining to determine in the percentages of live, apoptotic and
370 necrotic bovine peripheral blood lymphocytes and monocytes. Beside annexin V staining, the
371 measurement of caspases activity is also widely used to determine the apoptosis rate. Caspases are a
372 family of cysteine proteases that plays a key role in apoptosis and inflammation. These enzymes are
373 present in healthy cells as inactive zymogens, but when stimulated they undergo autolytic cleavage
374 to become fully active. Consequently, many of the cleaved fragments remain intact during apoptosis
375 and can be detected using specific antibodies. These antibodies can be used to specifically label cells
376 in which caspase cleavage has occurred, allowing to quantify these cells by flow cytometry. Several
377 antibodies that specifically recognize caspase-cleaved fragments have been generated, including
378 antibodies that recognize the cleaved form of caspase-3, a key protease activated during the early
379 stage of apoptosis [76].

380 For flow cytometric detection of active caspase3, 1×10^6 cells are washed with PBS and then
381 resuspended with 100 μL of fixation/permeabilization buffer (Cytofix/CytopermTM, BD Biosciences,
382 USA) for 20 min at room temperature. In the following step, cells are washed twice with 600 μL of
383 erm/WashTM (BD Biosciences, USA) buffer and stained with the PE- rabbit anti-active caspase-3
384 antibody (clone C92-605) for 40 min at 4°C. Cells are then washed and resuspended in Perm/WashTM
385 buffer for flow cytometric analysis.

386 Both pyroptosis and apoptosis are programmed cell death mechanisms but are dependent on different
387 caspases. Pyroptosis is triggered by caspase-1 after its activation by various inflammasomes and
388 results in lysis of the affected cell. Pyroptotic cell death could be assessed in PBMC and monocytes
389 by flow cytometry by measuring the double-positive staining of activated caspase-1 and PI [77].
390 Furthermore, Hussen and coworkers [20], analyzed the inflammasome activation in bovine
391 monocytes through the evaluation of caspase-1 activation. Authors used the FLICACaspase-1
392 detection KIT (Thermo-Fisher, USA), a fluorescent inhibitor of active caspase-1 (FAM-YVAD-
393 FMK), which binds activated caspase-1. First, bovine PBMC or purified monocytes (1×10^6) are
394 stimulated for 2 h with LPS (100 ng/mL). Then ATP (2 mmol/L) is added to the cell culture for a

395 further 30 min. After stimulation, cells are detached from the plates by Accutase™ (Sigma-Aldrich,
396 USA) treatment (incubation for 15 min at 37°C and 5% CO₂). Cells are then suspended in 300 µL
397 RPMI medium and 10 µL of the 30X FLICA solution is added. After the incubation (1 h at 37°C and
398 5% CO₂) with FLICA, cells were washed two times with the FLICA buffer and suspended in 200 µL
399 of the same buffer. For flow cytometric analysis PI (2 µg/mL final concentration) is added to exclude
400 dead cells.

401

402 4. Monocyte and macrophage chemotaxis

403

404 Chemotaxis is the ability of cells to migrate in response to a chemical signal or chemoattractant
405 gradient concentration. It is a crucial function of leukocytes, including monocytes, as they fulfil their
406 defensive functions by migrating from blood to affected tissues, where they produce pro-
407 inflammatory cytokines and reactive oxygen species (ROS) and eventually phagocyte and destroy the
408 invading pathogens [78].

409 Monocyte chemotaxis is determined by using the Boyden chamber assay, composed of an upper and
410 a lower compartment separated by a porous membrane (Fig. 5a). This assay consists mainly of
411 monitoring the migration of the cells through a microporous membrane. In general, the cells are
412 seeded in the medium-filled upper compartment and are allowed to migrate through the pores of the
413 membrane, towards the medium-filled lower compartment where chemoattractants are present [79].
414 To assure the correct migration of the cells through the membrane, this one must be selected with the
415 appropriate pore size for the cells of interest. Human monocyte chemotaxis is usually triggered by N-
416 Formylmethionine-leucyl-phenylalanine (N-fLMP), a formylated peptide derived from bacteria [80],
417 which specifically reacts with Formyl Peptide Receptors [81]. Bovine cells do not have N-fLMP
418 receptors [82,83]. In this species, therefore, chemotaxis should be triggered with other
419 chemoattractants, including, such as, for example, zymosan activated serum (ZAS) [84]. The *in vitro*
420 chemotactic capacity of bovine monocytes has been determined using this principle with a modified
421 Boyden chamber [84]. In this study, bovine monocyte migration was measured using a 48-well
422 microchamber, where the chemoattractant ZAS was added in the lower part of the chamber. A PVP-
423 free polycarbonate filter (5 µm pore size) was layered onto the lower wells and covered with a silicone
424 gasket and finally with the top plate above. In the upper wells, 1.5×10^6 cells/mL were seeded (50 µL
425 final volume) and the chamber was incubated at 37°C in 5% CO₂ for 105 min. The microchamber
426 was disassembled and the filters were removed carefully, the non-migrated cells on the upper part of
427 the filter were removed by scraping and the migrated cells found in the lower part of it were stained

428 with Romanowsky type stains including May-Grünwald-Giemsa and Wright's stains. Finally, cells
429 were counted in different high-power oil-immersion fields.

430 Modified Boyden chamber devices, allowing a more convenient and user-friendly methodology to
431 carry out the cell migration studies, are commercially available. Such is the case of the transwell
432 migration plates (Fig. 5b), that contain easy-to-use permeable cell culture membrane inserts of
433 different pore sizes that can be removed from the plates when needed to stain and count the cells that
434 have migrated through the membrane. The transwell migration assay has been described to assess the
435 chemotactic activity of human blood monocytes and the human monocyte cell line THP-1 [85] and
436 can be also used for bovine studies. In this study, 1×10^5 of THP-1 and blood monocytes were seeded
437 in the upper chamber of a 24-well transwell migration plate in a final volume of 125 μ L of serum-
438 free media. The monocyte chemoattractant protein-1 (MCP-1) and “platelet-releasate”, which is the
439 content released by secretory granules of activated platelets, were added as chemoattractants to the
440 lower chamber in a final volume of 1 mL and plates incubated for 2 h at 37°C. After incubation, the
441 upper chambers were removed and placed into 2.5% glycerinaldehyde for 15 min to fix the migrated
442 cells found on the underside of the membrane. The membranes were washed with PBS, and the upper
443 chambers were stained with 0.1% of crystal-violet for 45 min. Finally, cells were washed twice with
444 PBS and cells in ten 40x fields were counted using light microscopy.

445 Chemotaxis of monocyte-derived macrophages has been measured as well [86], using the same
446 technique, e.g. the migration through Boyden chamber, previously described.

447 The Boyden chamber technique has been modified over the years to allow the quantification of total
448 migrating leukocytes concurrently with the determination of their phenotype to associate specific
449 inflammatory responses with distinct leukocyte subsets. Gomez-Lopez and coworkers [87] validated
450 in humans a combined Boyden-flow cytometry chemotaxis assay (CBFCA), that allows the accurate
451 quantification and recovery of the migrating leukocytes for subsequent phenotyping and
452 characterization in a single assay, facilitating the understanding of leukocyte trafficking during the
453 inflammatory response. The combined chemotaxis assay is performed under the same conditions
454 described for the classic method, but only the polycarbonate membrane was used. Following
455 incubation, the leukocyte suspension in the lower well is recovered and cells counted by flow
456 cytometry. To our knowledge, the CBFCA assay has not yet been applied to study chemotaxis of
457 bovine monocytes.

