2021 年推免资格申请表

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所学外语语种	英语	通过最高级别	CET-6	成绩	463		
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2020 年 9 月 18 日

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免试攻读研究生专家推荐书

被推荐人姓名	倪思璐	性别	女	专业班级	动医1605班
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作为该生科研锻炼期间的导师，我已与该生接触，交流3年多。在实验室期间通过3年多的观察和沟通，对该生有了比较全面的了解。2016级本科生倪思璐，英语优秀，能够查找翻译以相关文献，具有较强的逻辑思维和创新思维的能力，能够提出自己独特的见解，掌握新技术的能力迅速。善于学习，勤奋刻苦，严格把握实验进度。经常利用周末假期在实验室工作，有时候通宵达旦，在科研方面表现优秀，并进一步深化了专业方面的理论学习，具备了较完善的知识结构和理论水平。

除此之外，我还了解到该生作为预备党员，自觉遵守实验室的规章制度，为人诚恳，尊敬师长，团结实验室师兄师姐积极进取，责任感强，具有良好的思想道德素质。热爱锻炼，身体素质优秀，能够适应高强度的学习和工作，平时积极参加社会实践活动，并取得不错的成绩。担任学生会部长

鉴于该生具有较大的发展潜力和培养前途，特推荐该生攻读研究生。

推荐意见	同意推荐 推荐人签字：陈德坤 年月日 2020.9.27

2021年西北农林科技大学动物医学院优秀应届本科毕业生
免试攻读研究生专家推荐书

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倪思璐同学在大二就到科研实验室进行科研锻炼，并参加了一些项目的科研工作。她对待科研工作认真而且很投入，经常在实验室加班加点工作到深夜。这种勤奋的工作，使得她很快就掌握了许多科研试验技术，为她后续科研成果的取得打下了很好的基础。不仅如此，她还积极与指导老师联系，学习英文文献的查阅和整理，锻炼自己的科研英文论文写作能力。通过她自己的勤奋努力，三年来她取得了以第一作者发表了1篇SCI论文、第二作者在西北农林科技大学学报发表了1篇论文的好成绩。

该生能够自觉遵守实验室的规章制度，为人诚恳，尊敬师长，团结实验室师兄师姐积极进取，责任感强，具有良好的思想道德素质。热爱锻炼，身体素质优秀，能够适应高强度的学习和工作，平时积极参加社会实践活动，并取得不错的成绩，大三期间，担任我院的学生会部长。

鉴于该生具有较大的发展潜力和培养前途，特推荐该生攻读研究生。

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在思想品德方面，倪思璐同学遵纪守法、热心关心和帮助他人，积极参与学校的志愿者活动。他时刻关注时政，通过了解和学习党的有关动态和精神，使自己在思想上和行动上与党中央保持一致，积极向党组织靠拢。

在学习方面，该生一直努力认真地学好每一门功课，熟练掌握了本专业有关知识。一份耕耘一分收获，每每看到自己可喜的成绩，她就会感叹只要有决心，没有什么事是不可能的。

在科研方面，该生工作热情，责任心强、勤恳踏实，积极思考。她遇到了比别人多的挫折与失败，但也取得了相对较多的进步，收获很多。鉴于该生具有较大的发展潜力，我推荐该生攻读硕士研究生。

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推 免 生：倪思璐，以下简称乙方。

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2020 年 9 月 27 日

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Review

Neutrophils: A Critical Participator in Common Diseases of Ruminants

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Abstract

Neutrophils, pivotal effector cells involved in innate immunity, play a central role in various infectious and inflammatory diseases. Using a powerful phagocytic killing mechanism, these cells protect the host by destroying the invading pathogens. However, these cells can also cause varying degrees of tissue damage if their activation is not finely controlled. In recent years, the involvement of neutrophils in human diseases has been extensively studied, while their roles in ruminant diseases have rarely been investigated. In the present review, we mainly summarize current knowledge regarding the characteristics and functions of neutrophils in ruminants such as goats and cattle. We emphasize the involvement of these cells in several common diseases such as mastitis, Brucellosis, *Mycoplasma bovis* infection and parasitic infections, among others. We also focus on discussing the relevant mechanisms and signaling pathways underlying these observations. In addition, we compare the phenotypes and functions of neutrophils of different ruminant species. The studies about ruminant neutrophils should help elucidate the pathogenesis of many ruminant diseases and ultimately shed light on the development of novel therapeutics for these diseases.

Keywords: *Neutrophil, Protective immunity, Tissue damage, Ruminant disease, Immunotherapy*

Nötrofiller: Ruminantların Yaygın Hastalıklarında Kritik Katılımcı

Öz

Doğal bağışıklıkla ilişkili önemli efektör hücreler olan nötrofiller, çeşitli bulaşıcı ve inflamatuvar hastalıklarda temel rol oynarlar. Bu hücreler güçlü bir fagositik öldürme mekanizması kullanarak istilacı patojenleri yok eder ve konağı korur. Bununla birlikte, aktivasyonları iyi kontrol edilmezse değişen derecelerde doku hasarına neden olabilirler. Son yıllarda, nötrofillerin insan hastalıkları ile ilişkisi kapsamlı bir şekilde incelenirken, ruminant hastalıklarındaki rolleri nadiren araştırılmıştır. Bu derlemede esas olarak keçi ve sığır gibi ruminantlarda nötrofillerin özellikleri ve fonksiyonları ile ilgili güncel bilgileri özetlenmiştir. Bu hücrelerin mastitis, Brusselloz, *Mikoplazma bovis* enfeksiyonu ve paraziter enfeksiyonlar gibi yaygın görülen çeşitli hastalıklardaki önemi vurgulanmıştır. Ayrıca, ilgili mekanizmalar tartışılmış ve bu gözlemlerin altında yatan yollara işaret edilmiştir. Ek olarak, farklı ruminant türlerin nötrofillerinin fenotipleri ve işlevleri karşılaştırılmıştır. Ruminant nötrofilleri ile ilgili çalışmalar, birçok ruminant hastalığının patogenezinin aydınlatılmasına yardımcı olmalı ve sonuç olarak bu hastalıklar için yeni terapötiklerin geliştirilmesine ışık tutmalıdır.

Anahtar sözcükler: *Nötrofil, Koruyucu bağışıklık, Doku hasarı, Ruminant hastalığı, İmmünoterapi*

INTRODUCTION

Neutrophils are polymorphonuclear cells that act as the first line of defense against invading pathogens [1]. By rapidly combating intrusive microorganisms, they limit infections during the initiation stage of an immune response. Neutrophils are the most abundant leukocytes in the blood, where they complete their maturation after migration

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from the bone marrow to the vasculature [2]. Although the lifespan of most neutrophils is very short, with a circulating half-life of only 6-8 h, their overall number is stably maintained due to the dynamic balancing of production, retention, mobilization, margination and clearance [1]. Unlike most other immune cells, senescent neutrophils are an exceptionally long-lived population that may play an important role in maintaining neutrophil heterogeneity and homeostasis. They can be identified by their surface antigenic profile of $CXCR4^{high}$ $CD11b^{high}$ $CD62L^{low}$ and their uniquely small size and excessive nuclear lobulation [3].

Once neutrophils receive signals related to pathogen invasion and inflammation, they immediately migrate to sites where they are needed [1]. Neutrophils have long been considered the terminal effector cells of acute inflammatory response since they basically devour invading pathogens. In addition, numerous studies have shown that neutrophils also release effector molecules including various cytokines, extracellular traps and other effector mediators [2]. These effector molecules are closely tied to the activation, regulation and effector functions of both innate and adaptive immune cells [1]. Additionally, neutrophils can release granules containing highly toxic molecules, products of reactive oxygen species (ROS) and inflammatory cytokines, or may undergo NETosis to trap microbial invaders [4]. Consequently, if neutrophil-mediated immune responses are not strictly regulated, detrimental inflammations and host tissue damages can be caused as a result [5].

In recent years, ruminant farming has been threatened and impacted partly due to a lack of knowledge regarding invading pathogens and host responses. Because neutrophils are central to disease control and prevention, understanding their roles should be a priority. What has been known is that the morphology, function and quantity of neutrophils vary among different ruminant species [6]. Moreover, among healthy dairy goats or cattle, differences in the viability and morphology of blood and milk neutrophils have been reported [7].

In this review, we mainly describe the basic characteristics of neutrophils and review the specific roles of neutrophils in ruminants. We also highlight and summarize the roles of reciprocal communication pathways linking neutrophils with common ruminant diseases.

CHARACTERISTICS AND FUNCTIONS OF NEUTROPHILS

Neutrophil Chemotaxis

Neutrophils are the first immune cells to reach sites of inflammation and infection. They are recruited to such sites via chemotaxis, a cellular process depending on the extracellular chemoattractant gradient [8]. Neutrophils undergoing chemotaxis are polarized, whereby actin filament (F-actin)-based protuberances on their leading edges moving in a synchronized fashion with cytoplasmic contractions and the trailing edge myosin-based movements to move the entire cell forward. Neutrophil movement within a chemoattractant gradient relies on the action of G protein-coupled receptor (GPCR) signaling pathways employing formylated peptide receptors (FPR1/2/3), classical chemoattractant receptors (BLT1/2, PAFR and C5aR) and chemokine receptors (CXCR1/2 and CCR1/2) [1]. After a chemical attractant binds to a GPCR, a receptor conformational change occurs that results in the activation of downstream signaling pathways, including the phospholipase C (PLC) pathway. The PLC pathway activation triggers the production of diacylglycerol (DAG) and inositol triphosphate (IP3) that activate protein kinase C (PKC) and protein kinase D (PKD), which ultimately induce an increase in intracellular calcium level. PKC has multiple isoforms that interact with different participants to promote F-actin activity and regulate cofilin activity [9]. PKD, a direct effector of PLC/PKC axis proteins, can phosphorylate the cofilin phosphatase SSH2 to ultimately regulate downstream cofilin activity during GPCR-mediated neutrophil chemotaxis [10].

