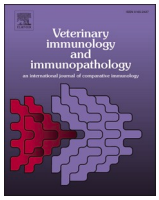
Veterinary Immunology and Immunopathology 251 (2022) 110461

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Rules of thumb to obtain, isolate, and preserve porcine peripheral blood mononuclear cells

Ivan Díaz [a,b,c]

[a] Centre de Recerca en Sanitat Animal, Institut de Recerca en Tecnologies Agroalimentàries (IRTA-CReSA), 08193 Bellaterra, Spain
[b] World Organization for Animal Health (OIE) Collaborating Centre for the Research and Control of Emerging and Re-Emerging Swine Diseases in Europe (IRTA-CReSA), 08193 Bellaterra, Spain
[c] World Organization for Animal Health (OIE) Reference Laboratory for Classical Swine Fever Virus (IRTA-CReSA), 08193 Bellaterra, Spain

ARTICLE INFO

Keywords:
PBMC
PBL (peripheral blood lymphocytes)
Isolation
Cryopreservation
Porcine

ABSTRACT

One of the most used biospecimens in immunology are peripheral blood mononuclear cells (PBMC). PBMC are particularly useful when evaluating immunity through responses of circulating B- and T-cells, during an infection, or after a vaccination. While several reviews and research papers have been published aiming to point out critical steps when sampling, isolating, and cryopreserving human PBMC -or even analyzing any parameter before sampling that could impair the immune assays' outcomes-, there are almost no publications in swine research dealing with these topics. As it has been demonstrated, several factors, such as stress, circadian rhythmicity, or the anticoagulant used have serious negative impact, not only on the separation performance of PBMC, but also on the ulterior immune assays. The present review aims to discuss studies carried out in humans that could shed some light for swine research. When possible, publications in pigs are also discussed. The main goal of the review is to encourage swine researchers to standardize protocols to obtain, manage and preserve porcine PBMC, as well as to minimize, or at least to consider, the bias that some parameters might induce in their studies before, during and after isolating PBMC.

1. Introduction

To measure B- and T-cell responses against a particular antigen it is paramount to analyze immune response during an infection, or after a vaccination. A broad variety of techniques have been established for humans, or classical model animal species used to study *basic science* or human diseases, with swine immunology being always begging behind.

The in vivo study of lymphocytes involves significant technical obstacles, and usually becomes a very tedious work. In swine immunology, some complications are unmanageable, mainly for two reasons: I) the techniques have not yet been developed, and II) the high economic costs cannot be assumed for the species. On the contrary, the *ex vivo* evaluation of isolated cells, such as peripheral blood mononuclear cells (PBMC), is simple and it is affordable for most research laboratories. Certainly, significant phenotypic differences between PBMC and tissue or mucosa lymphocytes isolated from the primary site of a disease exist. Also, it can be assumed that blood is basically a *highway,* where cells are transported and where most of the immunological processes do not occur. Indeed, in *ex vivo* experiments, cells lack the environmental stimuli they would have been exposed to in the organism. Despite these disadvantages, PBMC are largely used in human research, so that a plethora of articles have been published on their obtention and preservation. In swine research, although PBMC are also widely used to evaluate the immunity to infections or vaccinations, there are few publications aiming to evaluate procedures for handling and preserving them. Since these first steps are crucial, as they are the initial stage for the application of very sensitive and complex ulterior analyzes, it is of value to examine it.

The present article aims to review factors that influence the obtention and preservation of porcine PBMC, and more specifically peripheral blood lymphocytes (PBL)[1], discussing articles from human and swine research when available. The final objective is to define parameters that should be considered during B- and T-cell assays from pig blood, as well as those preanalytical factors that can impact on host immunity and therefore, on the assays (Table 1). This information is useful for swine researchers, as well as for researchers that use pig as an animal model in

E-mail address: ivan.diaz@irta.es.

[1] A great proportion of cells within PBMC corresponds to PBL and some studies evaluate immune responses corresponding to lymphocytes but use the nomenclature that encompasses all mononuclear cells (PBMC). Accordingly, throughout this review both nomenclatures will be used, being most times interchangeable.

https://doi.org/10.1016/j.vetimm.2022.110461
Received 13 May 2022; Received in revised form 13 July 2022; Accepted 15 July 2022
Available online 16 July 2022

Table 1
Main preanalytical factors in swine that can affect outcomes of the immunological assays and/or PBMC isolation and preservation.

Before sampling	• Stress: social (mixing/separation/hierarchy); environment (temperature, humidity, dust); animal handling. • Nutritional status (starvation and immunonutrients). • Age. • Genetics. • Immune status (immunizations or infections, especially with those pathogens with immunomodulation/suppression properties, such as Porcine circovirus 2, Porcine reproductive and respiratory syndrome virus or Classical swine fever virus).
During sampling	• Stress: animal handling, blood-drawing (restrained). • Circadian rhythmicity: sampling time of the day. • Anticoagulants.
Isolation	• Blood storage (time and temperature). • Separation tubes.
Cryopreservation	• Cryopreservation media and technique. • PBMC concentration. • Cooling rate. • Temperature. • Time of storage. • Thawing (overnight resting).

translational medicine, or as a potential donor in xenotransplants.

2. Factors before and during blood sampling that can influence the outcome of immune assays

In human, several factors before the blood sampling or directly related to when or how the blood sampling is made, can influence the immune cells and therefore, the outcome of the assays (Mallone et al., 2011; Betsou et al., 2019;). In this first section, the influence of some of these factors will be reviewed. In regards of swine, for the impact of other factors not reviewed in the present article, such as age, breed, or housing, mainly when related to stress, reading of Martínez-Miró's review (2016) is strongly advised.

2.1. Stress

Stress induces biological responses, releasing cortisol and catecholamines. As many immune cells express receptors for them, both hormones can interfere with the immune competence. In humans, a meta-analytic review from the 90′s revealed that stressors -conditions that induce stress- decrease PBL proliferation to mitogens (Herbert and Cohen, 1993). More recently, stressors have been related to the impairing of the cytokine synthesis and to the release of immunosuppressive cytokines (Elenkov and Chrousos, 2002; Padgett and Glaser, 2003).

In swine, an acute increase in cortisol concentration decreases the number of PBL and the production of antibodies (Wallgren et al., 1994), while chronic stress can dysregulate the immune function by promoting pro-inflammatory responses and shifting Th1 to Th2 responses (Salak-Johnson and McGlone, 2007).

As pigs are social animals that group in well-stablished social structures with dominance hierarchies, mixing stimulates fights to establish new status of hierarchy and cause a social stress, which seems to be higher in smaller than in larger groups, and in males compared to females (Martínez-Miró et al., 2016). The social stress due to mixing can be acute, immediately following regrouping, or chronic when the animals are socially subordinate. As soon as one hour after mixing, pigs have a peak of cortisol, which decrease the lipopolysaccharide (LPS)-induced TNF-α secretions (Bacou et al., 2017). In the long-term, social stress can have a negative impact on antiviral immunity (de Groot et al., 2001). To note, individual circumstances during, before and after a stressor can have a different outcome for the same stressor. Thus, a dominant pig may have enhanced immune activation after mixing, whereas subordinated animals, or those losing rank in the dominance hierarchy, can suffer an immunosuppression (Wallgren et al., 1994). Also, mixed dominants are more seriously affected than mixed subordinates (de Groot et al., 2001). Regarding environment, climate fluctuations, prolonged cold or heat stress, airborne pollutants, and dust, can affect cytokines, antibody production and/or white blood cell counts. For example, it has been described that both cold and heat stress can significantly dysregulate serum concentrations of cytokines, such as IL-1β and IL-6 increases in pigs housed in a cold environment (Frank et al., 2003) or the decrease of IL-2 in heat-stressed pigs (Ju et al., 2014). Also, IL-2 production in PBMC supernatants and leukocyte percentage significantly decrease during three consecutive weeks of heat stress (Ju et al., 2014). In contrast, constant exposure of pigs to high levels of airborne endotoxins in dust can result in high circulating B-cell counts, as well as higher levels of IgG, IgE, IFN-γ and IL-4, plus lower levels of IgA and TNF-α compared to pigs with low endotoxin exposure (Roque et al., 2018). Indeed, synergic negative effect on immune responses can be observed for some of these factors (Morrow-Tesch et al., 1994).

2.2. Nutritional status

It is well-documented that diet and nutritional state can influence immune responses for human and mouse models (Alwarawrah et al., 2018). This influence, associated with either acute starvation due to long-term general nutritional deprivation, or by lack of specific nutrients, can reduce the immune responses capacity of the organism to mount a response against pathogens or after vaccination (Bourke et al., 2016; Childs et al., 2019).