458

459 **5. Monocyte/macrophages phagocytosis and killing**

460

461 Phagocytosis and respiratory burst are two of the most important functions for
462 monocytes/macrophages and polymorphonuclear cells and are essential for both the elimination of
463 invading microorganisms and the presentation of antigens to T-cells. Phagocytosis is a process in
464 which target particles are engulfed and taken up by other cells, crucial to the innate immune response
465 and consequently regulating the adaptive immune response [88]. Phagocytic affinity is related to
466 particular marker expression patterns, directed by a complex range of cell surface receptors, and may
467 also change in different populations of monocytes [88]. Bovine monocytes mediate the inflammatory
468 response activated in phagocytosis and killing capability, which leads to different abilities to
469 phagocytose bacteria between the different monocyte's subsets – cM are the ones with the highest
470 phagocytosis, followed by intM and ncM with the lowest ability [18]. The phagocytosis ability of
471 monocytes can also relate to other factors such as age, breed, nutrition and the clinical status of the
472 animal [89–91]. **Lymphocytes in** neonatal calves have poorer phagocytosis ability but counterbalance
473 with higher absolute numbers, in contrast to monocytes of neonatal calves that showed a higher
474 bacterial uptake than 3-9 week old calves, suggesting that the monocytes contribute to the
475 compensation of impaired earlier innate immune defence in infancy [92]. A similar mechanism seems
476 to take place in the mammary gland, where a greater number of viable macrophages may partially
477 compensate for the functional deficit in polymorphonuclear cells in cows with high somatic cell
478 counts [93].

479 The *in vitro* phagocytosis assay of bovine monocytes can be performed by co-culturing the cells with
480 opsonised fluorescein-labelled bacteria and then incubating them at 37°C in a humidified atmosphere
481 of 5% CO₂ for 1 h. The cells are washed twice with sterile HBSS and then incubated with trypan blue
482 to quench non-internalised fluorescent bacteria, and after two subsequential washes with HBSS, the
483 intensity of the fluorescence, which can be correlated with the phagocytic ability, can be measured
484 [44]. Improved results have been achieved *in vitro* in the study of monocyte-derived macrophages
485 phagocytosis when opsonizing bacteria with naive-autologous sera than bacteria opsonized with fetal
486 bovine serum [94].

487 Phagocytosis assays of monocyte-derived macrophages can also be performed by co-incubating the
488 cells with opsonised bacteria in a ratio of one bacterium to one macrophage, in a water bath at 37°C
489 under continuous rotation. The suspension is removed and added to ice-cold gelatine-Hanks' salt
490 solution to stop phagocytosis, and the macrophages are centrifuged (110 x g for 4 min) to remove the
491 non-ingested bacteria, that remain in suspension in the supernatant fluid. The supernatant is then
492 seeded on tryptic soy agar plates, in dilutions between 10 and 100 colony counts, incubated in 8%
493 CO₂ at 37°C for 72 hours. The results are expressed as the percentage of survival of non-
494 phagocytized/non-cell-associated bacteria based on the formula [94]:

495

496

$$\text{Percent survival} = \frac{\text{CFU of bacteria after incubating for 120 min}}{\text{CFU of bacteria at time 0}} \times 100$$

497

498 The monocyte's and macrophage's ability to bind, internalize and kill bacteria occur as independent
 499 and sequential steps, and, as a result, a phagocyte may be able to ingest a pathogen and yet not manage
 500 to kill it. Thus, the phagocytosing and killing capabilities of monocytes and macrophages should be
 501 measured by separate assays [95]. The *in vitro* intracellular bacterial killing assay is performed by
 502 co-incubating bovine monocytes (5×10^5 cells) with opsonised bacteria (*E. coli*) for 60 min. The
 503 unbound bacteria are removed, by centrifuging **them** at $110 \times g$ for 5 min, and the cell pellet is
 504 resuspended in HBSS with $100 \mu\text{g/mL}$ gentamicin, to kill any remaining extracellular bacteria, and
 505 then incubated for 1 h at 37°C . Cells are washed with warm HBSS and then centrifuged again at 110
 506 $\times g$ for 5 min to remove the gentamicin. The cells are then lysed by incubation on ice with 0.5% Triton
 507 X-100 for 10 min and the surviving bacteria **are** seeded on MacConkey agar plates for CFU counting
 508 [44].

509 Flow cytometric assays have been developed to quantify phagocytosis and respiratory burst in whole
 510 blood, as well as isolated cells from blood, tissue or secretions [96,97]. Hussien and coworkers [98]
 511 reported a flow cytometric assay to evaluate phagocytosis capacity of bovine monocytes. FITC-
 512 conjugated and heat-killed *S. aureus* or *E. coli* were opsonized with heterologous bovine serum
 513 (diluted 1:10 with PBS) for 45 minutes at 37°C . MACS-separated bovine monocyte subsets were
 514 incubated for 24 h (37°C , 5% CO_2) in RPMI medium. After this resting period, cells were incubated
 515 with opsonized bacteria (50 bacteria/cell) for 40 minutes (37°C , 5% CO_2). Control samples were
 516 incubated without bacteria. After incubation, samples were analyzed using flow-cytometry after the
 517 addition of propidium iodide (2 mg/mL final concentration) to exclude dead cells. Phagocytic activity
 518 of different monocyte subsets is defined as the percentage of green fluorescing cells among viable
 519 cells.

520

521 **6. Monocyte/macrophages ROS production**

522

523 Generation of reactive oxygen species (ROS) is a critical factor in the antimicrobial activities of
 524 monocytes, though it may also lead to cellular cytotoxicity and/or tissue damage. The intracellular
 525 presence of ROS can result in damage to the intracellular constituents and oxidative stress, which
 526 may have consequential alterations in monocyte's function [88]. As other functional abilities, ROS
 527 production differ between different monocyte populations – bovine intM is reported to present the

528 strongest production between the subsets [18] – and virulent pathogens of cattle, such as *N. caninum*,
529 may also regulate ROS production of infected macrophages [88].
530 The production of oxidative burst activity of bovine monocytes can be measured *in vitro* using a
531 cytochrome C reduction assay, as similarly used in bovine and caprine neutrophils and goat
532 monocytes [99,100]. The measurement of cytochrome C reduction is commonly used for the
533 measurement of extracellular superoxide anion [101] and demonstrated a dose-dependent specificity
534 in detecting extracellular superoxide production in bovine neutrophils [99]. The extracellular ROS
535 production is measured by mixing 2×10^5 monocytes in RPMI-1640 medium (adjusted final volume
536 on wells of 200 μ L) and cytochrome C (1 mM). Parallel studies to assess the ROS production after
537 inducing a pro-inflammatory challenge to the cells, by adding phorbol 12-myristate-13-acetate
538 (PMA), can also be performed. PMA (2.5 μ g/mL final concentration) and cytochrome C (1 mM) are
539 added to the cells and the absorbance is measured immediately with a plate reader every 30 min for
540 2 h at a wavelength of 550 nm. Background values are measured from wells with cytochrome C
541 diluted in RPMI-1640 and subtracted from all values [102]. Analysis of ROS generation via
542 cytochrome C reduction assay has been used in bovine monocytes and alveolar macrophages (AM),
543 revealing that monocytes appear to be less responsive in ROS formation than AM, likely for being
544 more immature and thus possessing less cytoplasmic organelles enabling metabolic processes [103].
545 Production of ROS reduces substrates such as dichloro-dihydro-fluorescein-diacetate (DCHF) or
546 dihydro-rhodamine 123 (DHR 123) that become fluorescent and can be quantified by flow cytometry.
547 Hussen and Coworkers [98] reported a typical flow cytometric analysis of ROS generation on bovine
548 monocyte. The analysis is performed after stimulation of cells with PMA (50 nmol/L) or heat-killed
549 and serum opsonized *E. coli* (50 bacteria/cell) for 15 min (37°C, 5% CO₂) in the presence of the
550 fluorogenic substrate DHR 123 (750 ng/mL). ROS generation is calculated as Mean Fluorescence
551 Intensity (MFI) of gated monocyte region after subtracting the MFI of non-stimulated cells.