Cytotoxic Function

After neutrophils are recruited to sites of infection, they recognize and devour microbes. During this process, their cytotoxic function plays an important role in pathogen killing [8]. This function is made possible during neutrophil differentiation within the bone marrow whereby three types of granule proteins are formed in a stepwise fashion and assembled during maturation into a powerful pathogen-killing weapon. Meanwhile, within each neutrophil cytoplasm, numerous secretory vesicles are present that contain various types of plasma membrane receptors such as receptors for lipopolysaccharide (CD14), complement (CR1 and CR3/Mac-1), urokinase-type plasminogen activator, immune complex and chemoattractant (formyl peptide) [11]. At the infection site, neutrophil extracellular traps (NETs) also function as critical cytotoxins to promote "neutrophil apoptosis" through the process of "NETosis" to achieve extracellular entrapment of pathogens [4].

SPECIFIC FEATURES OF NEUTROPHILS IN RUMINANTS

Dairy Goat Neutrophils

During peak lactation, neutrophils in the blood and milk of dairy goats differ greatly in their morphological features and functions. Milk neutrophils are derived from migrating blood neutrophils that settle in the mammary gland, where they function to combat invading microbes that penetrate the physiological barriers of the papillary duct [12]. Occasionally, neutrophil band cells are found among blood neutrophils, but never among milk neutrophils. Notably,

milk neutrophils generally appear to be more mature than their blood counterparts. However, compared with blood neutrophils, milk neutrophils have impaired phagocytosis and oxidative burst functionality and lower viability, which may be due to spontaneous aging, interactions with milk components and/or diapedesis-based effects. Morphologically, milk neutrophils have a more ruffled appearance and possess a multi-lobed nucleus instead of the 2- to 3-lobed nucleus observed in the blood. In addition, milk neutrophils exhibit relatively lower ability to release gelatinase compared to blood neutrophils under both PMA stimulation or non-stimulation conditions [13].

In the 1960s, Paape introduced the term "somatic cells" (SCs) to refer to various types of cells found in mammalian milk [14]. As is well known, SCs are a handful of host cells found in animal milk that are predominantly leukocytes, including macrophages, neutrophils and lymphocytes, with a few epithelial cells. Thus, based on their origins, SCs can be categorized into two groups, blood-derived SCs and epithelial SCs [15]. The somatic cell count (SCC) in the milk of healthy dairy goats differs from that of cows, partially reflecting the differences in host processes that control normal mammary epithelial cell shedding and renewal [16]. Studies have shown that during mastitis, distinct neutrophil changes occur that differ between animal species, with neutrophils significantly outnumbering macrophages in the milk of mastitic sheep and goat, while macrophages predominate in healthy milk [17].

Bovine Neutrophils

Bovine neutrophils in the milk, similar to their goat counterparts, migrate from the blood to the mammary gland to provide the first line of defense against invading pathogens [7]. Although neutrophils newly migrating into the mammary gland are active phagocytic cells, they are continuously exposed to inhibitors in milk, such as fat globules and casein, resulting in decreased phagocytic capacity accompanied by morphological alteration [18]. Milk neutrophils possess large phagocytic vacuoles containing previously engulfed casein micelles with smooth surfaces and spherical shapes. These micelles are formed during loss of pseudopods by the internalization of pseudopod membrane material that results in milk fat globule formation.

Unlike other animal species in which neutrophils account for multitudinous blood leukocytes, bovine neutrophils make up only 25% of total blood leukocyte numbers. However, mature lactating Holstein cows have a potential pool of more than 100 billion circulating neutrophils that appear to be supplemented by a marginal pool of mature neutrophils adhering to vessel walls [19]. The makeup of neutrophil populations which respond to a particular mammary stimulus depends on the intensity of the stimulus and the strength of the chemotactic agent, with the number of neutrophils in bovine SCs determined by indirect immunofluorescence to be 3×10^4~3×10^6 cells/mL [20].

Akin to goat neutrophils, bovine neutrophils are also involved in the inflammatory process of mastitis. In fact, a wide variety of neutrophil β-defensins have been isolated that combat invasive pathogens [21]. However, these antimicrobial weapons also damage the fragile inner layer of the mammary gland and lead to permanent scar formation and decreased mammary epithelial cell participation in lactation. Interestingly, co-culture of neutrophils with cold-pressed terpeneless Valencia orange oil (TCO) has been shown to increase the chemotaxis of these cells *in vitro* without altering their phagocytic ability. Indeed, genes reflecting the pro-inflammatory immune response are generally down-regulated in the presence of TCO, resulting in the inhibition of bacterial growth without negatively altering neutrophil function [22], suggesting that TCO may serve as an effective therapy for mastitis.

Similarly, butyric acid, a short-chain fatty acid that can exert potent anti-inflammatory effects both *in vitro* and *in vivo*, could also serve as a mastitis treatment. Butyric acid acts via several mechanisms to regulate the innate immune response of ruminants: by activating neutrophils, inducing platelet activating factor (PAF), increasing CD63 expression, inducing the release of matrix metalloproteinase-9 (MMP-9) and lactoferrin, inducing NETs formation and through short-chain fatty acids (SCFA)-based pathways [23]. Previous studies have reported that increased production of SCFA is involved in subacute rumen acidosis and the activation of the inflammatory response [24]. In humans and rodents, SCFAs regulate the inflammatory response in the gut through free fatty acid receptor 2 (FFA2), which is activated by butyric acid in cattle. Researchers have found that butyrate activates bovine neutrophils to induce two second messenger events, Ca^{2+} influx and phosphorylation of mitogen-activated protein kinase (MAPK) that are involved in FFA2 activation. Butyric acid-induced Ca^{2+} influx is dependent on extracellular and intracellular Ca^{2+} sources and PLC activation. Therefore, butyric acid appears to be involved in SCFA regulation of inflammation through its effects on neutrophil activation [23].

RELATIONSHIP BETWEEN NEUTROPHILS AND COMMON DISEASES OF RUMINANTS

Role of Neutrophil Leucocyte in Cows and Goats During Mastitis

Mastitis is considered as one of the most complex diseases impacting dairy farming. The disease is caused by inflammation of the mammary gland initiated during several types of bacterial infections, among which *E. coli* infections, *M. agalactiae* infections and *S. aureus* infections are the most common ones [25]. Such infections cause damage or even necrosis of the mammary gland that eventually leads to low milk production and even the

removal of dairy production, seriously hindering the development of the livestock industry and imposing a great economic burden on farmers.

Studies about mastitis have shown that neutrophils can efficiently mount defenses against invading pathogens by migrating from the blood to the mammary gland, where they deploy a cascade of oxidative and non-oxidative response mechanisms to destroy pathogens [26]. In cows with mastitis, both SCC and milk neutrophil percentages are significantly increased while the viability of neutrophils is relatively low [27]. This phenomenon may result from pathogen-based signals that trigger both the release of neutrophils to blood from bone marrow and neutrophil migration from blood through the endothelial cell layer to the mammary gland, ultimately the increase of milk SCC. During this process, chemokine-mediated stimulation is a key determinant of SCC influx. Several potent chemoattractants that recruit milk neutrophils include C5a as well as LPS, interleukin-6 (IL-6), IL-17 and IL-8 [26,28]. Although they are present in increased numbers, milk neutrophils possess decreased viability in mastitis. In a previous study, it was suggested that improving the viability of milk neutrophils might prevent or reduce the severity of *E. coli* mastitis in dairy cows [29]. In fact, the phagocytic activity of milk neutrophils is higher than blood neutrophils during subclinical and clinical mastitis, with the opposite observed in healthy cows [27]. However, when compared to healthy cows, blood and milk neutrophils from cows with clinical mastitis showed a significantly reduced phagocytic activity. These observations imply that the defense mechanism against invading pathogens of the mammary gland greatly depends on the rate at which neutrophils enter the infection site, their ability to produce reactive oxygen intermediates (ROI) and the number of circulating neutrophils at the infection site [30]. In healthy cows, milk neutrophils could be considered inactive cells when compared to circulating cells, since the phagocytic capacity of milk neutrophils is regulated by ROS production and milk neutrophils presumably undergo apoptosis to reduce ROI production after diapedesis. Nevertheless, the study found that immunosuppression always relied on cortisol, not on apoptosis, regardless of the physiological state of the cows [31] *(Fig. 1)*.

Blood neutrophils change their shape as they pass through the mammary epithelial barrier by becoming spherical. Upon entering the mammary gland, they become irregular milk neutrophils with wrinkled outer surfaces. The latter state not only helps to better internalize the cell membrane during phagosome formation, but also increases their surface areas for optimal phagocytosis [30] *(Fig. 1)*.

