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8. Dietary spray-dried animal plasma alleviates mucosal inflammation in experimental models

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Abstract. The intestinal and bronchoalveolar mucosae contribute to homeostasis by preventing the entrance of biological and chemical agents that could alter the stability of the system. In this review, we summarise the main effects of dietary supplementation with spray-dried plasma (SDP), a complex mixture of biologically active functional components, on two models of acute inflammation; a murine model of intestinal inflammation, based on the administration of *S. aureus* enterotoxin B (SEB), and a model of acute lung inflammation, using mice challenged with lipopolysaccharide from *E. coli* (LPS). Oral SDP modulates the immune response of the intestinal mucosa and restores the barrier function of the epithelium, preventing most of the effects of SEB on defensin expression, tight-junction permeability and mucosal cytokine production. In the lung, SDP supplementation partially prevents the LPS-induced release of pro-inflammatory cytokines, an effect that involves the participation of the common mucosal immune system. In both models, the effects of SDP are mediated by an increased T-reg response and enhanced release of anti-inflammatory cytokines that contribute to mucosal homeostasis.

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Introduction

Spray-dried plasma (SDP) is a protein-rich product obtained from the industrial fractionation of blood from porcine and bovine animals slaughtered for human consumption. Blood is collected with an anticoagulant, centrifuged to separate blood cells and spray-dried using high pressure and a temperature over 80ºC for a very short period of time. With this procedure, proteins are not denaturalised and their biological activity is mostly preserved [1].

At the end of the last century, SDP was initially proposed as a protein source for piglets [2]. Since then, many studies have demonstrated that SDP improves piglet and calf performance, and today it is widely used as an alternative to the use of antibiotics as growth promoters [3]. Numerous studies have shown that feeding SDP of either bovine or porcine origin reduces mortality and morbidity in various animal species challenged with pathogenic bacteria (*E. coli*, *Salmonella*), viruses (rotavirus, coronavirus, white spot syndrome virus) or protozoa (*C. parvum*) [4;5;6].

In addition, a greater efficacy of SDP has been described in younger pigs which have a less mature immune system [7], or in pigs kept under less hygienic conditions [8].

1. Effects of SDP on acute intestinal inflammation

The gastrointestinal tract provides a protective interface between the internal milieu and the permanent challenge resulting from microorganisms and antigens derived from food present in the lumen. The intestinal mucosa regulates the penetration of luminal antigens and the generation of immunologic responses in the gut, and dysregulation of these barrier mechanisms causes intestinal inflammation [9].

Since the host's immune responses can be modulated by diet [10], the dietary approach offers a therapeutic potential in conditions associated with gut barrier dysfunction and inflammatory response.

1.1. Intestinal barrier

A key function of the intestinal epithelium is to serve as a selective barrier allowing the uptake of nutrients while excluding toxins and microorganisms. Mucosal permeability mainly depends on the capacity of tight-junctions to efficiently seal the apical poles of epithelial cells. The

space between cells is occupied by interlocking proteins such as claudins, occludin or E-cadherin that bind scaffolding proteins such as ZO-1 and β-catenin which, in turn, link them to the cellular cytoskeleton [11]. An acute change of the intestinal barrier function contributes to disease pathogenesis, especially when the intestine is challenged by luminal antigens. Several bacterial products, such as Clostridium and Vibrio toxins, change the localisation of several tight-junction proteins [12] or reduce the number of strands in the tight-junction [13].

Staphylococcal enterotoxin B (SEB) reduced the expression of β-catenin in a rodent model of intestinal inflammation [14] and SDP supplementation prevented this effect (**Figure 1A**). Moreover, SEB treatment significantly increased the flux of 4 kD FITC-dextran (FD4, a fluorescent tracer of paracellular permeability) across the intestinal wall and SDP supplementation prevented this effect (**Figure 1B**). The effects of SEB on intestinal permeability were similar to those described in SEB-injected mice (15). β-catenin expression was negatively correlated with FD4 flux, suggesting that the increases in dextran flux are paralleled by a reduction in β-catenin expression (**Figure 1C**). These results indicate that the increase in FD4 flux induced by SEB treatment is associated with a reduction in the tightness of the epithelial junction complex and that SDP dietary supplementation resulted in complete recovery. The effects of SDP supplementation in reducing a toxin-induced increase in mucosal permeability may prevent the passage of microbial and food antigens to the interstitial space, thereby avoiding local inflammation [16].