552

553 7. Monocyte/macrophages production of cytokines

554

555 Cytokines are important chemical messengers produced by both innate and adaptive immune cells,
556 that allow the communication between both types of immunity and within the adaptive immune
557 system [104]. Specifically, monocytes and macrophages play a critical role in immune regulation by
558 producing a wide range of pro and anti-inflammatory cytokines like TNF- α , IL-1 β , IL-6, IL-8, IL-
559 10, IL-12, and TGF- β 1 [105]. At the same time, these cytokines modulate their functions by
560 stimulating or inhibiting their microbial activities in an autocrine and paracrine manner [106] and **are**
561 **also** involved in cell migration, activation, proliferation and antibodies' production. **Moreover**,

562 monocytes and macrophages can be further classified into different subsets according to the cytokine
563 profile they produce [107]. **Therefore**, the measurement of monocyte and macrophage cytokine
564 production is a highly useful approach for phenotyping and characterizing their functionality.

565 Bovine monocytes' cytokine production can be measured mainly by two approaches: detection of
566 secreted cytokines at the protein level through enzyme-linked immunoabsorbent assays (ELISAs)
567 [64] or by measuring cytokines' mRNA abundance by real-time quantitative PCR (q-PCR)
568 [16,21,62,108].

569 **Several commercially available ELISA kits have been used for the quantification of bovine cytokines,**
570 **including IFN- γ [109,110], TNF- α [111], IL-1 β [112] and IL-4 [111]. However, the quantification of**
571 **secreted cytokines in monocytes/macrophages can be performed by other methods. Recently, type 1**
572 **IFN was measured in ovine alveolar macrophages' supernatant by using an IFN bioassay [113]. An**
573 **IFN bioassay is a method to quantify IFN biological activity units, meaning it measures the actual**
574 **biological power of an IFN preparation in a specific cell line or biological system [114]. It is well**
575 **known that IFNs types' cytokines induce a potent antiviral activity in vertebrate cells [115].**
576 **Therefore, this method mainly relies on type 1 IFN antiviral properties and determines the amount of**
577 **IFN in a solution- usually secreted by virus-infected monocytes/macrophages- according to its ability**
578 **to neutralize viral replication and protect the infected cells in culture from cytopathic effects. One of**
579 **the most common IFN bioassays is based on the quantification of the fluorescence intensity of viral-**
580 **encoded reporter proteins, like green fluorescent protein (GFP), during the virus replication in cell**
581 **culture [116]. The protective effects of IFN are then quantified by measuring the fluorescence**
582 **intensity and is determined by the ED50 that is the amount of IFN needed to reduce the fluorescence**
583 **by 50% [114]. Briefly in ovine, the bioactivity of IFN secreted by Sendai virus-infected ovine alveolar**
584 **macrophages was quantified by first serially diluting the IFN containing supernatant in DMEM**
585 **medium supplemented with FBS and antibiotics. This supernatant was then added to 2×10^4 ovine**
586 **fibroblasts that were seeded the day before in 96 well plates, and cells were incubated at 37 °C for 24**
587 **h. After incubation, supernatants were removed and ovine fibroblasts were infected with**
588 **Recombinant Vesicular Stomatitis virus expressing GFP (VSV-GFP) at a multiplicity of infection of**
589 **0.01 and incubated at 37 °C. After 18 h post-infection, the green fluorescence of VSV-GFP infected**
590 **cells was detected by using a plate reader with an excitation wavelength of 480 nm and emission of**
591 **518 nm [113]. This method gives extra information when compared to ELISA, as by quantifying IFN**
592 **with a bioassay one can measure the antiviral activity on a specific experimental condition without**
593 **needing absolute numbers [114].**

594 To **measure** bovine monocytes' cytokines mRNA abundance using qPCR [62], RNA from at least 1
595 $\times 10^6$ cells/well is extracted by using guanidinium-acid-phenol extraction methods, such as TRizol™

596 (Thermo-Fisher, USA) or Qiazol™ (Qiagen, Germany), or commercial kit [117]. After having
 597 assessed the quality and concentration of RNA using a NanoDrop ND-1000 UV–vis
 598 spectrophotometer, an Agilent Bioanalyzer or a Qubit fluorometer, genomic DNA is eliminated using
 599 DNase I, RNase free kit. Reverse transcription is then carried out on RNA using commercially
 600 available cDNA Synthesis kit. The cDNA is then used as a template to perform qPCR or microarray.
 601 To carry out a reliable relative quantification, at least three adequate reference genes should be
 602 identified and the geometric mean is used for normalization [118,119]. In the published MIQE
 603 (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines is
 604 indeed proposed the validation of RGs for all qPCR experiments [120]. Statistical softwares like
 605 geNorm™, NormFinder® and BestKeeper can be used to assess the appropriateness and stability of
 606 these genes [121–124]. The list of reference genes that have been used for quantitative real-time RT-
 607 PCR gene expression studies in monocytes and macrophages is reported in Table 4.

608

609 **Table 4.**

610 Validated reference genes for monocytes and macrophages used for qPCR.

Symbol	Name	Accession number	Cell target	Target specie	Ref.
HMBS	Hydroxymethyl-bilane synthase	NM_013551	Macrophages	Murine	[121]
B2M	β-2-microglobulin	NM_009735			
ACTB	β-actin	NM_001101.3	THP-1	Human	[122]
RPL37A	Ribosomal protein L37a	NM_000998.4	monocyte cell line		
SF3A1	Splicing factor 3 subunit 1	NM_001081510	Monocytes/	Bovine	[123]
ACTB	β-actin	BT030480.1	Other tissues		
HMBS	Hydroxymethyl-bilane synthase	NM_001046207.1			
H3F3A	H3 histone, family 3A (H3-3B)	NM_001014389.2	Monocytes	Bovine	[62]
SF3A1	Splicing factor 3 subunit 1	NM_001081510			
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	XM_025001429.1			

611

612 Additional studies have also reported the adequateness of some of the previously mentioned reference
 613 genes like YWHAZ and ACTB in different ovine tissues (brain, spleen, mesenteric lymph nodes)
 614 [125]; and of YWHAZ in goats' frozen whole blood [126], but not specifically in

615 monocytes/macrophages. Remarkably, the stability of all the genes tested in different ovine tissues
616 changed significantly within each tissue, suggesting that there is not a unique universal reference gene
617 for all tissues or cell types and that their stability must be evaluated prior the start of each study. Such
618 a statement can be further confirmed by the fact that in a previous study, ACTB was not an appropriate
619 reference gene for gene expression analyses in ovine interstitial cells[127].