Bacteria have been shown to undermine neutrophil functions at each of these steps. In mastitis caused by *S. aureus*, IFN-γ serves as a neutrophil priming agent by acting as a primary agonist to influence the early effector arm of the neutrophil response and modulate post-response trafficking [32]. However, several researchers have found that *S. aureus* produces membrane-damaging peptides such as α, β, γ, δ-hemolysins, phenol soluble modulins (PSMs) and bi-component leukocidins that directly punch holes in immune cell membranes to lyse the cells [33]. Moreover, *S. aureus* employs numerous virulence factors to restrain neutrophil activation, chemotaxis and phagocytosis and target key host effector proteins. For example, extracellular fibrinogen-binding (Efb) protein and staphylococcal complement inhibitor (SCIN) proteins

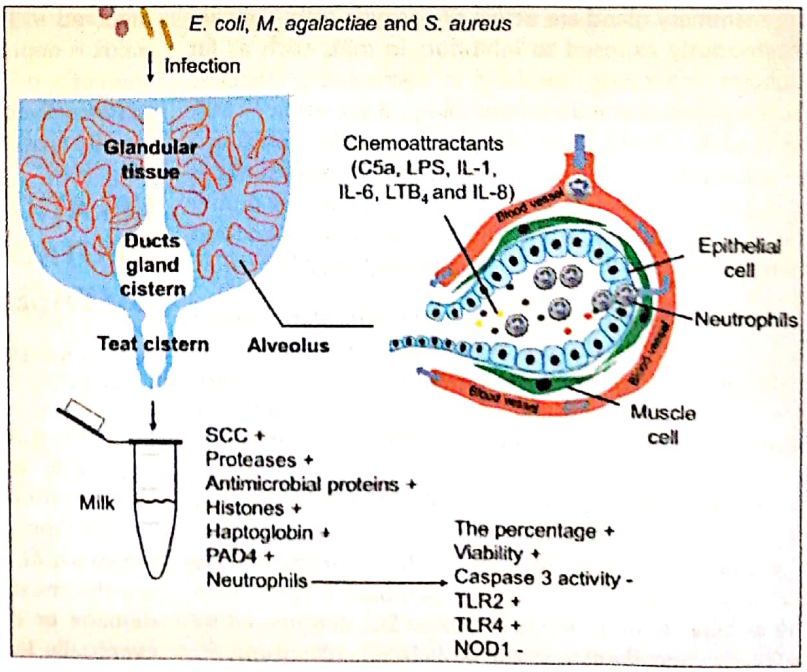

Fig 1. The role of neutrophils in mastitis. In glandular tissues affected by mastitis caused by *E. coli*, *M. agalactiae* and *S. aureus*, neutrophils patrolling in the bloodstream traverse the mammary endothelial cells and enter the mammary alveolus, where they destroy pathogens via oxidative and non-oxidative mechanisms. Neutrophils possess a wrinkled outer surface as they pass through endothelial cells. Within an alveolus, chemoattractants (C5a, LPS, IL-1, IL-6, LTB_4 and IL-8) tend to induce the participation of additional neutrophils and other immune cells during the inflammatory response. Changes in various milk proteins and cells are designated below the figure: "+" means increased or high expression; "-" means decreased or low expression

can target complement protein C3 convertase to prevent the formation of C3a and C3b, resulting in bacterial resistance to neutrophil phagocytosis [34]. Meanwhile, the *S. aureus* chemotaxis inhibitory protein (CHIPS) inhibits neutrophil migration and activation, preventing neutrophils from responding to both host- and bacterial-derived chemoattractants. Although toll-like receptor 2 (TLR2)-expressing cells can recognize staphylococcal glycan-related lipoproteins such as staphylococcal iron transporter C (SitC), *S. aureus* produces staphylococcus superantigen-like 3 (SSL3) protein that binds to TLR2 and inhibits the activation of neutrophils and other cells expressing TLR2. Crystal structure-based analysis demonstrates that the binding of SSL3 to TLR2 reduces the size of the available lipopeptide binding pocket by 50%, explaining the observed binding inhibition of TLR2 agonist Pam_2CSK_4. Moreover, *S. aureus* produces capsular polysaccharides and micro-capsules that may serve as yet another phagocytic escape strategy [35].

Abnormal apoptosis may also be a mechanism involved in neutrophil-based tissue damage in mastitis. Indeed, in the blood and milk of cows with mastitis, neutrophil caspase 3 activity is reduced, which may reflect reduced neutrophil apoptosis that promotes heightened neutrophil activity, ultimately causing tissue damage. Alternatively, the control of inflammation may involve extending neutrophil lifespan via the inhibition of NETosis [4]. In yet another possible scenario, higher neutrophil surface expression of TLR2 and TLR4, but not of TLR9, in cows with mastitis might enhance neutrophil pathogen recognition and immune responses, with crosstalk between C5a and neutrophil TLR4 signaling supporting a positive feedback loop that leads to severe mastitis responses *(Fig. 1)*. In this scenario, the onset of inflammatory reactions would somehow be linked to inefficient LPS detoxification that activates C5 cleavage to form C5a; if a large amount of C5a was produced, neutrophils would be activated via the C5a-C5aR pathway, a possible therapeutic target for mastitis treatment [36]. Another potential pathway in mastitis involves the interaction of neutrophils with the nucleotide-binding oligomerization domain-1 (NOD1), a key factor involved in the sensing of conserved bacterial peptidoglycan motifs that initiates pro-inflammatory and antimicrobial responses. In perinatal cows, neutrophil expression of NOD1 is decreased, which results in the inhibition of NOD1/NF-κB signaling, reduced neutrophil migration to *E. coli*-infected sites and impaired phagocytosis [37] *(Fig. 1)*. The NOD1/NF-κB pathway modulates neutrophil responses to reduce both neutrophil-mediated killing of bacteria and ROS production that in turn may control early inflammatory responses. Consequently, artificial restoration of neutrophil NOD1 function might be employed to either prevent or treat *E. coli*-induced mastitis.

Role of Neutrophil Leucocytes in Brucellosis

Brucellosis, caused by the genus *Brucella*, is a chronic global zoonosis [38]. Within infected macrophages and dendritic cells, pathogenic *Brucella* can replicate efficiently in the endoplasmic reticulum, a safe intracellular niche at the crossroads of many important host cell functions. There are many subspecies of *Brucella*, of which *B. melitensis*, *B. abortus* and *B. suis* are the three most common pathogens in humans and livestock [38]. Although *Brucella* spp. are closely related to each other genetically, they infect a broad range of livestock hosts including goats, cattle, camels, sheep, pigs and even wild animals such as bison, elk and feral swine [39]. Common symptoms of Brucellosis include high abortion rate, high mortality, infertility, low milk yield and a long interval between calving. The ability of *Brucella* spp. to evade the host immune system determines pathogen virulence, with one demonstrated mechanism involving escape from phagocytic killing. Thus, the mechanism by which neutrophils respond to pathogenic *Brucella* is a rather interesting and worthwhile research area.

Seminal studies have demonstrated that virulent smooth *B. abortus*, *B. melitensis*, *B. suis* and rough *B. canis* strains are resistant to neutrophil killing, regardless of whether they are resting or IFN-γ-activated. These results suggest that smooth strains may often be more virulent than rough strains. Bovine neutrophils counter infection by both smooth and rough *Brucella* spp. using oxidative burst-based killing [39]. This mechanism involves the inflammatory signaling regulator TAK1, a MAP3 kinase that is activated in response to cytokines, growth factors and TLR signals [39]. SYK kinase also plays a key role in the response of neutrophils to inflammations and is activated by Fc receptor binding. TAK1 is one of the major regulators of multiple kinase activation downstream of SYK in response to C-type lectin receptor stimulation. By inhibiting TAK1 or SYK, the degree of oxidative burst of neutrophils infected by *B. abortus* will be reduced, indicating a role of C-type lectin receptor in the response of bovine neutrophils to *B. abortus* infection [39] *(Fig. 2)*.

A *Brucella* virulence factor, β cyclic glucan (CβG), which has no toxicity for cells or animals, can induce dual pro-inflammatory and anti-inflammatory responses leading to transient neutrophil recruitment [40] *(Fig. 2)*. Notably, β-glucan is a polysaccharide of β-D-glucose extracted from cell walls of mushrooms, yeast, oats, barley, seaweed, algae and bacteria. Many researches and clinical studies have suggested that β-glucan acts as a biological response modifier that exhibits anti-tumor and anti-inflammatory properties. It is recognized by various pattern recognition receptors (PRR) expressed on dendritic cells, macrophages and neutrophils. In addition, complement receptor-3 (CR3), lactosylceramides, scavenger receptor and dectin-1 are also involved in β-glucan recognition, the outcome of which can trigger a series of signaling events that regulate the innate and the adaptive immune responses [41].

Effects of Mycoplasma bovis and Mycoplasma Lipoproteins on Neutrophils

Mycoplasma bovis (M. bovis) is the smallest bacterium

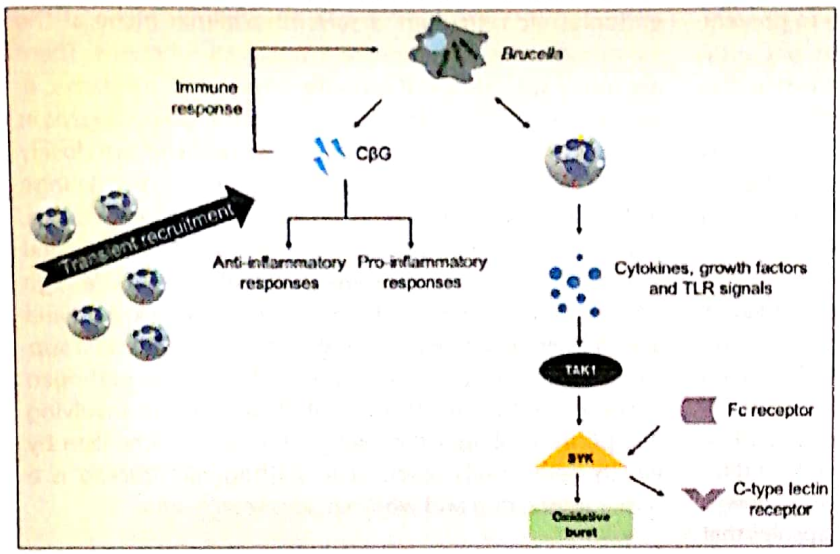

Fig 2. Neutrophils resist invasion by *Brucella*. *Brucella*-invading macrophages and other phagocytic cells interact with neutrophils and activate TAK1 in response to neutrophil TLR signals, as well as to multiple cytokines and growth factors. Downstream SYK is then activated by the action of the Fc receptor and further responds to signaling via the C-type lectin receptor. The neutrophil oxidative burst is eventually induced to resist *Brucella* infection. In addition, the virulence factor CβG produced by *Brucella* can induce transient neutrophil recruitment