Enterotoxins can also have indirect effects by inducing the release of pro-inflammatory cytokines such as IFN-γ and TNF-α. Both cytokines increase epithelial permeability by reducing the expression of β-catenin [17]. SEB also stimulates the secretion of IFN-γ and TNF-α from lymphocytes [18;19], which can disassemble tight-junction protein complexes [20] or reduce their expression [21;22], thus enhancing paracellular permeability of microvascular endothelial cells.

Mucosal homeostasis is also protected by mucosal defensins secreted by both Paneth cells and enterocytes. These are antimicrobial peptides that regulate the composition and number of luminal colonising microbes present in the small intestine, and they play an important role in reducing pathogen concentration in the intestinal lumen. Studies in humans indicate that reduced Paneth cell defensin expression may be a key pathogenic factor in ileal Crohn's disease, because it changes the profile of the colonising microbiota [23].

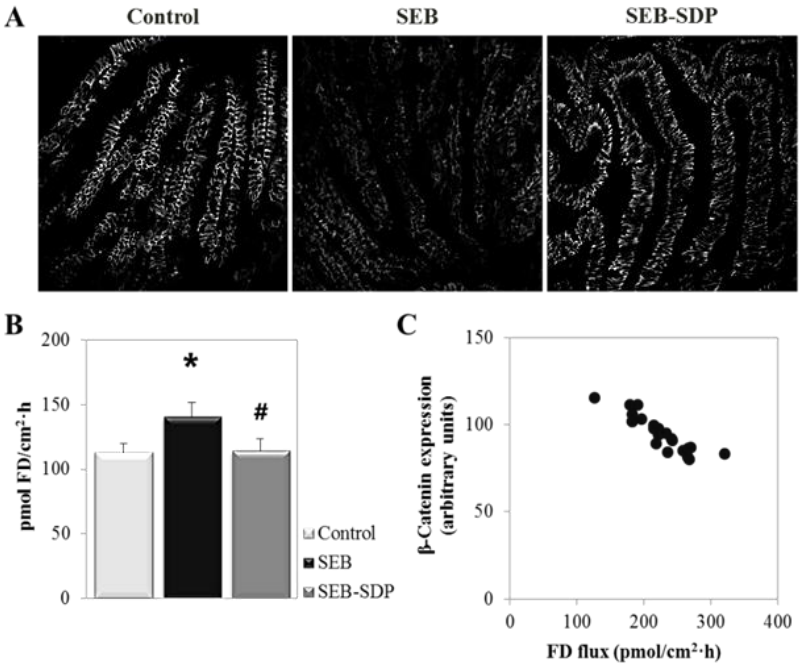

Figure 1. SDP effects on intestinal barrier function in acute inflammation (with permission from Pérez-Bosque et al., 2006)**.** Panel A shows representative images of β-catenin immunohistochemical localisation in jejunum from Control, SEB and SEB-SDP rats. Panel B shows the FITC-dextran (FD) flux across the intestinal wall of rat jejunum measured in an Ussing chamber. Results are expressed as means ± SEM (7-10 animals). Symbols indicate significant differences $P<0.05$; *SEB group vs Control group, #SEB-SDP group vs SEB group. Panel C shows the correlation between β-catenin expression and FD flux ($P<0.001$).

In rats, SEB reduces the expression of cryptdin 4 and β-defensin-1 (**Figure 2**). Since cryptdin 4 has the ability to block IL-1β release from LPS-activated monocytes [24], decreased expression of this defensin would result in increased intestinal IL-1β production, rendering the intestine more susceptible to SEB-induced damage and contributing to pathogenesis of inflammatory bowel diseases. β-defensin-1 is constitutively expressed by enterocytes, and when its expression is reduced, there is increased proliferation of several major components of the intestinal microbiota, including *Candida albicans*, *Bacteroides fragilis*, *Enterococcus faecalis* and *Escherichia coli* [25]. SDP restored the physiological production of mucosal