620 Intracellular cytokine staining (ICS) of stimulated whole blood or isolated PBMC followed by flow
621 cytometric analysis is a well-established method for detecting cytokines production at the single-cell
622 level. Recent studies developed a cytokine flow cytometric assay to detect intracellular IFN- γ in cattle
623 naturally infected with *M. bovis* [128]. The detection of intracellular IFN- γ may represent an
624 important alternative approach for an improved method of detection of cattle secreting IFN- γ below
625 levels of detection in the culture medium.

626 It is important to consider that the analysis of intracellular targets using flow cytometry presents
627 several technical challenges that are not generally encountered in the measurement of cell surface
628 epitopes. In general, cells must be first fixed to preserve and maintain both the structure and location
629 of target epitopes, then permeabilized to allow antibodies access to all cellular compartments, such
630 as the cytoplasm. In general, cell fixation is accomplished using either crosslinking fixatives, such as
631 formaldehyde, or low molecular weight alcohols (methanol), which generally act to coagulate
632 proteins. However, the fixation–permeabilization and methanol treatment need to be validated for the
633 complete set of antibody conjugates used for a new experiment [55].

634 Short-term stimulation of PBMC (6-16 h) with antigen or peptide mixtures is required to induce
635 cellular activation and production of cytokines. Cells can also be stimulated in 15 mL conical
636 polypropylene tubes or plates. Secretion inhibitors such as Brefeldin A and/or Monensin are added at
637 the time of stimulation (for peptides) or after 2 h (for proteins) to allow secreted cytokines and other
638 proteins to be retained intracellularly. At the end of the stimulation period, cells can be held at 4–
639 18°C until ready to process. They are then co-incubated with 0.2 mg/mL of EDTA at room
640 temperature to remove adherent cells, fixed, permeabilized and stained for intracellular determinants.
641 In some cases, surface marker staining is carried out jointly with intracellular staining. However, most
642 other cell-surface markers require staining before fixation, because the epitopes recognized by
643 staining antibodies are sensitive to fixation and/or permeabilization reaction [129].

644

645 **8. Phenotypic characterization of monocyte subsets: the flow cytometry approach**

646

647 The flow cytofluorimetric approach allows the immunophenotypic characterization and absolute
648 count of the monocyte subsets. Hussen et al. [17] demonstrated for the first time that bovine

649 monocytes can be distinguished in three subsets on the differential expression of CD14 and CD16.
 650 Similar to humans, bovine cM are CD14⁺CD16⁻ and intM CD14⁺CD16⁺. On the contrary, ncM are
 651 CD14⁻CD16⁺. Further evidence [19,130,131] suggested that CD14 is not a useful marker for the
 652 immunophenotyping studies and cell sorting experiments of non-classical monocytes.

653 CD172a, a member of signal regulatory protein (SIRP) family, is a marker for myeloid cells and
 654 neurons [132]. Two specific mAbs (Table 5), can be successfully used to identified bovine
 655 monocytes, granulocytes, macrophages and dendritic cells [133], suggesting their potential use as
 656 pan-monocyte markers. Interestingly, clone DH59B recognizes a conserved epitope across a wide
 657 range of other species including small ruminants [134], dog [135], water buffalo [136] and dolphins
 658 [137].

659 Although CD163 is a monocyte/macrophage differentiation antigen [138], and its expression in ncM
 660 is very low or absent [16,17], it is still regarded as an important functional marker.

661 A multicolour panel designed for a proper resolution of these subsets should, therefore, contain the
 662 markers: CD14, CD16, CD163, CD172a and a fluorescent dye for dead cell exclusion. Besides,
 663 CD335 (NKp46) should also be used to exclude NK cells. In Table 5 a list of monoclonal antibodies
 664 that can be used with either direct or indirect labelling protocols using anti-mouse secondary
 665 fluorescent antibodies is reported, although labelling of monocytes is still a hot topic within the flow
 666 cytometric community [55].

667 **Table 5.**

668 List of monoclonal antibodies reactive with bovine antigens

669

Specificity	Clone	Isotype	Target specie	Reference
CD14	CAM36A	IgG1	Bovine	[139]
	CAM66A	IgM	Caprine	[108]
	CC-G33	IgG1	Bovine	[140]
	M5E2	IgG2a	Human	[141]
	MM61A	IgG1	Bovine	[142]
	TÜK4	IgG2a	Human	[143]
	VPM65	IgG1	Ovine	[144]
CD16	KD1	IgG2a	Human	[145]
CD163	2A10/11	IgG1	Pig	[17]
	EDHu-1	IgG1	Human	[16]
	LND5A	IgG1	Bovine	[146]
	LND37A	IgG1	Bovine	[146]
	LND37A	IgG1	Bovine	[146]
CD172a	CC149	IgG2b	Bovine	[147]
	DH59B	IgG1	Bovine	[148]

670

671

672 A typical stain/lyse/wash protocol is the following:

- 673 1) 100 μ L of whole blood anticoagulated with Heparin [149] are labelled with saturating
674 concentrations of monoclonal antibodies (Table 5) in a final volume of 120 μ L with PBS (pH
675 7.2), in a 5 mL polypropylene flow cytometric tube for 30 min at 4°C in the dark.
- 676 2) Erythrocytes are lysed with 1.0 mL of Tris-buffered ammonium chloride solution (0.87% w/v,
677 pH 7.3) for 10 min, then 2 mL of cold PBS are added
- 678 3) The sample is centrifuged at 300 g for 5 min.
- 679 4) Pellet of labelled cells is re-suspended with 200 μ L of cold PBS and immediately acquired by
680 flow cytometry.

681 A simple indirect labelling protocol is done by introducing simple changes to the protocol previously
682 described. In brief, whole blood is incubated under the same conditions with 0.75 μ g of purified mAbs
683 and then is processed in according to step 2 a 3. Pellet is re-suspended in 100 μ L of fluorescent goat
684 anti-mouse isotype-specific secondary mAbs and incubated for 15 min at 4°C in the dark. After the
685 final wash, cells are re-suspended and acquired.

686 Appropriate controls are necessary to evaluate auto-fluorescence, background staining and proper
687 sample compensation, as well as to perform a careful antibody titration.

688 The gating strategy to be used to identify the three subsets of monocytes should include: 1) time
689 parameter *vs* FL or SSC to exclude event burst, 2) FSC-A *vs* FSC-H to exclude doublets, 3) exclusion
690 of dead cells in the fluorescent live/dead channel, 4) FSC *vs* SSC to exclude debris and granulocytes
691 from single live leukocytes, 5) CD172a *vs* CD16 dot plots to select CD172a positive mononuclear
692 cells (all monocytes), 6) CD16 *vs* CD14 to separate cM subsets from intM and ncM, 7) back gating
693 on dot plots 2, 3, and 4 to confirm the validity of the gating strategy.

694 To avoid false positives, the use of the fluorescence minus one (FMO) control for quadrant positions
695 of the dot plot CD16 *vs* CD14 is recommended.