Fasting causes a rapid decrease in leptin levels in serum (Houseknecht et al., 1998). This hormone has several functions that affect human PBL directly or indirectly. In innate immunity, it modulates the function of antigen presenting cells by licensing them towards Th1-cells priming. In adaptive immunity, it increases the proliferation of both naïve B- and T-cells, reduces the proliferation of regulatory T-cells, promotes the switch towards Th1, helps Th17 responses, and activates B-cells (Francisco et al., 2018). Altogether, results indicate that leptin bias responses towards Th1 (Lord et al., 1998). Regarding prolonged starvation, it diminishes drastically the leptin levels, consequently impairing T-cell priming and IFN-γ production (Faggioni et al., 2001; La Cava, 2017). Also, long starvation has been related with lower CD4 + T-cell levels and IL-2 production upon phytohemagglutinin (PHA) stimulation (Sävendahl and Underwood, 1997).

In pigs, although it seems that down regulation of leptin is much more inconspicuous compared to other species, Barb et al. (2001) have hypothesized that leptin is related to the regulation of immunity. Apart from a good general nutrition status, immune system requires specific nutrients, known as immunonutrients (Suchner et al., 2000). Usually, negative interferences with immune system could appear when some of them are lacking, when present in insufficient amount, or even when present in excess. In pigs, an excessive intake of Zinc or vitamin A has been related with a decrease in the effectiveness of the immune functions (Pluske et al., 2018).

2.3. Genetics

Genetic modifications to generate animals with lower or higher immune responses or even to obtain resistance to particular pathogens have been extensively applied in those species classically used for translational medicine. In pigs, some knock-out genetic lines have been created to be resistant to particular viruses (Whitworth et al., 2016; Xie et al., 2018), to obtain translational cancer models (Boettcher et al., 2018), or even to obtain genetically engineered donor pigs for xenotransplants (Carvalho-Oliveira et al., 2021). However, genetic selection has been classically focused on improving growth rates, feed efficiency, meat quality or sow prolificacy. Unfortunately, selecting pigs to improve immune responses seems almost diametrically opposed to currently

selected productive parameters (Wilkie and Mallard, 1999; Pluske et al., 2018). Nevertheless, few examples of selection over many generations to gain or loss intensity in the immune responses exist (Rauw et al., 1998; Crawley et al., 2005) and, in this sense, a general rule is impossible to determine.

2.4. Blood sampling

During blood sampling in pigs, classical methods of restraint the individual are very stressful (Tallet et al., 2017). In longitudinal studies, where animals suffer repetitive sampling, it is a great handicap. As vena cava, jugular veins and carotid artery are the typical venipuncture sites to obtain blood, such procedures of restrain seem to be virtually impossible to eliminate. To reduce the stress the number of neutral or positive interactions during handling should be increased, avoiding negative interactions (Tallet et al., 2017; Yang et al., 2021). Alternatively, restrained for bleeding could also be achieved chemically. In this last case, it must be checked that no interactions exist between sedation drugs and immunological evaluations carried out during the experiment, since it has been demonstrated in humans that some of these products can dysregulate the immune system (Yuki et al., 2011; Chen et al., 2022), including those used in pigs, such as midazolam (Smith et al., 2014). Also, a catheter for sequential blood sampling can be implanted; however, it can be only used when animals are housed individually, otherwise they will uproot it from each other.

2.5. The impact of the circadian rhythmicity: sampling time of the day

In humans, many aspects of the immune system show circadian rhythmicity (Scheiermann et al., 2013), including lymphocyte trafficking between blood and tissues; therefore, immune cell population counts may differ throughout the day (Suzuki et al., 2016). Due to the role of cortisol in both stress and circadian rhythmicity, differences in adrenocortical responses to stressors may depend upon when, during the circadian cycle, the stimulus is applied (Selmaoui and Touitou, 2003). This phenomenon has been also observed in pigs (Ruis et al., 1997).

Amounts of porcine PBL also differ throughout the day. T-cells peak during night, but, in contrary to humans in which B-cells peak during the resting phase (Born et al., 1997), no changes are found in porcine B-cells (Engert et al., 2018). The proportions of T-cell subtypes in porcine blood could also vary during the day (Engert et al., 2017). Therefore, in a given study, bleeding should be done at the same time of the day, to diminish the bias that circadian rhythmicity could cause.

3. Anticoagulants: Heparin, EDTA or citrate?

In humans, contradictory results have been published when comparing anticoagulants (Aziz et al., 2019; Biancotto et al., 2012; Bull et al., 2007; Vanham et al., 1998). Nevertheless, the use of sodium heparin as anticoagulant have been largely recommended for T-cell analyses (Nicholson et al., 1993; Mallone et al., 2011; Betsou et al., 2019). Ethylenediamine tetraacetic acid (EDTA) and citrate are not recommended, since they act as Ca^{2+}-chelating agents, which can interfere with the activation of T-cells (Kay, 1971). Furthermore, it seems that human blood collected in tubes with EDTA tends to deteriorate faster than blood collected in heparin or citrate, affecting both the PBL viability and functionality (Bull et al., 2007; Lin et al., 2009). In conclusion, heparin, and probably citrate anticoagulants, were apparently better than EDTA for human PBL preservation for downstream B- and T-cell analysis.

Regarding the suitability of anticoagulants for porcine, little information is available. Duvigneau et al. (2007) compared EDTA and heparin effects on the cytokine mRNA expression profiles (pro and anti-inflammatory, and Th1 or Th2 markers) after in vitro stimulation with concanavalin A or LPS. The main conclusion was that isolated cells from EDTA showed higher capacity to express cytokines than cells from heparinized blood. Hence, while heparin is strongly recommended as anticoagulant for lymphocyte assays in other species, there is a lack of data to determine it for pigs.

To fill more than one blood tube consecutively during immunological studies is a common procedure. In humans, it is recommended to take the tube/s with anticoagulants the last, to avoid contamination from anticoagulants to the serum tube/s. Sampling in pigs can be an extremely variable time-consuming procedure. This facilitates the formation of micro-clots, which could complicate the cells isolation and produce biases in the outcomes (Humann-Ziehank and Ganter, 2012). To avoid clotting, they recommend starting with the most sensitive sample, e.g., the tube with anticoagulant, followed by the serum tube. According to these authors, it seems that micro-clots are more frequent in EDTA-anticoagulated blood samples.

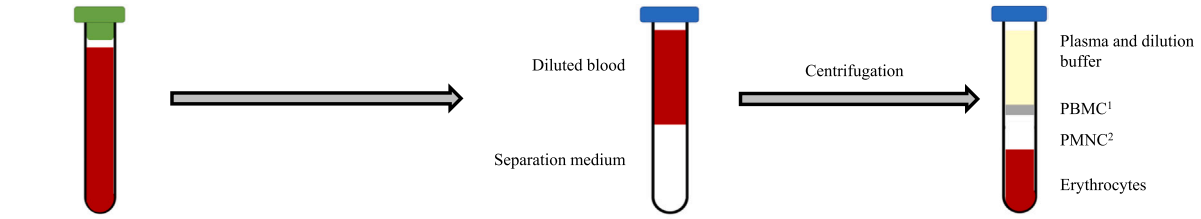
4. Blood storage. How and how long can the blood be stored before processing

Isolated PBMC are mainly used in immunity research because their immune responses are significantly higher than whole blood (Weinberg et al., 1998). Prior to isolation, time and temperature of blood shipping and storage can affect both the isolation and the ulterior assays. Apart from the loss of cell viability, granulocyte contamination is the main reason behind the loss of quality that samples suffer over time. The granulocytes activation, that occurs during prolonged storage periods ($>$ 12 h), provokes changes in the buoyancy, which in turn provokes a loss in the efficiency of PBMC isolated by density gradient centrifugation. Moreover, increased frequencies of activated CD11b+ CD15 + granulocytes reduce the capacity of human T-cells to proliferate in presence of PHA (McKenna et al., 2009) and their specific-responses against viral epitopes (Bull et al., 2007). To prevent granulocyte contamination, PBS or special stabilizers upon collection can be added (Olson et al., 2011; Bouwman et al., 2012). In regards of temperature, it has been demonstrated that sample exposure to 15ºC or 40ºC for more than 8 h has a negative impact on post-thaw viability and T-cell functionality (Olson et al., 2011).