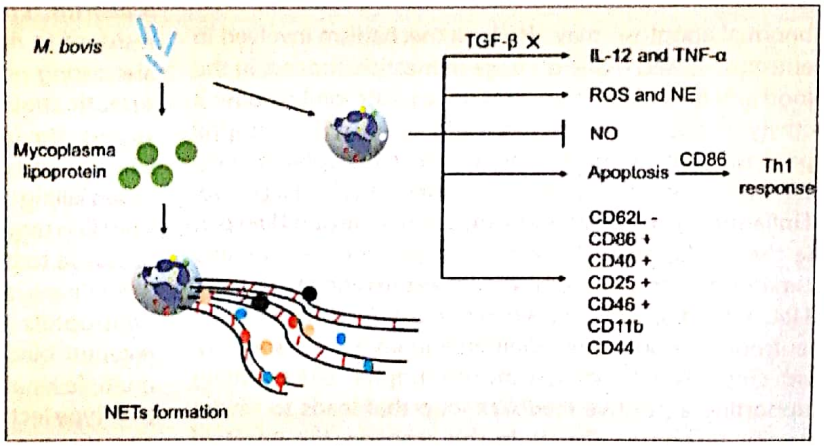

Fig 3. Effects of *M. bovis* and Mycoplasma lipoproteins on neutrophils. *M. bovis* invades the body and evades the immune response by accelerating neutrophil apoptosis and inducing ROS and NE production while inhibiting NO production. Subsequently, the Th1 response is induced by CD86-mediated signaling. During *M. bovis* infection, changes in molecules expressed on the neutrophil surface occur: "+" means increased or high expression; "-" means decreased or low expression; no mark means no change. Furthermore, neutrophils co-cultured with *M. bovis* could secrete IL-12 and TNF-α in the absence of TGF-β, while pathogen lipoproteins could induce the formation of neutrophil NETs

lacking a cell wall and often causes bovine chronic pneumonia and polyarthritis syndrome, conjunctivitis, otitis media, meningitis and mastitis [42]. These organisms require relatively rigorous cultural conditions for growth, posing a serious hindrance to vaccine preparation and disease prevention. Indeed, the lack of a vaccine has greatly influenced the health, welfare and productivity of dairy and beef cattle.

It has been shown that neutrophils exposed to *M. bovis* exhibit altered bactericidal function *in vitro* and that *M. bovis* inhibits the production of IFN-γ and TNF-α, but not IL-10 [43]. While little is known about how *M. bovis* evades host innate immunity, *M. bovis* has been shown to infect and persist within all PBMC subpopulations and erythrocytes [44]. Importantly, bacterial survival relies on the triggering of a series of pathogenic responses that delay the apoptosis of host cells. Such responses are carried out through the interaction of bacterial components with multiple proteases to activate cell survival pathways and prevent cytochrome C release. Conversely, *M. bovis* evades immune recognition by accelerating neutrophil apoptosis and inducing ROS production, while *in vitro* experiments have shown that the bacteria can also inhibit NO production, which has a dual biological role as a signaling molecule and cytotoxin. Meanwhile, IL-12 and TNF-α production in the absence of TGF-β has been observed when bovine neutrophils are infected with *M. bovis in vitro*. This observation suggests that an activation state is created based on inflammatory cytokines that may enhance the biological response of bovine neutrophils to *M. bovis* infection. In addition, host NE production is also important for the elimination of *M. bovis (Fig. 3)* [44].

As a unique survival mechanism during infection, *M. bovis* may drive neutrophils to a state of incompetence, as indicated by a decrease in CD62L expression with the up-regulation of CD86, CD40, CD25 and CD46 [44]. This mechanism appears to help *M. bovis* escape the host immune response and survive *in vitro* [44]. In this scenario, increased neutrophil apoptosis following *M. bovis* stimulation results in the activation of type 1 helper T cell responses, with increased expression of CD86 that is involved in antigen presentation to naive T cells [44] *(Fig. 3)*.

As another unique mechanism, mycoplasma lipoprotein, the most abundant component of the mycoplasma membrane, interacts with the host to promote cell

adhesion, determine strain virulence and induce NETosis. It is important that only fat-soluble mycoplasma proteins effectively induce NETs formation as an explanation for *M. agalactiae* evasion from NETs both in cultured sheep neutrophils and in mastitic mammary glands [45]. Using a different mechanism, *S. aureus* has been shown to induce macrophage apoptosis by disassembling NETs and converting them to deoxyadenosine, which induces caspase 3-mediated immune cell apoptosis [46]. In contrast, mycoplasma digests NETs DNA scaffolds, a mechanism requiring live bacteria that prevents the transmission of overlapping signals associated with neutrophil DNA and *M. agalactiae* within the mammary gland [45] *(Fig. 3)*.

Role of Neutrophil Leucocyte in Parasitic Infections

Parasitic infections are an enormous hazard to cattle and goat farming. Many studies have shown that NETs act as a novel effector mechanism in innate immunity against parasitic infections such as *Besnoitia besnoiti (B. besnoiti)*, *Eimeria arloingi (E. arloingi)* and *Cryptosporidium parvum (C. parvum)* infections in cattle and goats [47].

Cattle infected by *B. besnoiti* exhibit clinical symptoms such as systemic dermatitis, orchitis and vulvitis. *In vitro*, bovine neutrophils interact with *B. besnoiti* tachyzoites to induce rapid formation of NETs, which can be eliminated by DNase treatment or reduced by pre-incubation with NADPH oxidase inhibitors, neutrophil elastase (NE) and MPOs. It appears that neutrophils can immobilize parasites by forming an embedded structure so that other immune cells can be recruited quickly to synergistically kill the pathogen. *E. arloingi* coccidiosis in goats is mainly characterized by severe enteritis of 4- to 10-week-old goats, with infection rates as high as 100%. Once neutrophils contact *E. arloingi*, they form NETs during sporozoite or oocyst stages, whereby NETs effectively capture 72% of the sporozoites, greatly reducing the early infection rate [48]. *C. parvum* causes severe enteritis in neonatal livestock as well, triggering the formation of NETs in a time-dependent manner, whereby sporozoite-triggered NETs depend on intracellular Ca^{2+} concentration and ERK 1/2- and p38 MAPK-mediated signaling pathways. In fact, about 15% of parasites formed by *C. parvum* are immobilized in NETs [49].

Taken together, as the first line of immune defense, neutrophils play a unique role in parasitic infections. Neutrophils capture such pathogens mainly through the formation of NETs, delaying pathogen spread and promoting further elimination of invading parasites, although the detailed mechanisms are still unclear.

Other Modulators of Neutrophil Responses

In addition to diseases mentioned above, neutrophils also participate in other inflammatory ruminant diseases including bovine leukemia virus infection, *Pasteurella haemolytica* pneumonia, bovine respiratory disease, lung inflammation induced with *Mannheimia hemolytica*, pulmonary and systemic inflammation of fetal sheep and *Histophilus somni* infection, among others.

Host stress responses and viral invasion can adversely impact neutrophil responses and most typically affect neutrophil number without altering their recruitment, leukotoxin sensitivity or responses to bacterial infections. For example, non-cytogenetic bovine viral diarrhea virus infection leads to reduced production of neutrophils in the bone marrow and ultimately persistent neutropenia [50]. When occurring during abrupt weaning stress with increased neutrophil numbers, virus infections can recruits abundant neutrophils to sites of inflammation. However, the virus-triggered type I interferon response limits the production of CXC chemokines, leaving the host vulnerable to deadly secondary infections. Such infections include pulmonary *Streptococcus pneumoniae* or BRSV infections, the latter of which is characterized by neutrophil airway and alveolar infiltration with lower MPO levels and functionally immaturity [50].

Other factors that modulate neutrophil function include β-hydroxybutyrate, a mediator that can attenuate neutrophil phagocytosis and induce the formation of NETs [23]. Meanwhile, glucocorticoids can increase the survival and recruitment of neutrophils [50]. *H. somni* is able to inhibit an oxidative burst in both neutrophils and alveolar macrophages, while also inducing NETs production in a dose- and time-dependent manner that is not associated with lactate dehydrogenase release [50]. Taken together, both host and pathogen factors can significantly influence neutrophil function, with pathogens employing numerous mechanisms to escape killing by neutrophils and other cells of the immune system.

CONCLUSIONS AND PROSTECTS

We have discussed current knowledge regarding neutrophil chemotaxis and cytotoxic functions. We have also discussed specific features of ruminant neutrophils and the involvement of these cells in common diseases of ruminants. In recent years, studies revealing neutrophil origins, heterogeneity, circadian rhythms and epigenetic control of neutrophil activities have been widely discussed. However, most of these studies are based on humans or mice, researches on ruminant neutrophils are still lacking. Considering that neutrophils are actively involved in various ruminant diseases, further studies are required to explain and understand how neutrophils function during the progression of these diseases. In addition, how to better harness neutrophil activities during disease progression and how to optimally exploit these cells are also needed to explore in the future.

Conflict of Interest

The authors declare that no conflict of interest exists.