696 Pomeroy and coworkers [131] found a significant association between postpartum diseases (mastitis
697 and metritis) and counts of monocyte subsets. Therefore, it is **meaningful** to evaluate the enumeration
698 of absolute count of these subsets. Two different approaches can be used: dual-platform (flow
699 cytometry and haematology) or single platform (flow cytometry). In the first method, the percentage
700 of subsets (e.g. cM, intM, ncM) is **measured** with a flow cytometer while the absolute monocyte count
701 is assessed by automated haematology analyzer. The absolute subset counts are **therefore** indirect. In
702 the single-platform method, the flow cytometer **is therefore used for directly counting monocyte**
703 subset in a **given** sample volume.

704 In humans, the precise and accurate leukocyte subset count (e.g. CD4⁺ T cells in HIV patients, CD34⁺
705 for autotransplantation) is of primary importance, and the two methods have been extensively
706 described [150]. According to European Working Group on Clinical Cell Analysis (EWGCCA)

707 [135], the single-platform method should be regarded as the gold standard for absolute cell
708 assessments, and the use of stain/lyse/no-wash labelling protocols is recommended.

709

710 **9. Characterization of tissue macrophages via immunohistochemistry.**

711 Circulating monocytes migrate into various tissues and transform into macrophages. Macrophages
712 have several roles in tissues monitoring, inflammation and repair representing one of the dominant
713 cell types in chronic inflammatory reactions.

714 Under non-inflammatory conditions, circulating monocytes localize in tissues and organs becoming
715 resident macrophages to monitor healthy tissues. However, many resident macrophages are
716 established before birth and then self-maintained during adult life. Resident macrophages are found
717 in connective and adipose tissues, liver (Kupffer cells), splenic red pulp, bone marrow, lymph
718 nodes, lung, brain, serous fluids, skin [151].

719 Under inflammatory conditions, most of the macrophages that accumulate at diseased sites typically
720 derive from circulating monocytes and take on several functions according to the inciting factors
721 and milieu including antigen presentation, phagocytosis, and immunomodulation through the
722 production of various cytokines and growth factors. Macrophages can be classified based on their
723 function and activation pathways. According to this grouping, there are classically-activated (M1)
724 macrophages, wound-healing macrophages (also known as alternatively-activated (M2)
725 macrophages), and regulatory macrophages (Mregs) [23,152,153].

726 Macrophages can be activated directly by bacterial lipopolysaccharides, via TLR and extracellular
727 matrix proteins. Under T_H1 influence, macrophages react to $IFN-\gamma$, and $TNF-\alpha$ to become
728 classically activated M1 macrophages producing Nitric Oxide and Reactive Oxygen Species and
729 upregulating lysosomal enzymes to kill ingested organisms or digest foreign substances [23,152]. In
730 T_H2 conditions macrophages act in response to IL-4, IL-5 and IL-13 by becoming wound healing
731 M2 macrophages that secrete growth factors promoting angiogenesis, activating fibroblasts, and
732 collagen synthesis [23,152]; under T regulatory secretion of IL-10 become regulatory/anti-
733 inflammatory Mreg macrophages producing IL-10 to suppress inflammation [153].

734

735 *9.1 Morphological characterization of macrophages: cytology and histology*

736

737 Following tissue entry and activation, monocytes in tissue undergo functional and morphological
738 transformation into reactive macrophages that are morphologically readily recognizable.

739 Thus, macrophages can be identified morphologically on cytological and histological specimens.

740 Cytological specimens are obtained by several techniques including imprints from tissues, scrapings
741 to sample firm lesions which are less likely to exfoliate cells, swabs or by the needle aspiration
742 technique. Sampled material by any of the aforementioned sampling methods can be smeared and
743 air-dried [154]. Fluid specimens such as bronchoalveolar lavages, cerebrospinal fluid, articular fluid
744 can be collected into an EDTA or a plain (if bacteriology is required) tube. Fluids can be
745 cytocentrifuged or directly smeared according to their cellularity and again air-dried. Routine
746 cytological stains are represented by alcohol-based Romanowsky type stains including May-
747 Grünwald-Giemsa and Wright's stains [155].

748 On cytological specimens, macrophages are easy to identify:

749 a-Reactive macrophages (Fig. 6A) are cells characterized by round to irregular shape, variably
750 large size (averaging 18-30 μm), low N/C ratio, abundant light blue foamy to highly vacuolated
751 cytoplasm with a paracentral to eccentric round to oval/reniform nucleus with granular chromatin
752 and often one visible nucleolus. Macrophages phagocytize and degrade foreign exogenous or
753 endogenous material and several etiological agents such as mycobacteria thus, they are recognized
754 also because of their cytoplasmic content (Fig. 6B).

755 b-Multinucleated macrophagic giant cells are cells developing in persistent granulomatous
756 inflammation by fusion of two or more activated macrophages in the attempt to engulf and degrade
757 large or undigestible material or organisms. Multinucleated giant macrophages can be extremely
758 large (40-80 μm), have two to multiple nuclei that may be haphazardly arranged termed foreign
759 body type giant cells or have nuclei arranged at the periphery along the cell membrane in the so-
760 called horseshoe fashion. These cells are named Langhans cells and develop commonly but are not
761 exclusive of tuberculosis in humans as in cows.

762 c-Epithelioid macrophages develop in response to persistent intracellular pathogens such as
763 mycobacteria. Epithelioid macrophages are characterized by large size, round to polygonal to
764 spindle shape, abundant homogeneous light blue homogeneous, smooth cytoplasm with little to no
765 vacuoles, an oval variably eccentric nucleus with finely granular chromatin. These cells are
766 specialized in the production of cytokines and chemokines and do not have or have minimal
767 phagocytic functions.

768 In tissue specimens, macrophages, their architectural organization and possibly the engulfed
769 causative agent can be viewed by histology. For histological evaluation, tissues are fixed in 10%
770 neutral phosphate-buffered formalin. Fixation in formalin provides a standardized method to
771 immortalize tissues providing with optimal tissue architecture and morphology. Formalin is a
772 solution of Formaldehyde fixing by adducts crosslink (methylene bridges) firming the tissue.
773 Fixation times vary according to with type and size of tissues with a penetrance of 1mm per hour of