Unfortunately, there are no articles aimed to evaluate the impact of time and temperature of blood storage on porcine PBMC. In absence of them, the guidelines advised for human should be also followed for swine. Fig. 1 summarizes the critical points when isolating PBMC.

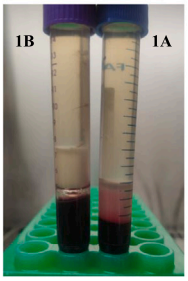
5. PBMC isolation

Since antigen-specific B- and T-cells are usually found in blood in low frequency, an ineffective PBMC isolation significantly affects the results of subsequent immune assays. Because granulocytes and erythrocytes have a higher density than mononuclear cells, isolation of PBMC is accomplished by means of density gradient media, that depends on the species and the age − 1.077 g/mL for porcine, 1.075 g/mL for piglets during the first seven weeks of life (Talker et al., 2013)-. The *classical* protocol to obtain PBMC is a labor-intensive and time-consuming process that requires certain technical expertise. In other species, PBMC isolation using specific tubes directly from the specimen is widely used. In this latter case, blood is collected in a tube system that contains separation polyester gel, density gradient media and sodium citrate (e.g., CPT™; BD); PBMC isolation is achieved after the tube centrifugation, without pre-dilutions or a gradient of density. If blood is collected using conventional tubes, there are alternatives to avoid the classical PBMC isolation such as Leucosep® (Greiner Bio-One), or SepMate™ (StemCell) (Fig. 1). They include an insert that avoids the mixture of the sample with the separation medium, making the layering process easier. In general, it seems that the abovementioned systems achieved similar, or even better results isolating human PBMC that the *classical* one, in terms of granulocyte contamination and performance of T-cells (Ruitenberg et al., 2006; Grievink et al., 2016). Grievink and collaborators (2016), compared CPT™, SepMate™ and typical density centrifugation in terms



- **Anticoagulant:** depending on the ulterior analysis. Usually, in heparin tubes.
- **Temperature of shipping/storage:** 22-30ºC. Never at 4ºC.
- **Time:** Samples must be processed as soon as possible (<6-8h). When not possible, blood should be diluted (1:1; e.g. PBS or RPMI-1640) (always <16h). In this case (8-16h), we must assume some alterations in the PBMC isolation and the assays outcomes.

- Although cell distribution depends on their density (each layer contains a specific cell population), cross-contamination can exist. It strongly depends on **operator expertise and sample quality.**
- **Tubes:** Instead of conical sterile tubes (1A), tubes including an insert that avoids the mixture of the diluted blood and separation medium can be used (e.g. Leucosep®; Greiner Bio-One, or SepMate™; StemCell) (1B). In humans, cell preparation tubes are also commonly used (e.g CPT™; BD). In this case, blood samples are drawn directly into the tube and cell separation is carried out on it. The tubes contain the anticoagulant, the liquid density medium and an inert gel barrier, which, after centrifugation, forms a physical barrier between PBMC and erythrocytes and granulocytes.



Irrespective of the tubes used, PBMC must be washed, and erythrocytes lysed, if needed. Contamination of erythrocytes can cause an inaccurate count, mainly in manual counting. Later, **Viability** is evaluated (>95% is mandatory for fresh cells) and cells counted.

(1) PBMC: Peripheral blood mononuclear cells (lymphocytes and monocytes)
(2) PMNC: Polymorphonuclear cells

Fig. 1. Critical points on PBMC isolation.

of recovery, viability, and functionality of human cells. Regarding recovery, the classical approach achieved the lowest values, whereas the higher erythrocyte contamination was observed for CPT™. Nevertheless, the cell viability was always 100% and the composition of the lymphocyte populations showed minor differences among the three techniques. When responses to a super antigen stimulation were measured, classically-isolated cells secreted lower amounts of IFN-γ than those isolated using CPT or SepMate™. The main recommendation from the authors was to use CPT or SepMate™, because they usually resulted in a 70% higher lymphocyte recovery compared to *classical* isolation. Also, it should be mentioned using this type of tubes processing time is significantly reduced, and they are more user-friendly, being strongly recommended to avoid variations due to different degrees of laboratory personnel expertise.

Although similar results could be expected for swine, no studies systematically comparing different tubes have been published. Nevertheless, alternative methods, as SepMate™, are slowly being implemented in procedure protocols for porcine samples (Li et al., 2021).

6. PBMC freezing and thawing

Although it is desirable, the use of fresh cells for immunological assays is not always possible. On many occasions we are forced to work with frozen PBMC, especially when lacking infrastructures or labor, or even having them, because the number of samples is too high. Also, additional analyses can be necessary later. Besides, B- and T-cell analyses usually require time-consuming processes that can fail. As these cells are a primary cell line of short path, to have a frozen aliquot would really act as an insurance policy. In conclusion, frozen cells alone, or in parallel with fresh ones, could be useful to do immunological assays and to protect important stocks that may be difficult to replace. However, the freeze-thaw process is stressful to all cells and tissues. Therefore, cryopreservation must be done accurately, since the process could alter, not only the lymphocyte viability and recovery, but also the phenotype and even their immunological responses.

Cells, and particularly lymphocytes, are very sensitive to the freezing process. During freezing, cells can be damaged mainly by two mechanisms: the formation of intracellular ice crystals, and the dehydration, shrinkage, and injury due to an osmotic imbalance (Rapatz et al., 1968; Mazur, 1984; Elliott et al., 2017). All these phenomena are strongly dependent on the cooling rate. As discussed later, a correct cooling rate should be slow enough to prevent the formation of intracellular ice crystals but fast enough to prevent osmotic damages. It has been demonstrated that many other processing factors may influence the frozen human and porcine lymphocyte viability and functionality, including cryopreservation media, storage conditions (time and temperature), and thawing process. Also, cell concentration in each cryovial is critical; all recommendations point out to $< 30 \times 10^6$ PBMC/mL (Disis et al., 2006; Mallone et al., 2011), or even much lower ($< 5 \times 10^6$) (Betsou et al., 2019).

6.1. Cryopreservation media

Cryoprotectants are compounds that protect cells during a slow freezing process, mainly by replacing water to diminish the osmotic imbalance and the formation of intracellular ice (Elliott et al., 2017). One of the most used is dimethyl sulfoxide (DMSO), usually diluted 1:10 in fetal bovine serum (FBS). Using this combination, lymphocyte viability reached highest values, irrespective of the type of anticoagulant and tube used during extraction and isolation (Consuegra et al., 2017). Apart from DMSO, several commercial media, both based or not on DMSO, have been developed. To note, it is widely accepted that lymphocyte viability below 70–80% should be avoided since seriously impaired the results of immunological assays (Weinberg et al., 2000; Maecker et al., 2005). However, as discussed later, the real effect of cryopreservation on lymphocyte functionality is a controversial issue, even obtaining a high percentage of viability.

Regarding the impact of cryopreservation media on porcine PBMC, three of them have been recently compared in terms of recovery rates and viability; the *classic* DMSO 10% + FBS 90% and two commercial products -PSC Cryopreservation kit (Thermo Fisher Scientific) and CryoStor CS10 (Stemcell Technologies), both containing DMSO (Li et al.,

2021). Recovery rates after thawing were similar among the three media and, in turn, they were like those described by Liang et al. (2019), who found a mean of 73.7% when comparing five cryopreservation media to freeze human PBMC. In regards of viability, it was on average higher than 89% for all three-cryopreservation media, fulfilling the requirements of viability and recovery.

6.2. Cooling rate, temperature, and time of storage

Several years ago, Leibo et al. (1978) evaluated different cooling rates (ºC/min) in DMSO frozen cells. They concluded that best rate to minimize impact on cells was − 1 ºC/min. At this cooling rate, cells lose water fast enough, with no intracellular ice crystal formation and avoiding the osmotic damage. This cooling rate can be achieved by freezers that can control it automatically or using specialized freezing containers, which are based on isopropyl alcohol (e.g. Mr Frosty) or on alcohol free (e.g. CoolCell).

Temperature in which frozen lymphocytes are stored has a great influence on PBMC viability and functionality. At − 80 ºC small traces of unfrozen media and water still exist, allowing molecular processes to injure cell continuously over time, even during a short-term storage (>3 days). However, at − 132 ºC water does not exist in liquid form and there is not enough energy, so molecular processes and chemical reactions are suspended, plus free radical generation is prevented (Baust et al., 2015).