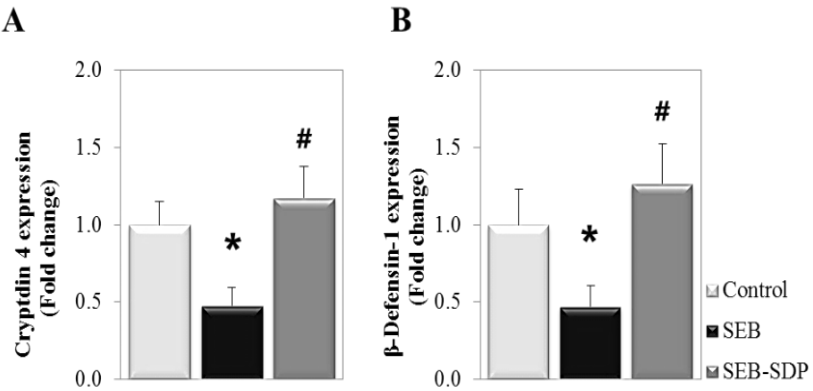

Figure 2. Effects of SDP supplementation on cryptdin 4 (A) and β-defensin 1 (B) expression in acute inflammation. Expression determined by real time PCR. Data are the mean ± SEM of 7-8 rats. Symbols indicate significant differences P<0.05; *SEB group vs Control group, #SEB-SDP group vs SEB group.

defensins, indicating that plasma protein supplementation may contribute to maintenance of intestinal immune homeostasis by maintaining the production of natural innate antibacterial agents, as well as by regulating the production of pro-inflammatory cytokines. The possible relationship between the current effects of SDP on defensin expression, and previous observations showing that dietary plasma proteins induce changes in the microbiota profile associated with a higher resistance to dysbiosis [26], should be further explored.

1.2. Intestinal immune response

Gut-associated lymphoid tissue (GALT) accounts for up to 80% of the mucosal immune system and is distributed along the intestine. It contains a broad network of secondary lymphoid organs, as well as a large number of lymphocytes, including several intestine-specific subpopulations [27]. Upon activation, the intestinal immune system coordinates a strong inflammatory response against invasive pathogenic bacteria (thus promoting protection) while providing inhibitory mechanisms to prevent an excessive response against commensal bacteria (thus promoting tolerance). However, if the immune system is stimulated and the response is not controlled, tissue may be damaged [18].

SEB administration induces a recruitment of neutrophils [28] and eosinophils [29]. The dietary inclusion of SDP does not modify the SEB-induced effects on neutrophil infiltration, but does reduce eosinophil infiltration and the degree of cell degranulation. The SEB challenge also increases the activation of intestinal T-helper lymphocytes present in Peyer's patches (PP), in mucosal *lamina propria* and in the intraepithelial compartment [28;30]. Furthermore, dietary supplementation with SDP prevents the SEB-induced activation of T-helper lymphocytes in all the above mentioned intestinal compartments (**Figure 3A**). SDP reduces the expression of mucosal pro-inflammatory cytokines [19], which is paralleled by a reduction in intestinal activated T cells (**Figure 3B**), consistent with the fact that activated T-helper lymphocytes release pro-inflammatory cytokines to amplify the immune response [31]. Bosi et al. [32] observed that pigs challenged with *E. coli* K88 and fed SDP had a lower intestinal expression of pro-inflammatory cytokines. The effect of SDP on the mucosal cytokine profile reduces mucosal inflammation and prevents changes in mucosal permeability and tight-junctional protein expression following SEB administration [14].

The inducible regulatory T cell (T-reg) population is another component of the mucosal immune system that maintains immunological unresponsiveness to self-antigens and suppresses excessive immune responses that can be deleterious to the host [33]. T-reg cells mediate peripheral T cell tolerance to antigens derived from dietary origin or from the commensal flora. In addition, after antigenic stimulation, T-reg lymphocytes can specifically inhibit the immune response of activated T-helper cells [34], through the expression of characteristic cytokines such as transforming growth factor-β and IL-10, distinct from either Th1 or Th2 cells.

SDP supplementation increased IL-10 production in SEB-challenged rats at both intestinal (Peyer's patches and intestinal mucosa) and systemic levels [19]. Dietary supplementation with plasma proteins increases the mucosal expression of IL-10, which suggests the involvement of this anti-inflammatory cytokine in regulating the production of pro-inflammatory cytokines (**Figure 3C**). This SDP effect on IL-10 contributes to intestinal homeostasis, since this cytokine is involved in the control of the intestinal pathology caused by T cell and innate immune cell activation. In view of the role of IL-10 in the amelioration of intestinal inflammation [35], it is worth noting that SDP can also increase mucosal IL-10 in absence of any challenge.