774 a 1 mm³ tissue specimen with a volume of 1:10-1:20 with general fixation times of 24-48 h.
775 Following adequate fixations, tissues are routinely processed, dehydrated in graded alcohol scales to
776 remove free water, followed by tissue clearing (alcohol removal) in xylene and embedding in
777 paraffin. Tissue sections of 3-6 µm are cut, punt onto glass slides, dried, deparaffinized and stained
778 routinely with hematoxylin and eosin. Macrophage morphology is similar to that described for
779 cytological specimens (see the previous section) with observation or foamy reactive macrophages
780 with pink to grey granular to vacuolated cytoplasm with variably distinct cell boundaries (Fig. 6C),
781 multinucleated giant cells (Fig.6C and Fig. 6D) and epithelioid cells. Histopathology provides with
782 reduced cellular detail compared with cytology, however, give relevant information regarding the
783 organization of the inflammation. Specifically, granulomatous inflammation is a chronic
784 inflammation in which monocyte-macrophages predominate. Granulomatous inflammation can be
785 diffuse (lepromatous) with macrophages randomly dispersed in sheets (Fig. 6E) or nodular
786 (tuberculoid) with macrophages arranged in distinct masses termed granulomas (Fig. 6F). Diffuse
787 granulomatous inflammation is currently believed to derive from T_H2 driven stimuli while nodular
788 granulomatous inflammation derives from the predominance of T_H1 immunologic responses [156–
789 159]. The prototypic diffuse granulomatous inflammation in bovines is seen in Johne' diseases
790 where lamina propria of the intestine is expanded by sheets of macrophages (Fig. 6G associating to
791 a heavy cytoplasmic burden of *M. avium subsp. paratuberculosis* (Fig. 6H) and often with minimal
792 lymphoplasmacytic associated inflammation. In contrast, well-formed granulomas have a specific
793 stratigraphy. The prototype of nodular tuberculoid granuloma develops in bovines and humans
794 following *M. bovis* and *M. tuberculosis* infection. Granulomas in these conditions are generally
795 paucibacillary. Histologically, granulomas are recognized as caseating that is with a central core of
796 necrosis or non-caseating. Non-caseating granulomas contain a core of macrophages with variable
797 epithelioid and multinucleated giant cells and may be surrounded by mature lymphocytes, plasma
798 cells and rimmed by fibroblasts and collagen. Caseating granulomas have a similar arrangement but
799 with variably extensive central areas of necrosis and can be seen in bovine (Fig. 6I) and human
800 tuberculosis. In granulomas developing in the context of inflammatory reactions to persistent and
801 poorly degradable antigens (e.g. *Nocardia* spp., *Actinobacillus* spp., *Actinomyces* spp., *S. aureus*)
802 central accumulation of organisms rimmed and/or embedded in intensely eosinophilic material can
803 be seen. This material represents the accumulation of immunoglobulins and is termed splendore-
804 Hoeppli phenomenon (Fig. 6J). **Furthermore, granulomas can be also formed after vaccine or alum**
805 **administration as described in many animal species including cats, dogs, swine, cattle and sheep.**
806 Histochemical stains for macrophage identification are occasionally applied and include lysozyme
807 and acid phosphatase 5 [160] that however, should not be considered specific for macrophages.

808

809 *9.2 Immunohistochemistry and Immunofluorescent characterization of macrophages.*

810

811 Immunohistochemistry and immunofluorescence are techniques that have been utilized to identify
812 macrophages and macrophage subpopulations by definition of their antigen surface expression. The
813 phenotype of macrophages has been evaluated on cytological specimens, [161] frozen tissues and
814 formalin-fixed, paraffin-embedded tissue specimens. Immunofluorescence is a technique that
815 provides the same information of immunohistochemistry (protein expression by a cell or a tissue)
816 but by activation of fluorescent dyes. Compared to immunohistochemistry, immunofluorescence
817 allows for single and double stains on tissues with an easier interpretation of co-expression of
818 antigens. However, immunofluorescence stained tissues need to be kept in the dark and must be
819 evaluated with a fluorescence microscope. Tissue sections stained with fluorescent dyes need to be
820 analysed promptly since fluorescence intensity decays rapidly (few days to few weeks). Also,
821 autofluorescence interferes with the detection of specific fluorescent signals, especially when the
822 signals of interest are very dim. Immunofluorescence is generally applied to frozen tissue sections
823 while is seldom applied to formalin-fixed and paraffin-embedded samples because of tissue
824 processing associates with increased autofluorescence that may interfere with positive
825 immunofluorescent signals especially if these are dim. Additionally, immunofluorescent expression
826 of markers on tissue macrophages should be interpreted with care since degradation components
827 such as lipofuscins accumulating in macrophage cytoplasm are autofluorescent.

828 Immunohistochemistry is the most frequently applied technique to assess bovine macrophage
829 phenotype in formalin-fixed tissue specimens, however, while formalin is the most common
830 substance used worldwide for tissue fixation, induces cross-links (methylene bridges) that can block
831 antibody access to antigenic epitopes or can damage surface antigens. To overcome antigen damage
832 or masking caused by formalin fixation, immunofluorescence and immunohistochemistry can be
833 applied to air-dried cytological specimens [162] or frozen tissue sections. Once the specimen is
834 adequately sectioned immunohistochemistry and immunofluorescence techniques are similar.
835 Immunohistochemistry on tissue sections has the advantage of visualizing protein expression in the
836 context of the tissue, tissue lesion and specific cell type. This is less so for frozen specimens that
837 bear inferior morphology and more so for immunofluorescence that reduces visualization of
838 morphology. However, immunofluorescence is still favoured in specific studies because allows with
839 easier double or multiple stains on tissues.

840 For immunohistochemistry, 3-6- μm sections are cut and put onto glued or charged slides to avoid
841 detachment. Sections are deparaffinized and rehydrated using xylene and graded ethanol series. For

842 formalin-fixed tissue specimens in specific cases, antigen retrieval may be necessary. Antigen
843 retrieval methods using enzymatic or heat-based protocols vary according to each molecular target
844 and comprise: heat-induced (heating by pressure cookers or microwave in citrate buffer for 10 to 20
845 min), enzymatic (0.1% trypsin or pepsin in phosphate-buffered saline for 30 min at 37°C) or
846 proteinase K (10 µg/mL proteinase K in Tris buffer for 15 min) may be necessary [163–168].
847 Endogenous peroxidase is quenched with 3% H₂O₂ in PBS or 3% H₂O₂ in methanol for 20-30
848 minutes to avoid false-positive results. Following washes in buffers, nonspecific antibody binding is
849 inhibited by incubating tissues with blocking agents that vary according to primary and secondary
850 antibody types and techniques. Primary antibody incubation temperatures (4-37°C) and times (1
851 hour to overnight) vary according to the reagents and techniques with longer incubation times
852 favoured for paraffin-embedded tissue sections [164]. Secondary biotinylated antibody incubation
853 and signal amplification with different systems such as avidin-biotin (ABC), streptavidin-
854 peroxidase complexes are followed by visualization with chromogens such as 3, 3'-
855 diaminobenzidine or 3-Amino-9-ethyl carbazole (AEC) carbazole and counterstained with
856 hematoxylin.

857 For immunofluorescence, tissue specimens should be snap-frozen in isopentane cooled at the freezing
858 temperature by immersion in liquid nitrogen. Frozen samples should be stored at -80°C or cut on a
859 cryostat immediately after freezing. For immunofluorescence, sections are dried and autofluorescence
860 is quenched with 3-3 DAB. The following steps parallel those described for immunohistochemistry.
861 Primary antibodies can be directly labelled with the fluorescent dyes thus the secondary antibodies
862 and steps involving amplification of the signal and visualization with chromogens can be avoided. If
863 primary antibodies are unconjugated, visualization is achieved by incubating the samples with the
864 specific secondary antibody generally conjugated to rhodamine or FITC. Nuclei can be counterstained
865 with 4',6- diamidino-2-phenylindole, dihydrochloride (DAPI) or Hoechst 33342.

866 Dual-colour immunofluorescence has been seldom applied to bovine tissues for macrophage
867 evaluation [169].

Table. 5

List and details of molecular targets utilized for the immunohistochemical or immunofluorescent examination of bovine macrophages in formalin-fixed or frozen tissue sections.