Even at ultra-low storage temperatures, there is a maximum time in which lymphocytes could be stored and eventually recovered with no damage. Although high percentages of viability and recovery rates have been confirmed after 12 years of storage (Kleeberger et al., 1999), it has been suggested that lymphocytes should not be cryopreserved more than six months (Owen et al., 2007). In that latter study, both CD4 + and CD8 + T-cell responses decreased, being more evident in cells stored for more than one year compared to less than six months, even when a high percentage of viability was maintained. Similar results could be expected for swine, but there are no published studies on the effect of all these parameters -cooling rates, temperatures, and long-term storage- on porcine lymphocytes.

The specific impact of cryopreservation on B- and T-cells phenotyping and functionality is extensively discussed below.

6.3. Thawing PBMC

Thawing is one of the most critical steps when working with frozen cells. To minimize osmotic variations that occurs during slow thawing, cells should rapidly be thawed in a dry thawer, or in a water bath at 37 ºC, being later washed and centrifuged to eliminate DMSO completely. Also, overnight resting at 37 ºC, 5% CO_2 before performing the immunological assays is advisable, since it improves results in ulterior immunological assays, particularly when measuring antiviral human T-cell responses (Kutscher et al., 2013; Santos et al., 2014). During this period, the fraction of cells undergoing death is eliminated and therefore, although the absolute number of recovered cells could fall, the percentage of viable ones improves and the interference that non-viable cells would have on the assays disappear (Mallone et al., 2011).

6.4. Impact of cryopreservation on human PBMC: recovery rate, viability, phenotyping, and functionality

To a greater or lesser degree, both viability percentage and recovery rate may be affected during freezing and thawing processes. Despite this, the viability percentage and recovery rates should not be a great issue when a proper protocol is applied. However, since freezing could affect the lymphocyte phenotyping and functionality, to evaluate the success of freezing and thawing processes only by checking cells viability is a serious mistake. As discussed in the next paragraph, even with viabilities higher than 90%, proportions of cell populations can vary, and their functionality can be impaired.

Some researchers have concluded that phenotype proliferation and functional assays should be always performed on fresh lymphocytes, instead of frozen ones. For instance, when Costantini et al. (2003) compared fresh versus frozen/thawed samples isolated from 19 healthy individuals and 21 *human immunodeficiency virus* (HIV)-infected patients they observed that, frozen PBL showed a significant decline of the proportions of naïve and central memory T-cells, an increase of the proportions of effector $CD8^+$ T-cells only in healthy subjects, and a loss of proliferative responses to some virus and HIV antigens. Other alterations due to cryopreservation, such as impairing the cell proliferation and altering the proportions of lymphocyte subsets and the releasing of cytokines have been also reported (Baust et al., 2015). However, for other authors the B- and T-cell functionality does not suffer significant alterations due to cryopreservation and thawing. In this sense, when immunophenotypes of lymphocytes have been evaluated among laboratories in multicenter trials, it has been reported that slight or no changes are observed when comparing fresh and frozen samples (Reimann et al., 2000). In that study, few specimens that showed positive lymphocyte proliferative responses to mitogens or antigens lost their responsiveness after cryopreservation. Accordingly, Reimann and collaborators (2000) argued that, in the light of these results, although losses can exist, frozen lymphocytes are still suitable for immunophenotyping and functional testing.

As stated, impact of cryopreservation on human lymphocyte functionality and phenotyping continues to be a controversial issue, since publications aimed to assess this topic reveals conflicting results (Table 2). Interestingly, an in-depth review of these publications reveals interesting findings that could shed light to this controversy. Firstly, although all freezing and thawing protocols followed the general rules, they were not the same. Little variations may greatly bias the final outcomes. For example, as discussed above, resting overnight can have a great impact on lymphocyte functionality. This could partially explain apparent contradictions among some articles evaluating B- and T-cell subsets (Costantini et al., 2003; Tompa et al., 2018). Also, the time passed between blood obtention, processing and freezing can affect the results. A 24 h lapsed-time results in slight decreases in the viability (−8%), but marker decreases in recovery (−32%) and in IFN-γ ELISPOT (from −36% to −56%), as compared to processing-freezing cells in less than 8 h (Bull et al., 2007). Apart from that, other factors, such as individual condition of the specimen (healthy or infected by a particular agent), immunologic parameter analyzed, specific immunoassay applied or the antigen or mitogen used, could explain the discrepancies. In this sense, articles done by the same laboratory team comparing different factors are quite interesting. Kvarnström and collaborators (2004) investigated the effect of freezing on spontaneous, auto-antigen, allergen, and mitogen induced cytokine secretion from several groups of patients. Results demonstrated that the process of cryopreservation and thawing affect by itself the expression of cytokines, but the magnitude varied among different cytokines, different stimuli, and different patient groups.

6.5. Impact of cryopreservation on porcine PBMC: recovery rate, viability, phenotyping, and functionality

While many articles aimed to evaluate the impact of cryopreservation on human lymphocytes, analyzing phenotyping and functionality by several points of view, only three articles aiming this issue on porcine ones: Koch and collaborators (1991), Li and collaborators (2009) and, more recently, Li and collaborators (2021) (Table 3). Among them, only Li's (2021) paper aimed to study the effect on antigen-specific responses. Regarding responses to mitogens, Koch and collaborators (1991) found that cryopreservation impaired the lymphocyte responses, and Li and collaborators (2009) observed that phorbol myristate acetate (PMA)-stimulated frozen cells produced significantly less IL-6 quantity than

Table 2
Impact of cryopreservation on human PBMC, in terms of immunophenotype and functionality. Brief summary of the plethora of articles published aimed to evaluate the impact of cryopreservation on human PBMC.

ARTICLE	ASSAY	CONCLUSIONS
Immunophenotype: B and T-cell subsets		
Reimann et al. (2000)	Flow cytometry / Nine laboratories	• Loss of B-cells, but no total loss of T-cells or $CD4^+$ or $CD8^+$ T-cells in HIV-1-infected patients.
Costantini et al. (2003)	Flow cytometry	• Changes in PBMC from healthy and HIV-infected individuals, e.g. decline of naïve and central memory T-cells and increase of effector $CD8^+$ T-cells.
Tompa et al. (2018)	Flow cytometry	• Although naïve and differentiated $CD4^+$ and $CD8^+$ T-cells were affected, subsets of CD4, CD8 and CD25hi were in general not influenced by long-term cryopreservation.
Functionality		
Cytokines		
Kreher et al. (2003)	ELISPOT	• Antigen-specific $CD4^+$ and $CD8^+$ T-cells maintain full functionality in ELISPOT assays (IL-2, IL-4, IL-5 and IFN-γ)
Kvarnström et al. (2004)	ELISA, ELISPOT, and mRNA	• Alteration of Th1 and TH2 cytokines expression, both at the protein and the mRNA level. • *The effect varies among different cytokines, different stimuli, and different patient groups.*
Mallone et al. (2007)	ELISPOT	• No alteration of net antigen-specific frequencies of IFN-γ-secreting cells, although more spontaneous secretion of IFN-γ is observed.
Owen et al. (2007)	Flow cytometry (intracellular cytokine staining)	• Short-term cryopreservation (6 months) may be acceptable for measuring CD4 + and CD8 + T-cell responses in HIV-infected individuals. • Long-term cryopreservation (1 year) may lead to the loss of $CD4^+$ T-cell responses and mild skewing of T-cell phenotypic marker expression.
Axelsson et al. (2008)	Multiplex fluorochrome technique (Luminex®) and mRNA	• Increased spontaneous secretion/expression: IL-6, IL-10, IL-12, IL-13, IFN-γ and MCP-1 (Luminex®), and FOXP3 and TGF-β (mRNA). • Antigen-specific response: higher levels of IL-6, IFN-γ, TNF-α and MIP-1α, and lower secretion of IL-10 and IL-13. • Lower secretion/expression after PHA stimulation: IP-10, MCP-1 and RANTES (Luminex®) and FOXP3 (mRNA). • *Frozen PBMC are suitable to assess immunological markers, even though their expression differs from fresh PBMC.*
Ford et al. (2017)	ELISPOT and flow cytometry (intracellular cytokine staining)	• 3–5-fold reduction in antigen-specific IFN-γ CD4 + . • Antigen-specific IFN-γ CD8 + relatively unaffected.
Proliferative responses		
Reimann et al. (2000)	Lymphocyte proliferation (thymidine) / Nine laboratories	• < 10% of specimens that showed positive PBMC proliferative responses to mitogens or antigens lose their responsiveness after cryopreservation.
Costantini et al. (2003)	Lymphocyte proliferation (thymidine)	• Reduced response to some HIV antigens and cytomegalovirus and influenza virus in HIV-infected patients.