Author Contributions

W-T.M. and F.G. designed the structure of this article, S-L.N., F.G., C-X.Z., X-D.T. and M-J.L. drafted the first version of the manuscript, J-J.C, Y.W., D-K.C. and W-T.M. revised the manuscript.

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检索报告

根据委托人倪思璐委托，通过网络检索，倪思璐发表的 1 篇论文被《科学引文索引》扩展版（SCI-Expanded）数据库收录。数据库具体检索结果如下：

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奶山羊 IL－6 与 TGF－β1 融合蛋白的制备及其活性检测

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[摘 要] 【目的】制备具有免疫活性的奶山羊白细胞介素 6(Interleukin-6,IL-6)与转移生长因子-β1(Transfer growth factor-β1,TGF-β1)融合蛋白。【方法】采集奶山羊外周血,分离外周血单个核细胞(Peripheral blood mononuclear cell,PBMCs),将 PBMCs 用刀豆蛋白 A(ConA)刺激后提取其总 RNA,RT-PCR 扩增 *IL-6* 与 *TGF-β1* 基因,构建其克隆载体及原核表达载体 pET-32a-TGF-β1 和 pET-32a-IL-6,然后进行 PCR 和测序鉴定。将原核表达载体 pET-32a-TGF-β1 和 pET-32a-IL-6 转化至大肠杆菌 BL21(DE3)中,用 IPTG 诱导表达,以镍柱纯化试剂盒纯化 IL-6 与 TGF-β1 重组蛋白,对表达产物和纯化后的蛋白进行 SDS-PAGE。用纯化的 IL-6、TGF-β1 蛋白刺激 PBMCs,以甘油醛-3-磷酸脱氢酶 *GAPDH* 基因为内参,采用 qRT-PCR 法检测 PBMCs *IL-17* mRNA 的表达量。【结果】RT-PCR 扩增获得了 627 bp 的 *IL-6* 基因片段和 1 137 bp 的 *TGF-β1* 基因片段,成功构建了 *IL-6* 和 *TGF-β1* 基因的克隆载体及 pET-32a-TGF-β1 和 pET-32a-IL-6 原核表达载体,并在大肠杆菌 BL21(DE3)中得到成功表达;获得了纯化的奶山羊 IL-6 与 TGF-β1 融合蛋白,该融合蛋白联合刺激能使 PBMCs 的 *IL-17* mRNA 表达水平显著升高。【结论】获得了具有免疫活性的奶山羊 IL-6 与 TGF-β1 重组蛋白。

[关键词] 奶山羊;白细胞介素 6;转移生长因子-β1;融合蛋白
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Preparation and function detection of purified fusion protein of dairy goat IL－6 and TGF－β1

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Abstract:【Objective】This study aimed to obtain immunologically active purified fusion protein of dairy goat interleukin-6(IL-6) and transfer growth factor-β1 (TGF-β1).【Method】Peripheral blood of dairy goats was collected and peripheral blood mononuclear cells (PBMCs) were isolated. PBMCs were stimulated by concanavalin A (ConA) and total RNA was extracted. The *IL-6* and *TGF-β1* genes were amplified by RT-PCR,and the cloning vector and prokaryotic expression vectors pET-32a-TGF-β1 and pET-32a-IL-6 were constructed and identified by PCR and sequencing. The prokaryotic expression vectors pET-32a-TGF-β1 and pET-32a-IL-6 were transformed into *E. coli* BL21(DE3) and induced by IPTG. The protein of IL-6 and TGF-β1 was purified by nickel column purification kit. The expression product and the purified protein were identified by SDS-PAGE. PBMCs were stimulated with purified IL-6 and TGF-β1 protein. The ex-

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pression of *IL-17* mRNA in PBMCs was detected by qRT-PCR with glyceraldehyde-3-phosphate dehydrogenase *GAPDH* gene as internal reference. 【Result】 A 627 bp *IL-6* gene fragment and a 1 137 bp *TGF-β1* gene fragment were obtained by RT-PCR. The cloning vectors of *IL-6* and *TGF-β1* genes and pET-32a-TGF-β1 and pET-32a-IL-6 were successfully constructed. Prokaryotic expression vector was successfully expressed in *E. coli* BL21 (DE3), and purified dairy goat IL-6 and TGF-β1 fusion protein was obtained. The IL-6 and TGF-β1 fusion protein combined stimulation promoted the *IL-17* mRNA expression level of PBMCs significantly. 【Conclusion】 The immunologically active purified fusion protein of dairy goat IL-6 and TGF-β1 was successfully obtained.

Key words: dairy goat; *IL-6*; *TGF-β1*; purified fusion protein

近几年来，国内奶山羊养殖数量不断增加，养殖地区也突破原有的省份并不断向国内多个地区辐射[1]。受自然牧草资源的限制，目前我国奶山羊饲养模式主要以舍饲养殖为主[2]，这种饲养模式有很多优点，但其养殖群体中多种疫病的发生率也明显高于放牧养殖群体，其中严重影响奶山羊养殖经济效益的疫病主要有乳房炎、传染性胸膜肺炎和羊口疮等[3-5]，这些疫病以奶山羊乳房、肺脏和口唇部呈现严重的病理性炎症反应为主要特征，在奶山羊舍饲养殖群体中普遍流行。国内奶山羊隐性乳房炎检出率为30%～85%[6]，羊口疮的检出率为15%～90%[4,7]，羊传染性胸膜炎的发病率为19%～90%[5]。造成这种现状的主要原因是针对性防控措施不得力，其根源在于对这些疫病病原的免疫防御机制不清楚。

在炎症性疫病发病过程中，受促炎性细胞因子和趋化因子的影响，多种免疫细胞被募集到炎症部位，通过吞噬和分泌作用清除病原微生物。伴随这一过程的是反应部位自身组织细胞也会受到损伤并发生病理学变化，进而影响到机体生理功能的正常发挥。大量研究表明，作为典型的促炎性细胞因子之一，白细胞介素6 (Interleukin-6, IL-6)水平在炎症过程中会显著升高，其增高幅度与炎症反应的严重程度呈正相关[8]。与此相反，转移生长因子-β (Transforming growth factor-β, TGF-β)则属于炎症反应抑制性细胞因子，TGF-β分为TGF-β1、TGF-β2和TGF-β3，其中TGF-β1在机体炎症反应过程中起重要的免疫调节作用[9]。令人感兴趣的是，当微环境中的IL-6和TGF-β水平同时有所升高时，就会介导一种新发现的T细胞亚群，即Th17细胞的产生[10-11]。

有研究指出，Th17细胞在炎症疾病发生过程中发挥着至关重要的作用，Th17细胞可以通过分泌IL-17等细胞因子来募集、活化中性粒细胞到达炎症部位并清除病原体，从而介导免疫反应维持机体自稳[12-13]。有研究证明，在小鼠模型中，TGF-β1和IL-6共刺激可使小鼠Th(辅助性T细胞)分化为Th17细胞[11,14]，而在体外只需要IL-1和IL-6细胞因子即可诱导人Th17的产生[15-16]。在奶山羊的炎症发病过程中，IL-6和TGF-β1等主要炎症相关细胞因子的作用及其与Th17细胞分化的关系等至今未得到阐明，尤其是奶山羊乳房炎、羊口疮等炎症疫病的病原免疫防御机制问题至今尚不清楚，给针对性地制定这些疫病的高效防控措施带来了困难。基于此，本研究对奶山羊*IL-6*和*TGF-β1*基因进行了克隆表达，并对其表达产物的活性进行了检测，旨在为IL-6和TGF-β1等细胞因子在奶山羊炎性疾病中的作用机制研究提供有益的参考资料，进而为奶山羊疫病防控措施的制定提供参考依据。

1 材料与方法

1.1 材 料

雌性22月龄健康萨能奶山羊，购自陕西咸阳某羊场。大肠杆菌DH5α和BL21 (DE3)、质粒pET-32a，由西北农林科技大学动物医学院兽医免疫学实验室保存。Total RNA提取试剂盒、限制性核酸内切酶(*Bam*H Ⅰ、*Xho* Ⅰ和*Eco*R Ⅰ)、T4连接酶、SYBR Premix Ex *Taq*™ Ⅱ试剂盒和镍柱纯化试剂盒，均购自TaKaRa生物技术有限公司。刀豆蛋白A(ConA)，购自Solarbio科技有限公司。SanPrep柱式DNA胶回收试剂盒、SanPrep柱式质粒DNA小量抽提回收试剂盒，均购自生工生物工程有限公司。FastKing RT Kit (With gDNase)，购自天根生化科技有限公司。pClone007 Blunt Simple Vector Kit，购自擎科生物技术有限公司。

1.2 试验方法

1.2.1 *IL-6*与*TGF-β1*基因的RT-PCR扩增 根据NCBI中收录的山羊*TGF-β1* (GenBank号为：

NM_001314142.1）和 IL-6（GenBank 号为：NM_001285640.1）mRNA CDS 区序列，分别用 NCBI 中 Primer blast 在线工具和 Primer 5.0 设计引物（表 1），并由 Invitrogen 公司合成。

表 1　用于扩增奶山羊 *IL-6* 和 *TGF-β1* 基因的引物

Table 1　Dairy goat *IL-6* and *TGF-β1* primer sequences used for amplification

基因 Gene	引物序列(5′→3′) Primer sequence (5′→3′)	退火温度/℃ Tm	预期目的片段长度/bp Expected segment length
TGF-β1	F:ATGCCGCCTTCGGGGCTGCGGCTGCTGC R:TCAGCTGCACTTGCAGGAGCGCACGATC	56	1 173
IL-6	F:ATGAACTCCCTCTTCACAAGCG R:CTACTTCATCCGAATAGCTCTCAGG	59	627