The observation that the effects of SEB on the release of systemic pro-inflammatory cytokines are small is in agreement with previous results in the spleen [28] and indicates that SEB has little effect on the peripheral immune system. However, the increase in IL-10 concentration in serum is highly correlated to a reduction in the TNF-α concentration (**Figure 3D**).

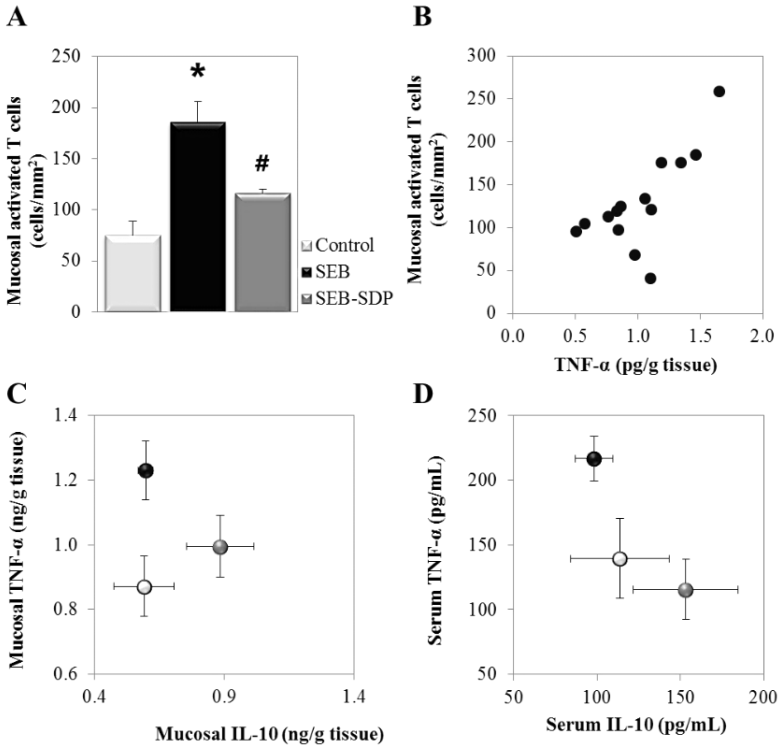

Figure 3. SDP effects on mucosal immune response in acute intestinal inflammation (with permission from Pérez-Bosque et al., 2008)**.** *Panel A* shows activated T lymphocytes in the intestinal lamina propria. Activated T cells were immunolocalised with specific antibodies on jejunal slides from Control, SEB and SEB-SDP rats. Results are expressed as means ± SEM (5-6 animals). Symbols indicate significant differences P<0.05; *SEB group vs Control group, #SEB-SDP group vs SEB group. *Panel B* shows the correlation between the number of activated T cells in the intestinal lamina propria and TNF-α mucosal concentration. The correlation coefficient was R^2=0.6971 (P<0.001). The correlation of TNF-α and IL-10 concentration in the intestinal mucosa and in serum is shown in panels C and D, respectively.

2. Effects of SDP on acute lung inflammation

The observation that SDP not only modulates GALT homeostasis [29] but also affects lymphoid tissue populations in peripheral tissues such as the spleen [36;28] and lung [37] has led to the hypothesis that plasma supplements may also modulate the immune response in non-intestinal mucosal tissues. This hypothesis is supported by the existence of the common mucosal immune system that connects the lymphoid tissue of the gut to the other mucosal areas, that is, nasopharyngeal, bronchoalveolar and genitourinary mucosae [38].

2.1. Common mucosal immune system

The respiratory and gastrointestinal tracts share some structural similarities. Both have an extensive luminal surface area, which is protected from commensal bacteria, pathogens and foreign antigens by a selective epithelial barrier [39] and an overlying mucus-gel layer [40]. These epithelial surfaces cover a mucosa-associated lymphoid tissue composed of resident lymphocytes. This lymphoid tissue regulates antigen sampling, lymphocyte trafficking and mucosal host defence [38]. Together with the genitourinary tract, they represent the main sites of intersection between the environment and the host.