Table 3
Impact of cryopreservation on porcine PBMC, in terms of viability, immunophenotype and functionality.

Article	Assay	Conclusions
Koch et al. (1991)	Concentration of free intracellular Ca^{2+} (PHA) PHA and alloantigen-induced blastogenesis Cell-mediated cytotoxicity	• Average recovery rates: 74.7% (61–94%). Average of viability: 94.5%. • Frozen PBMC weakly responded with an increase of Ca^{2+} after PHA stimulation = > severe disturbance of normal membrane functions. • Thawed PBMC retained or recovered some of their blastogenic potential during culture = > functional individual thawed cells reacted in the same way as fresh cells. • Almost complete failure of frozen PBMC to differentiate into cytotoxic effector cells. • *Some immunological functions are more affected by cryopreservation than others.*
Li et al. (2009)	ELISA	• The storage time (2, 5, 25, 50 days) did not significantly affect cell viability, being the average always > 89.8%. • After PMA stimulation, IFN-γ production from the frozen PBMC was significantly higher than that from the fresh ones. In contrast, IL-6 production was significantly lower from the frozen PBMC. No differences found for IL-4.
Li et al. (2021)	Comparison of three freezing media: viability, recovery rates, response to mitogens (ELISPOT).	• Average of viability and recovery rates for medium containing 90% FBS + 10% DMSO, PSC Cryopreservation kit, and CryoStor CS10 were: 96% and 70%, 89% and 70%, and 97.0% and 71%, respectively; all freezing media fulfilled requirements of viability and recovery. • ELISPOT IFN-γ (PHA) and IgG (R848 +IL-2) were like those obtained using fresh PBMC.
	Immunophenotyping (flow cytometry), proliferation of B- and T-cells (responses to recall antigen and mitogens; flow cytometry). Responses to recall antigen and mitogens (ELISPOT).	• Compared to fresh PBMC, frozen ones accounted proportions equivalent for $CD3^+$, $CD4^+CD8^-$ and $CD4^+CD8^+$, increased for $CD4^-CD8^-$, and decreased for $CD4^-CD8^+$. • Reduction of proliferation using recall antigen (PRRSV), mainly for B-cells and $CD4^+CD8^+$ T-cells. • Reduction of PRRSV-specific IFN-γ-SC; on average, − 41% compared to fresh cells (individually, from −21% to −54%) • *Frozen PBMC are mostly suitable for immunophenotyping and functional testing, as far long as mitogens are used. For recall antigen stimulation, freezing had a significant impact on the magnitude of the response, although responding animals could be identified.*

PMA-stimulated fresh ones. In this case, no differences in viability were observed among storage periods − 2, 5, 25 and 50 days-, indicating that in short-term cryopreservation losses are due to the freezing-thawing process, rather than the duration of storage. Conversely, in Li's article (2021), in which thawed cells rested overnight before assays, frozen lymphocyte responses to mitogens were like those obtained using fresh ones, for both IFN-γ and IgG production measured by ELISPOT. This observation was valid for three different freezing products-methods. Nevertheless, in that article was demonstrated that cryopreservation impairs antigen-specific responses. In that case, samples were obtained from *porcine reproductive and respiratory syndrome virus* (PRRSV)-vaccinated pigs. Then, as the sensitivities of different cell subset of PBL to cryopreservation could be different, recovered cells were phenotyped by flow cytometry and used to assess PRRSV-specific responses, by a proliferation assay and an IFN-γ ELISPOT. The PRRSV-specific responses of frozen cells were significantly impaired in the proliferation assay, particularly for $CD4^{+}CD8^{+}$ double positive T-cells, which accounted a large proportion of memory cells in pigs (Zuckermann and Husmann, 1996). Recall responses in IFN-γ ELISPOT were also significantly lower in frozen lymphocytes compared to fresh ones. Interestingly, as $CD4^{+}CD8^{+}$ double positive T-cells are the primary source of IFN-γ when responded to the PRRSV recall stimulation (Meier et al., 2003), lower recall responses in IFN-γ ELISPOT observed for frozen lymphocytes could be due to the impairment of $CD4^{+}CD8^{+}$ T-cell subset. To note, although antigen-specific responses were impaired by the cryopreservation and thawing process, we could detect responder pigs -vaccinated ones- as such (Li et al., 2021).

In human research, a pre-stimulation step analyzing antigen-specific responses of frozen cells may improve the ELISPOT sensitivity (Smith et al., 2009). However, this enhancement depended on the donor of cells, as well as the antigen. In Li's article (2021), the pre-stimulation step caused a significant enhancement -up to three times higher compared to no-pre-stimulated ELISPOT- in 40% of the pigs. As the enhancement could also depend on the antigen used (Smith et al., 2009), we strongly recommend evaluating this strategy for other antigens.

7. Discussion/conclusion

The great effort done by researchers around the world underscore the importance of developing standardized procedures when bleeding to obtain PBMC. As seen, the impact of several factors prior to bleeding should also be considered when analyzing immune responses. Once cells are obtained, several studies have highlighted the importance of optimizing and standardizing cells handling to the specific conditions of each research. In human, this has become especially apparent during the development of clinical trials and studies across multiple sites, such as for HIV, or more recently for *severe acute respiratory syndrome coronavirus 2*. Particularly, when frozen cells are used, it is essential to develop protocols with a high reproducibility between samplings and among experiments, to ensure a minimum loss compared to fresh lymphocytes, not only in terms of viability. As discussed, this requirement could be of special importance for antigen-specific responses.

For some of the abovementioned issues, knowledge from human can be adapted to swine research. In other cases, human research could be helpful, but some gaps still need to be filled. Thus, original research specifically focused on porcine is mandatory. One clear example are the few studies done evaluating the impact of cryopreservation on lymphocyte functionality. According to them, it seems that porcine frozen lymphocytes are, as human ones, suitable for phenotyping and functional testing using mitogens, but cryopreservation may have a negative impact on the antigen-specific responses. Although it seems that responding animals could be identified using frozen cells, the loss of sensitivity could have a huge impact in particular situations, such as: 1) evaluating vaccines, as T-cell responses are generally lower compared to natural infections; 2) evaluating T-cell responses in those antigens where low responses are expected, such as peptides or particular pathogens; or, 3) evaluating cell responses long time after the infection/vaccination. Either way, after the controversy that exists in humans and the few studies that exist in pigs, further experiments should be done to improve our knowledge about the precise impact of cryopreservation on porcine cells. To note, there is room for improvement on different antigen-specific responses and/or using animals at different times of immunization to analyze naïve, central memory and effector memory cells.

As largely discussed in the present review, the use of PBMC requires standardization at several steps. Undoubtedly, there is still a long way to go in swine research. To improve the outcomes assays and their interpretations, swine immunologists should head towards the principle suggested for human studies: "*rigorous quality assurance and quality control in all laboratories involved in PBMC isolation and cryopreservation is crucial to uniform processing of specimens*" (Bull et al., 2007) and to obtain the most accurate result.

Funding

This work was partially supported by the National Institute of Research and Agricultural and Food Technology, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain (INIA, reference E-RTA2015–0003-C02–01).

Declarations of interest

The author declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

To those who laid the groundwork to use PBMC as a tool to evaluate immunity. To all swine immunologists. I would like to especially thank members from UAB involved in PRRS immunology. CERCA Program from the Generalitat de Catalunya is also acknowledged.

References

Alwarawrah, Y., Kiernan, K., MacIver, N.J., 2018. Changes in nutritional status impact immune cell metabolism and function. Front. Immunol. 9, 1055. https://doi.org/10.3389/fimmu.2018.01055.

Axelsson, S., Faresjö, M., Hedman, M., Ludvigsson, J., Casas, R., 2008. Cryopreserved peripheral blood mononuclear cells are suitable for the assessment of immunological markers in type 1 diabetic children. Cryobiology 57, 201–208. https://doi.org/10.1016/j.cryobiol.2008.08.001.

Aziz, N., Butch, A.W., Ryner, T.C., Martinez-Maza, O., Detels, R., 2019. The influence of EDTA Vacutainer blood collection tube on the level of blood interleukin-1 receptor antagonist. J. Immunol. Methods 464, 114–118. https://doi.org/10.1016/j.jim.2018.10.009.

Bacou, E., Haurogné, K., Mignot, G., Allard, M., De Beaurepaire, L., Marchand, J., Terenina, E., Billon, Y., Jacques, J., Bach, J.M., Mormède, P., Hervé, J., Lieubeau, B., 2017. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. Physiol. Behav. 169, 1–8. https://doi.org/10.1016/j.physbeh.2016.11.012.