采集奶山羊外周血，常规方法[17]分离外周血单个核细胞（Peripheral blood mononuclear cell，PBMCs），加入 ConA 至终质量浓度为 20 μg/mL，培养 72 h。收集 PBMCs，按照 Total RNA 提取试剂盒说明书提取总 RNA。以提取的总 RNA 为模板，采用 RT-PCR 方法扩增 *IL-6* 和 *TGF-β1* 基因，试验以 ddH_2O 代替模板的处理为阴性对照。RT-PCR 体系为 25 μL，具体体系按照 FastKing RT Kit 试剂说明书进行配制。RT-PCR 反应条件为：50 ℃反转录 30 min；扩增反应程序为：94 ℃ 5 min；94 ℃ 60 s，56 或 59 ℃（*TGF-β1* 56 ℃，*IL-6* 59 ℃）退火 30 s，72 ℃延伸 90 s，共 32 个循环；72 ℃延伸 10 min。取 5 μL PCR 产物，用 2%琼脂糖凝胶进行电泳，紫外凝胶成像系统观察记录结果后，切下含目的片段的琼脂胶，采用 SanPrep 柱式 DNA 胶回收试剂盒进行胶回收。

1.2.2　*IL-6* 与 *TGF-β1* 基因克隆载体的构建　将 RT-PCR 扩增的 *IL-6* 和 *TGF-β1* 基因片段与 pClone007 Blunt-end Simple Vector 连接，具体操作按照 pClone007 Blunt Simple Vector 说明书进行。将连接产物转化感受态 DH5α，培养后挑取单菌落，接种于 4 mL 含 50 μg/mL 氨苄（Amp）抗性的 LB 液体培养中，37 ℃振荡培养 12 h，取 1 μL 进行 PCR 鉴定，试验同时以 ddH_2O 代替模板的处理为阴性对照。将菌液 PCR 为阳性的单克隆菌液送擎科生物技术有限公司测序。

1.2.3　*IL-6* 与 *TGF-β1* 基因原核表达载体的构建　根据 NCBI 中收录的山羊 *TGF-β1* 和 *IL-6* 基因 mRNA CDS 区序列，结合载体图谱及 Primer 5.0 软件预测的信号肽序列，用 Primer 5.0 软件设计引入酶切位点的引物，其扩增产物不包含信号肽。引物由 Invitrogen 公司合成，其相关信息见表 2。

表 2　用于扩增奶山羊去信号肽 *IL-6* 和 *TGF-β1* 基因的引物

Table 2　Dairy goat *IL-6* and *TGF-β1* without signal peptide primer sequences used for amplification

基因 Gene	引物序列(5′→3′) Primer sequence (5′→3′)	退火温度/℃ Tm	预期目的片段长度/bp Expected segment length
去信号肽 *TGF-β1* *TGF-β1* without signal peptide	F:TAAGGATCCGCCCTGGACACCAACTACTGCTTCA R:TTACTCGAGTCAGCTGCACTTGCAGGAGCGCACGATC	56	339
去信号肽 *IL-6* *IL-6* without signal peptide	F:CCGGAATTCTTCCCTACCCCGGGTCCCCT R:CCGCTCGAGCTACTTCATCCGAATAGCTCTCAGG	59	552

注：GGATCC、CTCGAG、GAATTC 分别为 *Bam*H Ⅰ、*Xho* Ⅰ、*Eco*R Ⅰ限制性内切酶酶切位点。

Note：GGATCC、CTCGAG、GAATTC is *Bam*H Ⅰ，*Xho* Ⅰ，*Eco*R Ⅰ restriction enzyme cutting site respectively.

将克隆载体阳性菌液 8 000 r/ min 离心 3 min，收集菌体沉淀，用 SanPrep 柱式质粒 DNA 小量抽提回收试剂盒提取质粒。以提取的质粒为模板，PCR 扩增去信号肽的 *TGF-β1* 和 *IL-6* 基因，PCR 体系为 25 μL：Mix 12.5 μL，上、下游引物各 1 μL，DNA 模板 1 μL，ddH_2O 9.5 μL，反应程序同 1.2.1 节。用限制性内切酶 *Bam*H Ⅰ/*Xho* Ⅰ双酶切 *TGF-β1* PCR 扩增产物和表达载体 pET-32a，用 *Xho* Ⅰ/*EcoR* Ⅰ双酶切 *IL-6* PCR 扩增产物和表达载体 pET-32a，分别纯化回收 PCR 产物与表达载体 pET-32a 片段，T4 连接酶连接，构成原核表达载体 pET-32a-TGF-β1 和 pET-32a-IL-6。将连接产物转化至感受态大肠杆菌 BL21（DE3），用 Amp 抗性 LB 平板（Amp 50 μg/mL）筛选、PCR 筛选，获得阳性克隆送擎科生物技术有限公司测序。

1.2.4　重组蛋白 IL-6 与 TGF-β1 的诱导表达和纯化　将鉴定含有正确重组质粒的大肠杆菌 BL21（DE3）菌液，按体积比 1∶100 的比例接种到含 50 μg/mL Amp 的 LB 培养基中，37 ℃培养箱中过夜培养；取过夜培养菌液按体积比 1∶50 接种至 4 mL

LB培养基,37 ℃振荡培养出现云雾样,加入IPTG至终浓度为1 mmol/L,37 ℃诱导4 h;收集3 mL菌液,8 000 r/min离心3 min后取上清,用PBS洗涤沉淀2次,最后用100 μL PBS(pH 7)悬浮。取5 mL诱导重组菌液,8 000 r/min离心3 min,取沉淀的菌体用PBS洗涤2次,将沉淀重悬于0.5 mL热解缓冲液中,超声(300 W,工作10 s,间歇10 s)裂解20 min,12 000 r/min离心15 min,将上清液移入新的EP管中。取收集的上清液用镍柱纯化试剂盒进行蛋白纯化,具体操作按镍柱纯化试剂盒说明书进行。取诱导表达样品及纯化后的样品,加入5×SDS上样缓冲液,水浴煮沸10 min,进行SDS-PAGE电泳鉴定,同时以未诱导重组菌液、诱导空载体菌液为对照。

1.2.5 IL-6与TGF-β1重组蛋白的免疫学活性检测 调整PBMCs密度为1×10^{6} mL^{-1},加入24孔细胞培养板,每孔100 μL,然后每孔再加入20 μg/mL的ConA 50 μL。将上述PBMCs分为对照组、IL-6组、TGF-β1组和IL-6+TGF-β1组,其中对照组加入50 μL的培养液,IL-6组细胞每孔加入25 μL的1 ng/mL重组蛋白IL-6和25 μL培养液,TGF-β1组细胞每孔加入25 μL的10 ng/mL重组蛋白TGF-β1和25 μL培养液,IL-6+TGF-β1组细胞每孔加1 ng/mL重组蛋白IL-6和10 ng/mL重组蛋白TGF-β1各25 μL。各组细胞培养72 h后,提取总RNA,检测核酸质量浓度。调整核酸质量浓度为200 ng/μL,使用600 ng RNA反转录得到cDNA。以甘油醛-3-磷酸脱氢酶基因(*GAPDH*)为内参,采用SYBR实时荧光定量PCR法(qRT-PCR)检测*IL-17* mRNA的表达水平。根据NCBI中收录的*IL-17*(GenBank号为:NM_002190.3)和*GAPDH*(GenBank号为:NM_014364.5) mRNA CDS区序列,在线设计*IL-17*和*GAPDH*基因引物(表3)。qRT-PCR反应体系为15 μL,具体体系按照SYBR Premix Ex *Taq*™ Ⅱ试剂盒说明书进行配制。qRT-PCR反应条件为两步法,先95 ℃预变性15 min,再进行PCR反应(95 ℃ 10 s,60 ℃ 32 s,40个循环)。

表3 用于*IL-17*与*GAPDH*基因扩增的引物

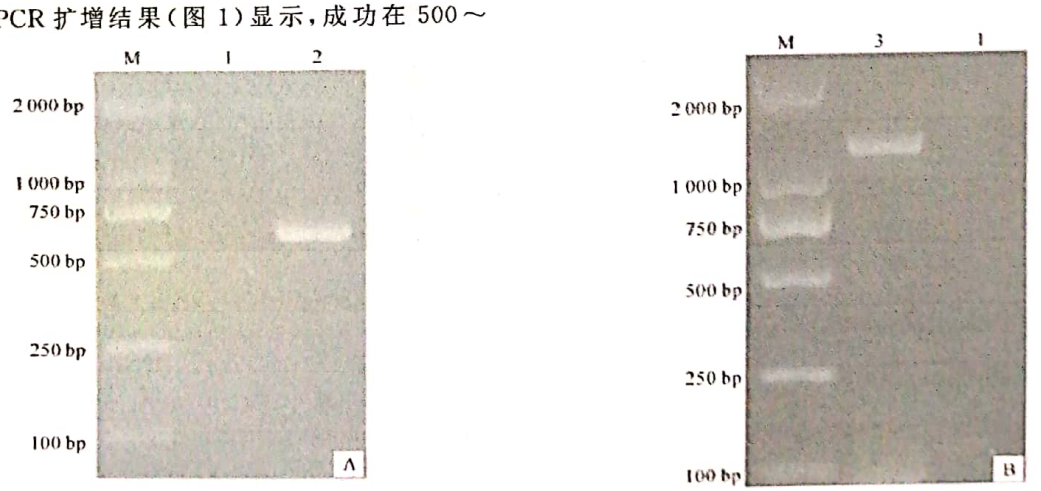
Table 3 *IL-17* and *GAPDH* primer sequences used for amplification