An additional feature of mucosal barrier tissues is their contact with beneficial microbiota. Therefore, these tissues must protect the host from pathogenic challenges while at the same time maintaining a *peaceful coexistence* with the resident microbiota [41].

There is much evidence suggesting that the mucosal immune system is a system-wide organ. Studies have demonstrated that stimulation in one compartment of the mucosal immune system can lead to changes in distal areas. For example, intranasal immunisation results in vaginal protection against genital infection with herpes simplex virus type 2 [42]. Furthermore, the use of antibiotics in neonates has been associated with a greater risk of developing asthma [43], which suggests that alterations in the gut microflora can have an effect on the lungs. Collectively, such studies suggest that the mucosal immune system is actually a large interconnected network with individual components efficiently sharing information [44].

2.2. Pro-inflammatory immune response

In a mouse model of acute lung inflammation induced by inhalation of LPS, the pulmonary response is characterised by leukocyte migration

accompanied by a massive release of pro-inflammatory cytokines and chemokines (**Figure 4A**), which recruit monocytes and neutrophils into the lung airway and into the lung tissue [45]. Dietary inclusion of SDP reduces the innate immune response to LPS inhalation. The reduction in leukocyte numbers in bronchoalveolar lavage fluid (BALF) and lung tissue, the lower concentration of pro-inflammatory cytokines and chemokines in BALF and the lower iNOS expression in lung tissue all suggest a dietary-dependent reduction in the chemical mediators responsible for acute lung injury.

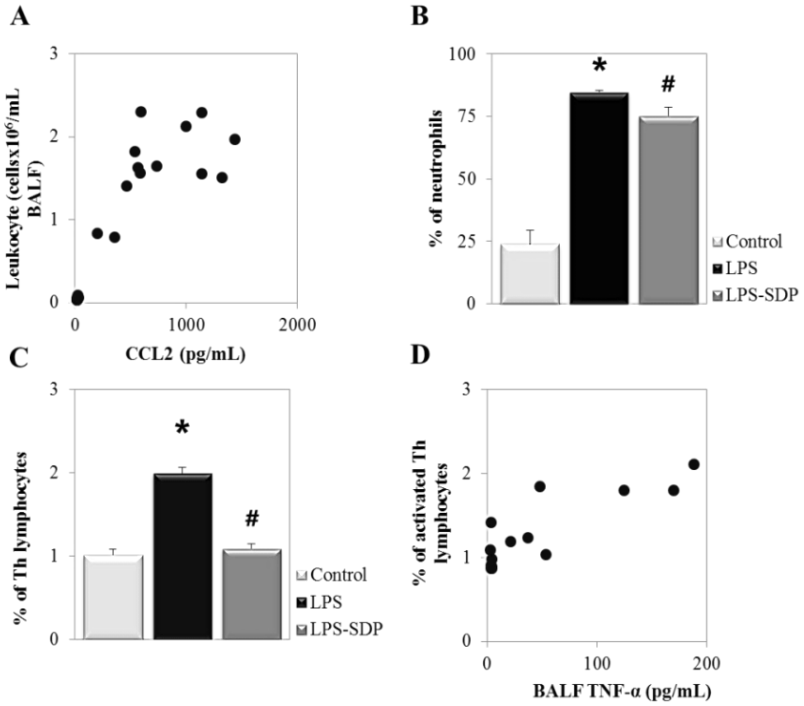

Figure 4. SDP effects on mucosal pro-inflammatory immune response in acute lung inflammation (with permission from Maijò et al., 2012a,b)**.** *Panel A* shows the correlation between leukocyte recruitment into lung airway and chemokine CCL2 concentration in bronchoalveolar lavage fluid (BALF). *Panel B and panel C* show the percentage of activated neutrophils (B) and activated Th lymphocytes (C) in the lung airway. Results are expressed as means ± SEM (5-6 animals). Symbols indicate significant differences P<0.05; *LPS group vs Control group, #LPS-SDP group vs SEB group. *Panel D* shows the correlation between the number of activated Th lymphocytes in BALF and IL-2 BALF concentration.