Barb, C.R., Hausman, G.J., Houseknecht, K.L., 2001. Biology of leptin in the pig. Domest. Anim. Endocrinol. 21, 297–317. https://doi.org/10.1016/s0739-7240(01)00123-0.

Baust, J.M., Corwin, W.L., VanBuskirk, R., Baust, J.G., 2015. Biobanking: the future of cell preservation strategies. Adv. Exp. Med. Biol. 864, 37–53. https://doi.org/10.1007/978-3-319-20579-3_4.

Betsou, F., Gaignaux, A., Ammerlaan, W., Norris, P.J., Stone, M., 2019. Biospecimen science of blood for peripheral blood mononuclear cell (PBMC) functional applications. Curr. Pathobiol. Rep. 7, 17–27. https://doi.org/10.1007/s40139-019-00192-8.

Biancotto, A., Feng, X., Langweiler, M., Young, N.S., McCoy, J.P., 2012. Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects. Cytokine 60, 438–446. https://doi.org/10.1016/j.cyto.2012.05.019.

Boettcher, A.N., Loving, C.L., Cunnick, J.E., Tuggle, C.K., 2018. Development of severe combined immunodeficient (SCID) pig models for translational cancer modeling: future insights on how humanized SCID pigs can improve preclinical cancer research. Front. Oncol. 8, 559. https://doi.org/10.3389/fonc.2018.00559.

Born, J., Lange, T., Hansen, K., Mölle, M., Fehm, H.L., 1997. Effects of sleep and circadian rhythm on human circulating immune cells. J. Immunol. 158, 4454–4464.

Bourke, C.D., Berkley, J.A., Prendergast, A.J., 2016. Immune dysfunction as a cause and consequence of malnutrition. Trends Immunol. 37 (6), 386–398. https://doi.org/10.1016/j.it.2016.04.003.

Bouwman, J.J., Thijsen, S.F., Bossink, A.W., 2012. Improving the timeframe between blood collection and interferon gamma release assay using T-Cell Xtend®. J. Infect. 64, 197–203. https://doi.org/10.1016/j.jinf.2011.10.017.

Bull, M., Lee, D., Stucky, J., Chiu, Y.L., Rubin, A., Horton, H., McElrath, M.J., 2007. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. J. Immunol. Methods 322, 57–69. https://doi.org/10.1016/j.jim.2007.02.003.

Carvalho-Oliveira, M., Valdivia, E., Blasczyk, R., Figueiredo, C., 2021. Immunogenetics of xenotransplantation. Int. J. Immunogenet. 48, 120–134. https://doi.org/10.1111/iji.12526.

Chen, R., Sun, Y., Lv, J., Dou, X., Dai, M., Sun, S., Lin, Y., 2022. Effects of dexmedetomidine on immune cells: a narrative review. Front. Pharmacol. 13, 829951 https://doi.org/10.3389/fphar.2022.829951.

Childs, C.E., Calder, P.C., Miles, E.A., 2019. Diet and immune function. Nutrients 11, 1933. https://doi.org/10.3390/nu11081933.

Consuegra, I., Rodríguez-Aierbe, C., Santiuste, I., Bosch, A., Martínez-Marín, R., Fortuto, M.A., Díaz, T., Martí, S., Muñoz-Fernández, M.Á., 2017. Isolation methods of peripheral blood mononuclear cells in Spanish biobanks: an overview. Biopreserv. Biobank. 15, 305–309. https://doi.org/10.1089/bio.2016.0105.

Costantini, A., Mancini, S., Giuliodoro, S., Butini, L., Regnery, C.M., Silvestri, G., Montroni, M., 2003. Effects of cryopreservation on lymphocyte immunophenotype and function. J. Immunol. Methods 278, 145–155. https://doi.org/10.1016/s0022-1759(03)00202-3.

Crawley, A.M., Mallard, B., Wilkie, B.N., 2005. Genetic selection for high and low immune response in pigs: effects on immunoglobulin isotype expression. Vet. Immunol. Immunopathol. 108, 71–76. https://doi.org/10.1016/j.vetimm.2005.07.006.

de Groot, J., Ruis, M.A., Scholten, J.W., Koolhaas, J.M., Boersma, W.J., 2001. Long-term effects of social stress on antiviral immunity in pigs. Physiol. Behav. 73, 145–158. https://doi.org/10.1016/s0031-9384(01)00472-3.

Disis, M.L., DeLaRosa, C., Goodell, V., Kuan, L.Y., Chang, J.C., Kuus-Reichel, K., Clay, T. M., Kim Lyerly, H., Bhatia, S., Ghanekar, S.A., Maino, V.C., Maecker, H.T., 2006. Maximizing the retention of antigen specific lymphocyte function after cryopreservation. J. Immunol. Methods 308, 13–18. https://doi.org/10.1016/j.jim.2005.09.011.

Duvigneau, J.C., Sipos, W., Hartl, R.T., Bayer, M., Moldzio, R., Stevenson, L., Adair, B., Gemeiner, M., 2007. Heparin and EDTA as anticoagulant differentially affect cytokine mRNA level of cultured porcine blood cells. J. Immunol. Methods 324, 38–47. https://doi.org/10.1016/j.jim.2007.04.012.

Elenkov, I.J., Chrousos, G.P., 2002. Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity. Ann. N. Y. Acad. Sci. 966, 290–303. https://doi.org/10.1111/j.1749-6632.2002.tb04229.x.

Elliott, G.D., Wang, S., Fuller, B.J., 2017. Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology 76, 74–91. https://doi.org/10.1016/j.cryobiol.2017.04.004.

Engert, L.C., Weiler, U., Stefanski, V., Schmucker, S.S., 2017. Data characterizing diurnal rhythms in the number of peripheral CD8α$^-$ and CD8α$^+$ γδ T cells in domestic pigs. Data Brief. 16, 843–849. https://doi.org/10.1016/j.dib.2017.12.013.

Engert, L.C., Weiler, U., Pfaffinger, B., Stefanski, V., Schmucker, S.S., 2018. Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs. Dev. Comp. Immunol. 79, 11–20. https://doi.org/10.1016/j.dci.2017.10.003.

Faggioni, R., Feingold, K.R., Grunfeld, C., 2001. Leptin regulation of the immune response and the immunodeficiency of malnutrition. FASEB J. 15, 2565–2571. https://doi.org/10.1096/fj.01-0431rev.

Ford, T., Wenden, C., Mbekeani, A., Dally, L., Cox, J.H., Morin, M., Winstone, N., Hill, A. V.S., Gilmour, J., Ewer, K.J., 2017. Cryopreservation-related loss of antigen-specific IFNγ producing CD4+ T-cells can skew immunogenicity data in vaccine trials: lessons from a malaria vaccine trial substudy. Vaccine 35, 1898–1906. https://doi.org/10.1016/j.vaccine.2017.02.038.

Francisco, V., Pino, J., Campos-Cabaleiro, V., Ruiz-Fernández, C., Mera, A., Gonzalez-Gay, M.A., Gómez, R., Gualillo, O., 2018. Obesity, fat mass and immune system: role for leptin. Front. Physiol. 9, 640. https://doi.org/10.3389/fphys.2018.00640.

Frank, J.W., Carroll, J.A., Allee, G.L., Zannelli, M.E., 2003. The effects of thermal environment and spray-dried plasma on the acute-phase response of pigs challenged with lipopolysaccharide. J. Anim. Sci. 81, 1166–1176. https://doi.org/10.2527/2003.8151166x.

Grievink, H.W., Luisman, T., Kluft, C., Moerland, M., Malone, K.E., 2016. Comparison of three isolation techniques for human peripheral blood mononuclear cells: cell recovery and viability, population composition, and cell functionality. Biopreserv. Biobank. 14, 410–415. https://doi.org/10.1089/bio.2015.0104.

Herbert, T.B., Cohen, S., 1993. Stress and immunity in humans: a meta-analytic review. Psychosom. Med. 55, 364–379. https://doi.org/10.1097/00006842-199307000-00004.

Houseknecht, K.L., Baile, C.A., Matteri, R.L., Spurlock, M.E., 1998. The biology of leptin: a review. J. Anim. Sci. 76, 1405–1420. https://doi.org/10.2527/1998.7651405x.

Humann-Ziehank, E., Ganter, M., 2012. Pre-analytical factors affecting the results of laboratory blood analyses in farm animal veterinary diagnostics. Animal 6, 1115–1123. https://doi.org/10.1017/S1751731111002679.