Primary Ab Target	Main cellular expression	Known or proposed functions	IHC/IF	Ref.
CD14	Monocytes, Macrophages	Coreceptor for bacterial LPS, recruiting LPS to TLR, clearance of apoptotic cells,	IF	[169,170]
CD11b	Monocytes, Macrophages, natural killer cells, neutrophil granulocytes,	Heterodimer binding to beta integrin CD18 (Mac-1 complex) mediating leukocyte adhesion and migration	IF	[169]
CD68	Monocytes, Macrophages, Osteoclasts	Lysosomal membrane activated phagocytosis	IHC, IF	[138, 141–143]
CD107b	Placenta, lung and liver (wide tissue distribution), activated neutrophil granulocytes, activated platelets, activated T cells, activated endothelium	Modulates chaperone-mediated autophagy. Decreases presentation of endogenous antigens by MHCII Maintenance of lysosomal integrity, pH and catabolism	IF	[160]
CD163	Monocytes, Macrophages, M2 polarization	Acute phase-regulated receptor, clearance and endocytosis of haemoglobin/haptoglobin complexes by macrophages (protect tissues from free haemoglobin-mediated oxidative damage). Innate immune sensor for bacteria inducer of proinflammatory cytokine production	IF, IHC	[136–138,144–147]
CD172a	Neutrophil granulocytes, Monocytes, Macrophages M2 polarization	Receptor-type transmembrane glycoprotein negative regulator of receptor tyrosine kinase-coupled signalling	IHC	[175,176]
CD204	Myeloid cells	Endocytosis of modified/oxidized low-density lipoproteins	IHC	[163]
CD206	Macrophages, Immature Dendritic cells	Scavenger receptor that binding and internalization and lysosomal transport of microbial high mannose oligosaccharides	IF	[173]
Iba-1	Activated macrophages,	Actin and calcium-binding protein with a role in Chemotaxis. Induced by cytokines and interferon promote	IHC	[163,166,167,171]

	Microglia, activated endothelial cells	macrophage activation and growth of vascular smooth muscle cells and T-lymphocytes.		
Mac387	Neutrophils Granulocytes, Monocytes, Macrophages	calcium- and zinc-binding protein with proinflammatory, antimicrobial, oxidant-scavenging and apoptosis-inducing activities.	IHC	[166]
Nramp-1	Monocytes, Macrophages	Role in resistance to intracellular bacterial <u>pathogens</u>	IHC	[165]

CD14 (Monocyte Differentiation Antigen CD14), CD11b (Integrin alpha), CD68 (Scavenger Receptor Class D, LAMP4, SCARD1), CD107b (Mac3, LAMP2-lysosomal associated membrane protein 2), CD163 (AM-3K, Scavenger Receptor Cysteine-Rich Type 1 Protein M130), CD172a (SIRPA, Signal Regulatory Protein Alpha), CD204 (MSR1, Macrophage scavenger receptor 1), CD206 (Mannose receptor MRC1), Iba-1 (Ionizing binding adaptor molecule 1, Allograft inflammatory factor 1 - AIF-1), Mac387 (Calprotectin beta chain), Nramp-1 (Natural resistance-associated macrophage protein 1)

Table 6. List of molecular targets used for the immunohistochemical or immunofluorescent identification of bovine macrophage M1/M2 polarization in formalin-fixed or frozen tissue sections.

Cytokine target	M1/M2 Polarization	Technique	Disease	Ref.
IFN γ	M1	IF, IHC	Paratuberculosis	[166,173]
IL1Ra	M2	IF	Paratuberculosis	[173]
IL1- β	M1	IF, IHC	Paratuberculosis	[173,177]
			Contagious pleuropneumonia	
IL-10	Mreg	IF	Paratuberculosis	[165,173]
IL-12p40	M1	IF	Paratuberculosis	[173]
IL-17A	M1	IHC	Contagious pleuropneumonia	[177]
IL-23	M1	IF	Paratuberculosis	[173]
iNOS	M1	IHC	Paratuberculosis	[165]
TGF- β	M2	IF, IHC	Paratuberculosis	[165,173]
TLR4 (CD284)	M1	IF	Paratuberculosis	[173]
TNF- α	M1	IF, IHC	Paratuberculosis	[165,173,177]
			Contagious pleuropneumonia	
uNOS (all NOS isoforms)	M1	IF	Paratuberculosis	[173]

Several molecular targets have been utilized for the identification of bovine macrophages in formalin-fixed (Fig. 6K) or frozen tissue specimens. The molecular targets for macrophage recognition in cattle, their function and corresponding references are listed in Table 5.

Immunohistochemistry and immunofluorescence have been utilized to identify, enumerate and characterize tissue macrophages in formalin-fixed [160,163–167,175–177] or frozen [168–170,172–174] sections of a variety of bovine tissue samples including adipose tissue [160,167,168,175], in the intestinal tissue of cattle with spontaneous paratuberculosis at different stages of the disease [165,166,172–174], the endometrium of pregnant and post parturient dairy cows [169,170,176], fibrotic liver lesions in bovines infected with *Fasciola hepatica* [163], lung lesion in bovine contagious pleuropneumonia [177] and early lesions during experimental *M. bovis* aerosol infection [164].

Polarization of M1-host defence macrophages versus M2-resolution/tissue repair macrophages has been investigated on tissue sections in bovine paratuberculosis and contagious bovine pleuropneumonia [165,173,177]. The target molecules assessed to study bovine macrophage polarization in tissues comprise several cytokines (Fig. 6L) and are listed in Table 6. Regarding macrophage polarization, the evaluation of CD163 expression as a specific marker for M2 polarization has been questioned [174] because of its widespread expression in macrophages independently of disease stage of bovine paratuberculosis. Thus, CD163 is now favoured as a marker for all bovine macrophages and this reason has been listed in Table 5 and not in Table 6. Studies on paratuberculosis have demonstrated significantly higher numbers of M2 macrophages in the ileum of bovines with the clinical stage multibacillary lesions [165,173]. On the contrary, significant polarization versus M1 phenotype has been observed in paucibacillary lesions [166]. In lung lesions of contagious bovine pleuropneumonia, a general M1 polarization has been detected [177].

10. Conclusions

The present work reviews the current methods to classify in subpopulations and study the main functions of monocyte and macrophages in bovine species. The concept of monocyte/macrophage polarization in subsets has been validated and accepted in bovine species, and monocyte distribution in blood, and macrophage in tissues, mostly resembles that of human species. Indeed, new knowledge on monocyte and macrophage function basic biology and functions is needed to understand their impact on immune response in cows. Further studies are needed to demonstrate if monocytes can be further classified in subsets and to determine what is the function of each sub-set in bovine species as well. Among the various techniques applied so far, flow cytometry is the most promising and rapid method to identify new monocyte populations in bovine species and study their functions. The

application of flow cytometry in veterinary medicine has lagged behind human medical uses: the number of specific antibodies is limited, and the cross-reactivity of most of those available for human and murine studies is not validated in cows. The lack of specific reagents is what holds major challenges in advancing the knowledge in bovine monocytes. **Lacking a labelled anti-Cd16 antibody allowing purification of ncM and intM is what prevents a full understanding of the functions of these important sub-classes of monocytes in the cow. Transcriptome analysis of resting and activated monocytes and macrophages is also limited to few studies: the role of different monocyte subset would be much more clear with a system biology approach by integrating transcriptome, metabolome, proteome and microRNAome of each subset after inflammatory activation.**

This information would provide important advancement in diagnosis and investigation of the pathogenesis of important cattle diseases, such for example tuberculosis, brucellosis or Johne's disease that affect human health as well.

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Figure Captions

Fig. 1 Bovine peripheral blood smears.

A. Normal round monocyte with a deeply indented nucleus and light blue homogeneous cytoplasm is present in a background of normal erythrocytes. Note the non-vacuolated cytoplasm, not all peripheral blood monocytes contain cytoplasmic vacuoles. Bar=7,2 μ m.