The LPS challenge increased the percentage of neutrophils by 70% [45]. The primary function of neutrophils is to contain and kill invading microbial pathogens [46]. In BALF and lung tissue, LPS increased the proportion of activated neutrophils (**Figure 4B**) and of activated monocytes [45], as a consequence of the release of large amounts of chemokines. SDP supplementation reduces the percentage of activated neutrophils and monocytes in the lung airway. The effects of SDP on the response of the innate immune system present in the lung are relevant because this system plays an important role in mediating defence against pathogens, detecting tissue damage and regulating tissue health and integrity [47]. Therefore, the lower cell migration and diminished activation of inflammatory cells in pulmonary tissue may reduce potential damage in respiratory epithelium and vascular endothelium associated with the inflammatory response.

LPS challenge also promotes the activation of Th lymphocytes at both local (lung tissue; **Figure 4C**) and systemic (blood) levels. These effects are accompanied by enhanced release of IL-2 in the lung (**Figure 4D**); this cytokine (almost exclusively produced by activated Th cells) promotes proliferation of lymphocytes, macrophages and NK cells [48]. SDP supplementation also reduces the percentage of activated Th lymphocytes and prevents the release of IL-2. This effect is consistent with the anti-inflammatory response previously described for plasma supplements [19].

2.3. Regulatory response during acute lung inflammation

The LPS challenge does not modify the percentage of T-reg cells (**Figure 5A**); however, dietary SDP inclusion increases the percentage of these cells and also reduces the T-activated:T-reg cell ratio (**Figure 5B**). T-reg cells reduce inflammation by counteracting the effects of other Th cells and contribute to suppression of innate and adaptive immune responses [49;50].

SDP promotes IL-10 production in the lung of inflamed mice, and the increases in the concentration of this cytokine are paralleled by increases in the percentage of T-reg cells (**Figure 5C**). Other studies carried out using a LPS model of acute lung inflammation in rats have demonstrated that treatment with IL-10 after endotoxin instillation protects against acute lung injury, possibly by suppressing pulmonary infiltration of activated neutrophils [51]. In the LPS-induced lung inflammation model, as in the

SEB model of intestinal inflammation, it has been shown that the dietary modulation of intestinal inflammation is mediated by an increase in mucosal IL-10 expression, which reduces the release of pro-inflammatory cytokines [19;52] (**Figure 5D**).