Ju, X.H., Xu, H.J., Yong, Y.H., An, L.L., Jiao, P.R., Liao, M., 2014. Heat stress upregulation of Toll-like receptors 2/4 and acute inflammatory cytokines in peripheral blood mononuclear cell (PBMC) of Bama miniature pigs: an in vivo and in vitro study. Animal 8, 1462–1468. https://doi.org/10.1017/S1751731114001268.

Kay, J.E., 1971. Interaction of lymphocytes and phytohaemagglutinin: inhibition by chelating agents. Exp. Cell. Res. 68, 11–16. https://doi.org/10.1016/0014-4827(71)90580-5.

Kleeberger, C.A., Lyles, R.H., Margolick, J.B., Rinaldo, C.R., Phair, J.P., Giorgi, J.V., 1999. Viability and recovery of peripheral blood mononuclear cells cryopreserved for up to 12 years in a multicenter study. Clin. Diagn. Lab. Immunol. 6, 14–19. https://doi.org/10.1128/CDLI.6.1.14-19.1999.

Koch, E., Larak, M., Ellendorff, F., 1991. Comparative studies on in vitro reactivity of fresh and cryopreserved pig lymphocytes. Cryobiology 28, 405–412. https://doi.org/10.1016/0011-2240(91)90048-s.

Kreher, C.R., Dittrich, M.T., Guerkov, R., Boehm, B.O., Tary-Lehmann, M., 2003. CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. J. Immunol. Methods 278, 79–93. https://doi.org/10.1016/s0022-1759(03)00226-6.

Kutscher, S., Dembek, C.J., Deckert, S., Russo, C., Körber, N., Bogner, J.R., Geisler, F., Umgelter, A., Neuenhahn, M., Albrecht, J., Cosma, A., Protzer, U., Bauer, T., 2013. Overnight resting of PBMC changes functional signatures of antigen specific T- cell responses: impact for immune monitoring within clinical trials. PLoS One 8 (10), e76215. https://doi.org/10.1371/journal.pone.0076215.

Kvarnström, M., Jenmalm, M.C., Ekerfelt, C., 2004. Effect of cryopreservation on expression of Th1 and Th2 cytokines in blood mononuclear cells from patients with different cytokine profiles, analysed with three common assays: an overall decrease of IL-4. Cryobiology 49, 157–168. https://doi.org/10.1016/j.cryobiol.2004.06.003.

La Cava, A., 2017. Leptin in inflammation and autoimmunity. Cytokine 98, 51–58. https://doi.org/10.1016/j.cyto.2016.10.011.

Leibo, S.P., McGrath, J.J., Cravalho, E.G., 1978. Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. Cryobiology 15, 257–271. https://doi.org/10.1016/0011-2240(78)90036-6.

Li, X., Zhong, Z., Liang, S., Wang, X., Zhong, F., 2009. Effect of cryopreservation on IL-4, IFNgamma and IL-6 production of porcine peripheral blood lymphocytes. Cryobiology 59, 322–326. https://doi.org/10.1016/j.cryobiol.2009.09.004.

Li, Y., Mateu, E., Díaz, I., 2021. Impact of cryopreservation on viability, phenotype, and functionality of porcine PBMC. Front. Immunol. 12, 5011. https://doi.org/10.3389/fimmu.2021.765667.

Liang, X., Hu, X., Hu, Y., Zeng, W., Zeng, G., Ren, Y., Liu, Y., Chen, K., Peng, H., Ding, H., Liu, M., 2019. Recovery and functionality of cryopreserved peripheral blood mononuclear cells using five different xeno-free cryoprotective solutions. Cryobiology 86, 25–32. https://doi.org/10.1016/j.cryobiol.2019.01.004.

Lin, Y., Gallardo, H.F., Ku, G.Y., Li, H., Manukian, G., Rasalan, T.S., Xu, Y., Terzulli, S.L., Old, L.J., Allison, J.P., Houghton, A.N., Wolchok, J.D., Yuan, J., 2009. Optimization and validation of a robust human T-cell culture method for monitoring phenotypic and polyfunctional antigen-specific CD4 and CD8 T-cell responses. Cytotherapy 11, 912–922. https://doi.org/10.3109/14653240903136987.

Lord, G.M., Matarese, G., Howard, J.K., Baker, R.J., Bloom, S.R., Lechler, R.I., 1998. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 394, 897–901. https://doi.org/10.1038/29795.

Maecker, H.T., Moon, J., Bhatia, S., Ghanekar, S.A., Maino, V.C., Payne, J.K., Kuus-Reichel, K., Chang, J.C., Summers, A., Clay, T.M., Morse, M.A., Lyerly, H.K., DeLaRosa, C., Ankerst, D.P., Disis, M.L., 2005. Impact of cryopreservation on tetramer, cytokine flow cytometry, and ELISPOT. BMC Immunol. 6, 17. https://doi.org/10.1186/1471-2172-6-17.

Mallone, R., Martinuzzi, E., Blancou, P., Novelli, G., Afonso, G., Dolz, M., Bruno, G., Chaillous, L., Chatenoud, L., Bach, J.M., van Endert, P., 2007. CD8+ T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. Diabetes 56, 613–621.

Mallone, R., Mannering, S.I., Brooks-Worrell, B.M., Durinovic-Belló, I., Cilio, C.M., Wong, F.S., Schloot, N.N., 2011. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clin. Exp. Immunol. 163, 33–49. https://doi.org/10.1111/j.1365-2249.2010.04272.x.

Martínez-Miró, S., Tecles, F., Ramón, M., Escribano, D., Hernández, F., Madrid, J., Orengo, J., Martínez-Subiela, S., Manteca, X., Cerón, J.J., 2016. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet. Res. 12, 171. https://doi.org/10.1186/s12917-016-0791-8.

Mazur, P., 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol. 247, 125–142. https://doi.org/10.1152/ajpcell.1984.247.3.C125.

McKenna, K.C., Beatty, K.M., Vicetti Miguel, R., Bilonick, R.A., 2009. Delayed processing of blood increases the frequency of activated CD11b+ CD15+ granulocytes which inhibit T cell function. J. Immunol. Methods 341, 68–75. https://doi.org/10.1016/j.jim.2008.10.019.

Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F. A., 2003. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309, 18–31. https://doi.org/10.1016/s0042-6822(03)00009-6.

Morrow-Tesch, J.L., McGlone, J.J., Salak-Johnson, J.L., 1994. Heat and social stress effects on pig immune measures. J. Anim. Sci. 72, 2599–2609. https://doi.org/10.2527/1994.72102599x.

Nicholson, J.K., Green, T.A., Laboratories, C., 1993. Selection of anticoagulants for lymphocyte immunophenotyping: effect of specimen age on results. J. Immunol. Methods 165, 31–35.

Olson, W.C., Smolkin, M.E., Farris, E.M., Fink, R.J., Czarkowski, A.R., Fink, J.H., Chianese-Bullock, K.A., Slingluff Jr., C.L., 2011. Shipping blood to a central laboratory in multicenter clinical trials: effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability, and immunologic function. J. Transl. Med. 9, 26. https://doi.org/10.1186/1479-5876-9-26.

Owen, R.E., Sinclair, E., Emu, B., Heitman, J.W., Hirschkorn, D.F., Epling, C.L., Tan, Q.X., Custer, B., Harris, J.M., Jacobson, M.A., McCune, J.M., Martin, J.N., Hecht, F.M., Deeks, S.G., Norris, P.J., 2007. Loss of T cell responses following long-term cryopreservation. J. Immunol. Methods 326, 93–115. https://doi.org/10.1016/j.jim.2007.07.012.

Padgett, D.A., Glaser, R., 2003. How stress influences the immune response. Trends Immunol. 24, 444–448. https://doi.org/10.1016/s1471-4906(03)00173-x.

Pluske, J.R., Turpin, D.L., Kim, J.C., 2018. Gastrointestinal tract (gut) health in the young pig. Anim. Nutr. 4, 187–196. https://doi.org/10.1016/j.aninu.2017.12.004.

Rapatz, G., Sullivan, J.J., Luyet, B., 1968. Preservation of erythrocytes in blood containing various cryoprotective agents, frozen at various rates and brought to a given final temperature. Cryobiology 5, 18–25. https://doi.org/10.1016/s0011-2240(68)80139-7.