B. Large peripheral blood monocyte with light blue non-vacuolated cytoplasm with an irregularly bean-shaped indented nucleus. Bar= 6 μ m.

C. Large peripheral blood monocyte with irregular membrane contour and abundant light blue cytoplasm characterized by multiple, variably sized discrete cytoplasmic vacuoles. Bar= 4,9 μ m. May-Grünwald Giemsa stain.

Fig. 2 Phase-contrast photomicrographs of cultured monocytes following MACS purification.

A. Monocytes the first day in culture (10%FBS supplemented RPMI media). Bar= 20 μ m;

B. Monocytes maintained for two days demonstrating a slight increase in size and shape and size variability. Bar=20 μ m.

C. Two monocytes maintained for four days in culture and characterized by variably increased size and presenting with cytoplasmic processes indicative of activation and initial transformation into macrophages. Bar= 10 μ m.

Fig. 3. Flow cytometry evaluation of monocytes, before and after sorting.

PBMC are showed in a representative dot plot FSC vs SSC based on size and granularity (complexity) before (A) and after MACS separation (D). Monocytes staining with FITC-anti-CD14 antibody is showed before (B) and after sorting (E). Representative histograms of the relative percentage of lymphocytes and monocytes are showed before (C) and after sorting (F). MACS purification gives an enrichment of CD14⁺ monocytes population > of 98%.

Fig. 4. Flow cytometry detection, by annexin V-FITC/PI, of apoptosis and necrosis on bovine PBMC incubated for 1 h at 37 °C.

On dot plot FSC vs SSC, a gate around lymphocytes and monocytes regions was drawn and apoptosis and necrosis in gated events were determined. Live lymphocytes (A) and monocytes (B) are Annexin V and PI double negative (lower left quadrant). At the early stage of apoptosis, the cells bind annexin V while still excluding PI (lower right quadrant). Double positive events are necrotic cells (upper right quadrant). The PI-positive - annexin V negative cells (upper left quadrant) are considered necroptotic cells [178].

Fig. 5. *In vitro* methods to measure chemotaxis

(A) Schematic illustration of the Boyden chamber assay. Cells seeded in the upper chamber migrate through a porous membrane, towards the lower chamber in the response of a chemoattractant gradient concentration. (B) Schematic illustration of a migration transwell plate. The multiwell plate is packaged with permeable and removable cell culture inserts with a porous membrane that allows the migration of the cells towards the medium/chemoattractant-filled lower well. Images were created with BioRender.com".

Fig. 6 Morphological and Immunohistochemical features of bovine macrophages in cytological and tissue specimens

Fig. 6 A Cytology of synovial fluid in a calf with arthritis. Moderately cellular cytological sample illustrating numerous foamy reactive macrophages with variably abundant cytoplasm with numerous cytoplasmic vacuoles admixed with non-degenerate neutrophils in a background of confluent erythrocytes. Joint tap, May-Grünwald Giemsa stain. Bar= 25 µm

Fig. 6 B Cytology of synovial fluid in a calf with arthritis. Large reactive macrophage with heavily vacuolated cytoplasm containing phagocytized apoptotic nuclear material. The macrophage is surrounded by moderately degenerated erythrocytes (crenation) and non-degenerated neutrophils in a granular background of synovial mucins. Joint tap, May-Grünwald Giemsa stain. Bar=7,5 µm

Fig. 6 C Histology of a bovine mesenteric lymph node in a case of paratuberculosis. Sheets of reactive macrophages characterized by varying size, pink to grey cytoplasm and oval nuclei with granular to reticular chromatin and evidence of 1-2 small, basophilic nucleoli are admixed with mature lymphocytes and rare plasma cells. A single multinucleated macrophage with haphazardly arranged nuclei is evident (arrowhead). Haematoxylin and eosin stain. Bar= 14 µm

Fig. 6 D Histology of a bovine thoracic lymph node in a case of Tuberculosis. At the centre of the image, there is a large multinucleated giant macrophage characterized by several nuclei arranged in

a horse-snow fashion beneath the cell membrane. The cell is surrounded by occasional epithelioid cells, foamy reactive macrophages with large cytoplasmic well distinct vacuoles and elevated numbers of lymphocytes (left) and lesser plasma cells. Haematoxylin and eosin stain. Bar= 32 μm

Fig. 6 E Bovine small intestine in a case of paratuberculosis. Lamina propria of intestinal villi is substituted and expanded by massive infiltration of large reactive macrophages displacing peripherally and compressing intestinal glands. Haematoxylin and eosin stain. Bar= 170 μm

Fig. 6 F Bovine tongue in a case of wooden tongue (*Actinobacillus lignieresii* infection). Large granuloma composed of reactive macrophages admixed with lesser numbers of neutrophils, eosinophils and lymphocytes and surrounded by fibroblasts and collagen). Haematoxylin and eosin stain. Bar= 75 μm

Fig. 6 G Bovine small intestine in a case of paratuberculosis. Diffuse granulomatous inflammation expanding and substituting the intestinal lamina propria with separation of the epithelial mucosal lining. Large reactive macrophages predominate and associate with lesser numbers of lymphocytes. In the left lower part of the image, an intestinal gland is compressed with gland epithelium lightly infiltrated by eosinophils (arrowhead). Haematoxylin and eosin stain. Bar= 64 μm

Fig. 6 H Bovine small intestine in a case of paratuberculosis. High numbers of acid-fast positive mycobacteria (*Mycobacterium avium* sbsp. paratuberculosis) in the cytoplasm of macrophages and free in the lamina propria. Ziehl – Neelsen stain. Bar= 22 μm

Fig. 6 I Bovine lung in a case of tuberculosis. The image shows the typical stratigraphy of a tuberculoid granuloma substituting the pulmonary parenchyma. The right and central parts of the image represent the extensive central area of caseous necrosis characterized by amorphous eosinophilic and granular basophilic tissue debris. In the left part of the image, necrosis is rimmed by neutrophils, macrophages, multinucleated giant cells of the Langhans cell type and mature lymphocytes. The external portion of the granuloma is composed of fibroblasts and collagen delimiting the granuloma. Haematoxylin and eosin stain. Bar= 196 μm

Fig. 6 J Bovine tongue in a case of wooden tongue (*Actinobacillus lignieresii* infection). Discrete granuloma with central intensely eosinophilic material corresponding to the accumulation of immunoglobulin/immunocomplexes and termed Splendore-Hoeppli phenomenon. Haematoxylin and eosin stain. Bar= 110 μm

Fig 6 K Immunohistochemical stain of a bovine reactive thoracic lymph node. Numerous Iba-1 positive reactive foamy macrophages in the medullary cords. Anti-Iba-1 immunoperoxidase stain, AEC chromogen, hematoxylin counterstain. Bar= 70 μm

Fig 6 L Immunohistochemical stain of a calf lung with bronchopneumonia. Alveolar lumen containing numerous reactive macrophages and rare neutrophils. Two M1 type macrophages

intensely expressing $\text{TNF-}\alpha$. Anti- $\text{TNF-}\alpha$ immunoperoxidase stain. AEC chromogen, hematoxylin counterstain. Bar= 18 μm

Highlights

Monocytes are a complex population of cells including subsets with specific functions
Understanding individual subset functions requires specific purification methods
Monocytes main roles are in both triggering inflammation and restoring homeostasis
The review presents methods to isolate and characterize bovine monocytes and macrophages.