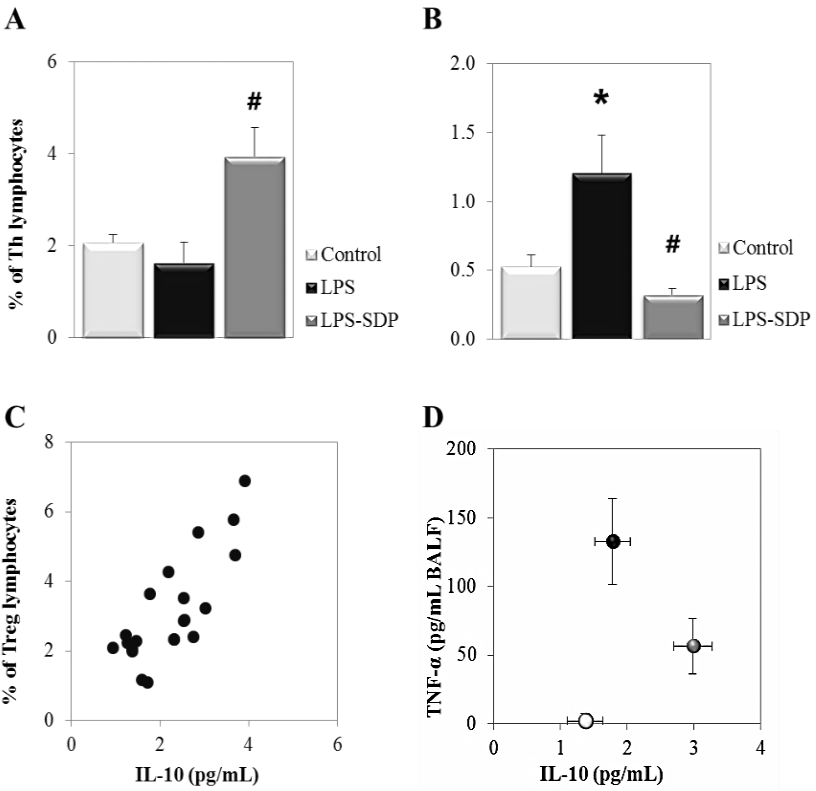

Figure 5. SDP effects on mucosal anti-inflammatory immune response in acute lung inflammation (with permission from Maijò et al., 2012b)**.** *Panel A and panel B* show the percentage of the regulatory T lymphocytes (T-reg) (A) and the ratio between activated Th lymphocytes and T-reg cells (B) in the lung airway. Results are expressed as means ± SEM (5-6 animals). Symbols indicate significant differences P<0.05; *LPS group vs Control group, #LPS-SDP group vs SEB group. *Panel C* shows the correlation between T-reg into lung airway and the concentration of the anti-inflammatory cytokine IL-10 in bronchoalveolar lavage fluid (BALF). *Panel D* shows TNF-α and IL-10 concentrations in BALF.

3. Mode of action of SDP

SDP is a highly complex mixture of functional peptides and proteins such as immunoglobulins and growth factors, with a high proportion of albumin [53]. As summarised by Petschow et al. [54], plasma supplements contribute to homeostasis by neutralising endotoxin in the intestinal lumen, promoting a stable microbiota, maintaining the gut barrier function and preserving the immune balance both in intestinal and peripheral tissues.

The mechanism by which oral plasma supplements modulate peripheral inflammation is not completely understood. However, there is increasing evidence that signals initiated in the intestinal lumen of different origin (dietary functional components, changes in the microflora, the presence of microbial cell wall components and even bacterial secreted products) can interact with the intestinal mucosa and have the capacity to regulate immune responses outside the gastrointestinal tract [55]. Plasma supplements ameliorate the inflammatory response by increasing the number of T-regs in the inflamed colon as well as by enhancing IL-10 release [56]. Therefore, there is evidence indicating that plasma supplements modulate the abundance of T-regs in the intestine (the inductor site) and stimulated blood and lung T-regs in the lung model (the effector site), both interconnected by the common mucosal system [55].

The mechanism of action of SDP is probably not unique. SDP contains a high proportion of immunoglobulins that can bind a variety of potential antigens in the lumen, preventing their attachment to the mucosa [32]. This is the mechanism claimed to explain the beneficial effects of SDP in the prevention of viral gastroenteritis in children [57], and in the reduction of diarrhoea in pigs [58] and in acquired immune deficiency syndrome patients infected with *C. parvum* [59]. However, it must be considered that over 250 peptides have been identified in plasma [53] and most of them will retain some biological function after spray-drying [1]. SDP may contain a fraction of natural antibodies that will contribute to immune homeostasis, enhancing anti-inflammatory IL-10 production, as suggested by Petschow et al. [60]. The presence of bioactive peptides in SDP may also cause changes in the intestinal microbiota profile. For example, SDP can inhibit the growth of pathogenic bacteria [61;58].

Animal plasma supplementation can also change the microbiota profile. Ovine Ig may alter the intestinal environment through a specific enrichment of Lactobacillus strains and depletion of enterobacteria [62], although

studies in piglets have yielded conflicting results [6;63]. In rats, the analysis of caecal microbiota showed that animals fed porcine SDP presented increased richness of the intestinal ecosystem [26]. Finally, it is worth noting that animal plasma supplements contain growth factors, cytokines and biologically active compounds that may also directly interact with mucosal receptors present both in enterocytes and ib dendritic cells, or that can reach the subepithelial compartment across the Peyer's patch M cells, as happens with food-derived peptides [64]. This is a largely unexplored area that deserves further attention.

4. Conclusions

Supplements prepared from animal plasma of porcine, bovine or ovine origin have been shown to contribute to gut homeostasis and act at luminal and mucosal levels. The main targets are the regulation of the intestinal barrier and the gut-associated immune system, which connects and modulates other mucosal areas, promoting the proliferation of regulatory lymphocytes and the expression of anti-inflammatory cytokines which presumably mediate most of its beneficial physiological effects. The mechanism by which plasma supplements initiate the regulatory responses is probably not unique but involves luminal mechanisms, with changes in the microbiota profile, direct or indirect interaction with enterocytes or with immune intestinal cells, and steps connecting the gut-associated phenomena with peripheral mucosal areas also exposed to the external environment. A better insight into the mechanisms implicated and a deeper knowledge of the specific plasma components involved are necessary to gain acceptance of these products.

Acknowledgements

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