Rauw, W.M., Kanis, E., Noordhuizen-Stassen, E.N., Grommers, F.J., 1998. Undesirable side effects of selection for high production efficiency in farm animals: a review. Livest. Prod. Sci. 56, 15–33. https://doi.org/10.1016/S0301-6226(98)00147-X.

Reimann, K.A., Chernoff, M., Wilkening, C.L., Nickerson, C.E., Landay, A.L., 2000. Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type 1-infected donors: implications for multicenter clinical trials. ACTG Immunol. Adv. Technol. Lab. Clin. Diagn. Lab. Immunol. 7, 352–359. https://doi.org/10.1128/CDLI.7.3.352-359.2000.

Roque, K., Shin, K.M., Jo, J.H., Lim, G.D., Song, E.S., Shin, S.J., Gautam, R., Lee, J.H., Kim, Y.G., Cho, A.R., Kim, C.Y., Kim, H.J., Lee, M.S., Oh, H.G., Lee, B.C., Kim, J.H., Kim, K.H., Jeong, H.K., Kim, H.A., Heo, Y., 2018. Association between endotoxin levels in dust from indoor swine housing environments and the immune responses of pigs. J. Vet. Sci. 19, 331–338. https://doi.org/10.4142/jvs.2018.19.3.331.

Ruis, M.A., Te Brake, J.H., Engel, B., Ekkel, E.D., Buist, W.G., Blokhuis, H.J., Koolhaas, J. M., 1997. The circadian rhythm of salivary cortisol in growing pigs: effects of age, gender, and stress. Physiol. Behav. 62, 623–630. https://doi.org/10.1016/s0031-9384(97)00177-7.

Ruitenberg, J.J., Mulder, C.B., Maino, V.C., Landay, A.L., Ghanekar, S.A., 2006. VACUTAINER CPT and Ficoll density gradient separation perform equivalently in maintaining the quality and function of PBMC from HIV seropositive blood samples. BMC Immunol. 7, 11. https://doi.org/10.1186/1471-2172-7-11.

Salak-Johnson, J.L., McGlone, J.J., 2007. Making sense of apparently conflicting data: stress and immunity in swine and cattle. J. Anim. Sci. 85 (2007), E81–E88 doi: 10.2527/jas.2006-538.

Santos, R., Buying, A., Sabri, N., Yu, J., Gringeri, A., Bender, J., Janetzki, S., Pinilla, C., Judkowski, V.A., 2014. Improvement of IFNg ELISPOT Performance Following Overnight Resting of Frozen PBMC Samples Confirmed Through Rigorous Statistical Analysis. In: Cells, 4, pp. 1–18 doi: 10.3390/cells4010001. Erratum in: Cells. 2015;4 (2):133-4.

Sävendahl, L., Underwood, L.E., 1997. Decreased interleukin-2 production from cultured peripheral blood mononuclear cells in human acute starvation. J. Clin. Endocrinol. Metab. 82, 1177–1180. https://doi.org/10.1210/jcem.82.4.3865.

Scheiermann, C., Kunisaki, Y., Frenette, P.S., 2013. Circadian control of the immune system. Nat. Rev. Immunol. 13, 190–198. https://doi.org/10.1038/nri3386.

Selmaoui, B., Touitou, Y., 2003. Reproducibility of the circadian rhythms of serum cortisol and melatonin in healthy subjects: a study of three different 24-h cycles over six weeks. Life Sci. 73, 3339–3349. https://doi.org/10.1016/j.lfs.2003.05.007.

Smith, S.G., Joosten, S.A., Verscheure, V., Pathan, A.A., McShane, H., Ottenhoff, T.H., Dockrell, H.M., Mascart, F., 2009. Identification of major factors influencing ELISpot-based monitoring of cellular responses to antigens from Mycobacterium tuberculosis. PLoS One 4, e7972. https://doi.org/10.1371/journal.pone.0007972.

Smith, M.A., Hibino, M., Falcione, B.A., Eichinger, K.M., Patel, R., Empey, K.M., 2014. Immunosuppressive aspects of analgesics and sedatives used in mechanically ventilated patients: an underappreciated risk factor for the development of ventilator-associated pneumonia in critically ill patients. Ann. Pharmacother. 48, 77–85. https://doi.org/10.1177/1060028013510698.

Suchner, U., Kuhn, K.S., Fürst, P., 2000. The scientific basis of immunonutrition. Proc. Nutr. Soc. 59, 553–563. https://doi.org/10.1017/s0029665100000793.

Suzuki, K., Hayano, Y., Nakai, A., Furuta, F., Noda, M., 2016. Adrenergic control of the adaptive immune response by diurnal lymphocyte recirculation through lymph nodes. J. Exp. Med. 213, 2567–2574. https://doi.org/10.1084/jem.20160723.

Talker, S.C., Käser, T., Reutner, K., Sedlak, C., Mair, K.H., Koinig, H., Graage, R., Viehmann, M., Klingler, E., Ladinig, A., Ritzmann, M., Saalmüller, A., Gerner, W., 2013. Phenotypic maturation of porcine NK- and T-cell subsets. Dev. Comp. Immunol. 40, 51–68. https://doi.org/10.1016/j.dci.2013.01.003.

Tallet, C., Brajon, S., Devillers, N., Lensink, J., 2017. Advances in Pig Welfare. Woodhead Publishing, pp. 381–398. https://doi.org/10.1016/B978-0-08-101012-9.00008-3.

Tompa, A., Nilsson-Bowers, A., Faresjö, M., 2018. Subsets of CD4+, CD8+, and CD25hi lymphocytes are in general not influenced by isolation and long-term cryopreservation. J. Immunol. 201, 1799–1809. https://doi.org/10.4049/jimmunol.1701409.

Vanham, G.L., Penne, G., Vereecken, C., Vingerhoets, J., Kestens, L., 1998. Interleukin-2 receptor subunit expression and function on human peripheral T cells is not dependent on the anticoagulant. Blood 92, 2989–2993.

Wallgren, P., Wilén, I.L., Fossum, C., 1994. Influence of experimentally induced endogenous production of cortisol on the immune capacity in swine. Vet. Immunol. Immunopathol. 42, 301–316. https://doi.org/10.1016/0165-2427(94)90075-2.

Weinberg, A., Betensky, R.A., Zhang, L., Ray, G., 1998. Effect of shipment, storage, anticoagulant, and cell separation on lymphocyte proliferation assays for human immunodeficiency virus-infected patients. Clin. Diagn. Lab. Immunol. 5, 804–807. https://doi.org/10.1128/CDLI.5.6.804-807.1998.

Weinberg, A., Zhang, L., Brown, D., Erice, A., Polsky, B., Hirsch, M.S., Owens, S., Lamb, K., 2000. Viability and functional activity of cryopreserved mononuclear cells. Clin. Diagn. Lab. Immunol. 7, 714–716. https://doi.org/10.1128/CDLI.7.4.714-716.2000.

Whitworth, K.M., Rowland, R.R., Ewen, C.L., Trible, B.R., Kerrigan, M.A., Cino-Ozuna, A. G., Samuel, M.S., Lightner, J.E., McLaren, D.G., Mileham, A.J., Wells, K.D., Prather, R.S., 2016. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat. Biotechnol. 34, 20–22. https://doi.org/10.1038/nbt.3434.

Wilkie, B., Mallard, B., 1999. Selection for high immune response: an alternative approach to animal health maintenance. Vet. Immunol. Immunopathol. 72, 231–235. https://doi.org/10.1016/s0165-2427(99)00136-1.

Xie, Z., Pang, D., Yuan, H., Jiao, H., Lu, C., Wang, K., Yang, Q., Li, M., Chen, X., Yu, T., Chen, X., Dai, Z., Peng, Y., Tang, X., Li, Z., Wang, T., Guo, H., Li, L., Tu, C., Lai, L., Ouyang, H., 2018. Genetically modified pigs are protected from classical swine fever virus. PLoS Pathog. 14, e1007193 https://doi.org/10.1371/journal.ppat.1007193.

Yang, H., Galang, K.G., Gallegos, A., Ma, B.W., Isseroff, R.R., 2021. Sling training with positive reinforcement to facilitate porcine wound studies. JID Innov. 1, 100016 https://doi.org/10.1016/j.xjidi.2021.100016.

Yuki, K., Soriano, S.G., Shimaoka, M., 2011. Sedative drug modulates T-cell and lymphocyte function-associated antigen-1 function. Anesth. Analg. 112, 830–838. https://doi.org/10.1213/ANE.0b013e31820dcabb.

Zuckermann, F.A., Husmann, R.J., 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87, 500–512.