基因 Gene	引物序列(5′→3′) Primer sequence (5′→3′)	退火温度/℃ Tm	预期目的片段长度/bp Expected segment length
GAPDH	F:GAGGGACTTATGACCACTGTCC R:CCAGTAGAAGCAGGGATGATGT	52	122
IL-17	F:CCCACCTACTGAGGACAAGAAC R:GTACCTCTCAGGGTCCTCATTG	57	151

2 结果与分析

2.1 *IL-6*与*TGF-β1*基因的RT-PCR扩增

RT-PCR扩增结果(图1)显示,成功在500～750 bp扩增出了*IL-6*的预期片段(图1-A),在1 000～2 000 bp扩增出了*TGF-β1*的预期片段(图1-B)。



A. *IL-6*基因;B. *TGF-β1*基因。M. 2000DL Marker;1. 阴性对照;2. *IL-6*基因扩增产物;3. *TGF-β1*基因扩增产物

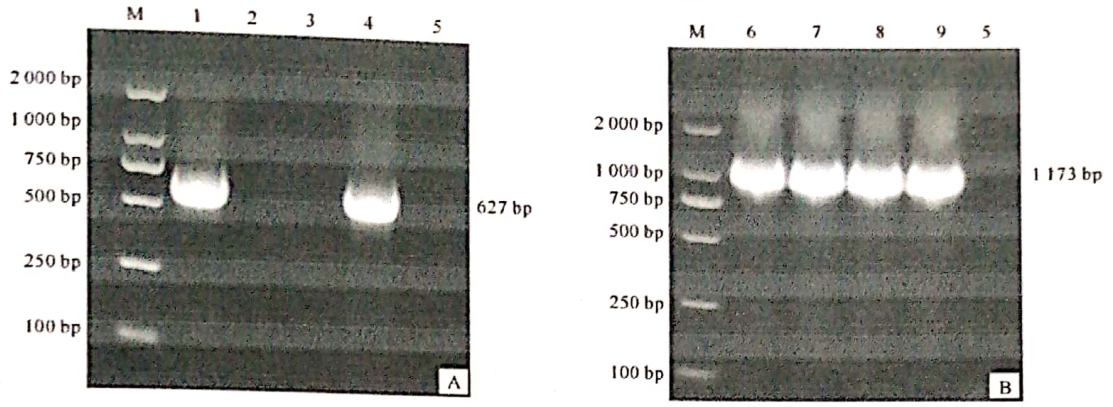
A. *IL-6* gene;B. *TGF-β1* gene. M. 2000DL Marker;1. Negative control;2. Amplification of *IL-6* gene;3. Amplification of *TGF-β1* gene

图1 奶山羊*IL-6*与*TGF-β1*基因的RT-PCR扩增

Fig. 1 PT-PCR amplification of dairy goat *IL-6* and *TGF-β1* gene

2.2　*IL-6* 与 *TGF-β1* 基因克隆载体的鉴定

PCR 鉴定结果(图 2)显示，成功获得了 627 bp 的 *IL-6*(图 2-A)和 1 173 bp 的 *TGF-β1*(图 2-B)目的片段，测序鉴定结果表明与预期长度相符。由此表明，*IL-6* 和 *TGF-β1* 核酸片段已成功插入到克隆载体中，获得了碱基序列和片段长度与预期结果完全一致的克隆载体，*IL-6* 和 *TGF-β1* 基因克隆载体构建成功。



A. *IL-6* 克隆载体；B. *TGF-β1* 克隆载体。M. 2000DL Marker；1，4. 阳性菌落；2，3. 假阳性菌落；5. 阴性对照；6～9. 阳性菌落

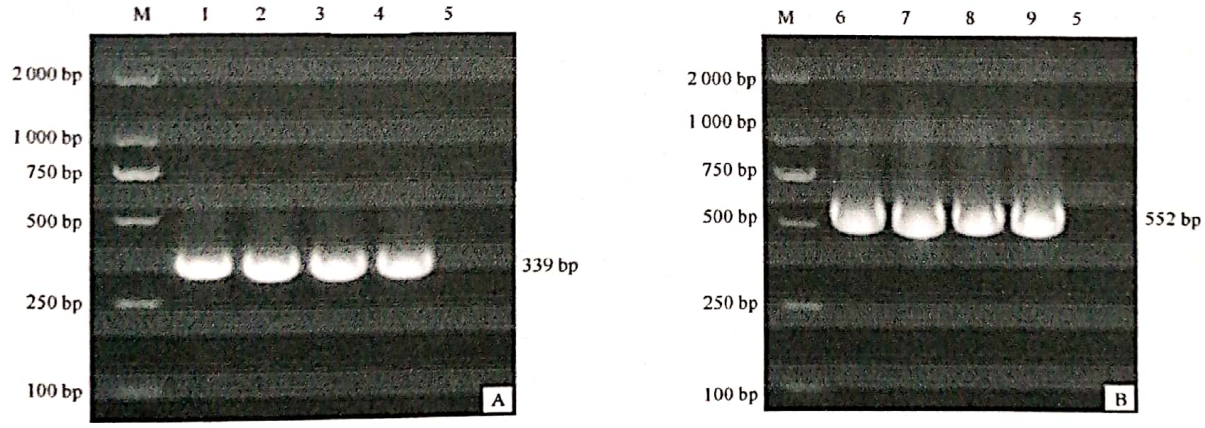
A. Clone vector of *IL-6*；B. Clone vector of *TGF-β1*. M. 2000DL Marker；1，4. Positive colony；2，3. False positive colony；5. Negative control；6－9. Positive colony

图 2　奶山羊 *IL-6* 与 *TGF-β1* 基因克隆载体的 PCR 鉴定

Fig. 2　PCR of dairy goat *IL-6* and *TGF-β1* colony

2.3　*IL-6* 与 *TGF-β1* 基因原核表达载体的鉴定

pET-32a-IL-6 表达载体经 PCR 鉴定获得了 339 bp 的去信号肽 *IL-6* 目的片段(图 3-A)，pET-32a-TGF-β1 表达载体经 PCR 鉴定获得了 552 bp 的去信号肽 *TGF-β1* 目的片段(图 3-B)。测序鉴定结果表明获得了预期长度的序列。上述结果表明，*IL-6* 和 *TGF-β1* 基因片段已成功插入到表达载体 pET-32a 中，获得了碱基序列和片段长度与预期结果完全一致的表达载体，pET-32a-IL-6 和 pET-32a-TGF-β1 表达载体构建成功。



A. *IL-6* 表达载体；B. *TGF-β1* 表达载体。M. 2000DL Marker；1～4. 阳性菌落；5. 阴性对照；6～9. 阳性菌落

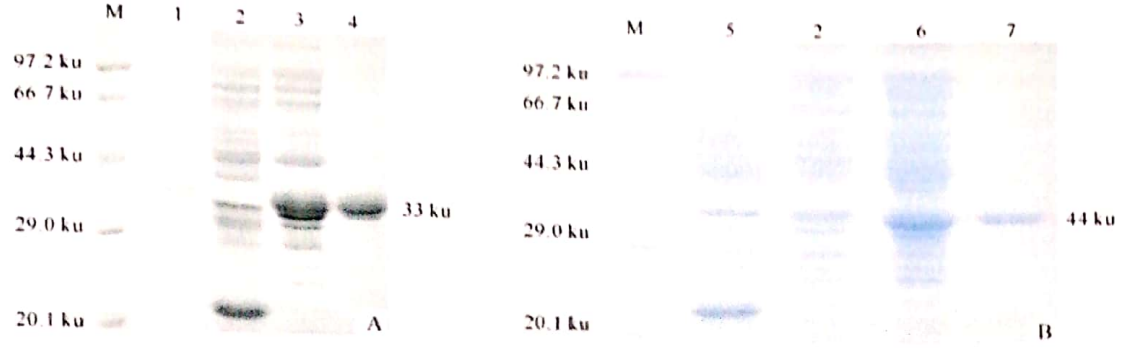
A. Expression vector of *IL-6*；B. Expression vector of *TGF-β1*. M. 2000DL Marker；1－4. Positive colony；5. Negative control；6－9. Positive colony

图 3　奶山羊 *IL-6* 与 *TGF-β1* 基因表达载体的 PCR 鉴定

Fig. 3　PCR identification of *IL-6* and *TGF-β1* gene expression vector in dairy goat

2.4　IL-6 与 TGF-β1 重组蛋白的表达与纯化

SDS-PAGE 鉴定结果(图 4)显示，IL-6 与 TGF-β1 诱导表达产物分别在分子质量约 33 和 44 ku 处出现目的条带，与预期结果一致，表明重组菌能够表达出奶山羊 IL-6 和 TGF-β1 融合蛋白；纯化结果(图 4)表明，获得了较纯的 IL-6 和 TGF-β1 融合蛋白。



M. 蛋白 Marker;1. 未诱导的 pET-32a-IL-6 重组菌;2. 诱导的 pET-32a 空载体;3. 诱导的 pET-32a-IL-6 重组菌;4. 纯化的 IL-6 重组蛋白;5. 未诱导的 pET-32a-TGF-β1 重组菌;6. 诱导的 pET-32a-TGF-β1 重组菌;7. 纯化的 TGF-β1 重组蛋白

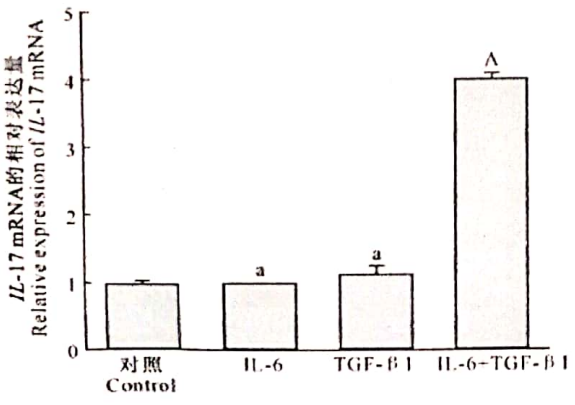
M. Protein Marker;1. Uninduced pET-32a-IL-6 recombinant bacteria;2. Induced empty vector pET-32a;3. Induced pET-32a-IL-6 recombinant bacteria;4. IL-6 purified protein;5. Uninduced pET-32a-TGF-β1 recombinant bacteria;6. Induced pET-32a-TGF-β1 recombinant bacteria;7. TGF-β1 purified protein

图4 重组 IL-6 蛋白(A)和 TGF-β1 蛋白(B)的诱导表达与纯化

Fig. 4 Induced expression and purification of recombinant IL-6 protein (A) and TGF-β1 protein (B)

2.5 IL-6 与 TGF-β1 重组蛋白对 PBMCs 的体外诱导作用

从 real-time PCR 结果(图2)可以看出,用重组蛋白 IL-6 和 TGF-β1 分别单独刺激奶山羊 PBMCs,均不能使 *IL-17* mRNA 相对表达量升高,而 TGF-β1 和 IL-6 共刺激则可使 *IL-17* mRNA 相对表达量极显著升高,表明制备出了可明显促进 PBMCs 的 *IL-17* mRNA 表达水平升高的奶山羊 IL-6 和 TGF-β1 重组蛋白。



与对照组相比,图柱上标 a 表示差异不显著($P>0.05$),标 A 表示差异极显著($P<0.01$)

Compared with the control group, a indicates insignificant difference ($P>0.05$), and A indicates extremely significant difference ($P<0.01$)

图5 IL-6 与 TGF-β1 联合刺激对 PBMCs *IL-17* mRNA 相对表达量的影响

Fig. 5 Effect of IL-6 and TGF-β1 stimulation on relative expression of *IL-17* mRNA in PBMCs

3 讨 论

IL-6 和 TGF-β 是机体免疫反应过程中非常重要的2种细胞因子[18-20]。IL-6 主要由纤维母细胞、单核/巨噬细胞、T 淋巴细胞、B 淋巴细胞、上皮细胞等分泌,在 T、B 淋巴细胞和单核细胞的分化增殖以及抗感染反应、炎症反应等过程中发挥着重要作用[21-23]。TGF-β 可由机体多种细胞分泌,单独的 TGF-β 在机体免疫调节中发挥着重要作用[24-25],其与 IL-6 协同作用则可诱导 Th17 分化,产生效应 Th17 细胞,分泌 IL-17 从而介导组织局部或者系统性的炎症反应[26-28]。IL-17(IL-17A)是一种重要的促炎性细胞因子,其与带有 IL-17 受体的靶细胞结合,可以激活天然免疫细胞和组织细胞,诱导促炎性细胞因子和趋化因子的分泌,募集大量中性粒细胞到达病原所在组织并清除病原体[12-13]。有充分的研究资料表明,在 TGF-β1 和 IL-6 的共同作用下,小鼠 Th 细胞能够分化形成 Th17 细胞[11-14]。也有研究结果显示,体外培养系统中,只需要 IL-1 和 IL-6 就足以诱导人效应 Th17 细胞的产生[15-16]。但更深入的研究结果揭示,TGF-β 仍是人 Th17 细胞产生必不可少的细胞因子[28-29]。本研究结果表明,原核表达的奶山羊 IL-6 和 TGF-β1 在体外协同作用,具有促进奶山羊 PBMCs 合成产生 *IL-17* mRNA 的效应,且 *IL-17* mRNA 的表达量显著升高,提示原核表达的 IL-6 和 TGF-β1 具有免疫活性。同时也表明,在奶山羊 Th17 细胞的分化中,TGF-β1 和 IL-6 仍属于关键的细胞因子,与小鼠、人 Th17 细胞分化

条件基本一致，这为进一步探讨奶山羊抗感染免疫、炎症性疫病等机理提供了很好的试验材料。

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西北农林科技大学
NORTHWEST A&F UNIVERSITY

本科生成绩单

姓名：倪思璐　　性别：女　　学号：2016011019　　入学时间：2016年9月

学院：动物医学院　　专业：动物医学　　班级：动医1605　　学制：五年

课程名称	成绩	性质	绩点	学分	备注
2016年秋季					
军训	87	必修	3.70	1.0	
食品安全与日常饮食	96	任选	---	1.0	
国防教育-军事理论	97	必修	4.00	1.0	
动物学	83	必修	3.30	2.5	
无机及分析化学	83	必修	3.30	5.0	
中国近现代史纲要	84	必修	3.30	1.5	
大学计算机基础（乙）	77	必修	2.70	2.5	
高等数学（乙）	77	必修	2.70	5.5	
体育 I	84	必修	3.30	1.0	
大学英语 I	73	必修	2.30	3.0	
工程训练（丙）	78	必修	3.00	1.0	
新生研讨课	优秀	限选	---	1.0	
2017年春季					
动物解剖学	66	必修	1.70	3.0	
动物解剖学实验	95	必修	4.00	1.0	
动物解剖学实习	95	必修	4.00	1.0	
有机化学	77	必修	2.70	4.0	
无机及分析化学实验	87	必修	3.70	1.5	
体育 II	89	必修	3.70	1.0	
线性代数 I	76	必修	2.70	2.0	
大学英语 II	72	必修	2.30	3.0	
概率论	73	必修	2.30	2.0	
程序设计基础（VB）	73	必修	2.30	3.0	
思想道德修养与法律基础	91	必修	4.00	2.5	
器械练习与健美	合格	任选	---	1.0	
职业素养提升	良好	任选	---	1.0	
2017年夏季					
思想政治理论课实践	良好	必修	3.70	4.0	
2017年秋季					
大学物理（丙）	94	必修	4.00	3.0	
有机化学实验	88	必修	3.70	2.0	
知识产权法律基础	84	任选	---	1.0	
动物组织胚胎学实验	91	必修	4.00	1.0	
体育III	90	必修	4.00	1.0	
动物组织胚胎学	87	必修	3.70	3.0	
动物生理学实验	95	必修	4.00	1.0	
动物生物化学实验	92	必修	4.00	1.0	
动物生理学	86	必修	3.70	3.0	
动物生物化学	90	必修	4.00	3.0	
大学英语III	81	必修	3.00	3.0	
2018年春季					
大学英语IV	82	必修	3.30	3.0	
大学物理实验（乙）	90	必修	4.00	1.0	
马克思主义基本原理	78	必修	3.00	2.5	
体育IV	92	必修	4.00	1.0	
动物病理生理学	89	必修	3.70	3.0	
兽医微生物学实验	93	必修	4.00	1.0	
兽医药理学实习	90	必修	4.00	1.0	
兽医微生物学	80	必修	3.00	3.0	
兽医微生物学实习	97	必修	4.00	1.0	

课程名称	成绩	性质	绩点	学分	备注
兽医药理学	91	必修	4.00	4.0	
创践-大学生创新创业实务	92	任选	---	1.0	
音乐鉴赏	合格	任选	---	1.0	
形势与政策	及格	必修	1.70	2.0	
2018年秋季					
兽医药剂学●	90	限选	4.00	2.0	
兽医免疫学	90	限选	4.00	3.0	
毛泽东思想和中国特色社会主义理论体系概论	85	必修	3.70	3.5	
创业人生	97	任选	---	1.0	
情绪管理	93	任选	---	1.0	
动物病理解剖学实习	94	必修	4.00	1.0	
兽医临床诊断学实习	94	必修	4.00	1.0	
动物病理解剖学实验	83	必修	3.30	1.0	
动物病理解剖学	89	必修	3.70	3.0	
兽医临床诊断学	87	必修	3.70	3.5	
2019年春季					
兽医内科学	92	限选	4.00	3.5	
兽医外科手术学	92	限选	4.00	1.0	
兽医法规	92	限选	4.00	1.0	
兽医外科学	81	限选	3.00	2.5	
兽医外科手术学实验	97	限选	4.00	1.0	
中兽医学	79	限选	3.00	3.5	
畜牧学概论	90	限选	4.00	3.0	
兽医外科手术学实习	85	必修	3.70	1.0	
生物统计学	81	必修	3.00	2.0	
2019年秋季					
兽医产科学※	87	限选	3.70	3.0	
兽医寄生虫学实验	96	限选	4.00	1.0	
兽医寄生虫学※	88	限选	3.70	2.5	
动物性食品卫生学实习	97	必修	4.00	1.0	
动物性食品卫生学	84	限选	3.30	3.0	
兽医传染病学实习	97	必修	4.00	2.0	
兽医寄生虫学实习	95	必修	4.00	1.0	
兽医传染病学实验	96	限选	4.00	1.0	
兽医传染病学	86	限选	3.70	3.5	
大学生创业导论	93	任选	---	1.0	
2020年春季					
动物中毒病与毒理学	98	限选	4.00	2.0	
兽医流行病学	95	限选	4.00	2.0	
兽医临床病理学	93	限选	4.00	2.0	
兽医生物制品学	87	限选	3.70	2.0	
以下空白					

应修学分：200	已获学分：168	学分成绩：85.28	GPA：3.43

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2020年9月21日

成绩审核专用章

高等学校国家级实验教学示范中心联席会

NATIONAL DEMONSTRATION CENTER FOR EXPERIMENTAL EDUCATION

证 书

为表彰第五届全国大学生生命科学创新创业大赛优秀成果奖获得者，特颁发此证书。

作品名称：在乳房炎患羊的乳品质量检测中羊乳αs1-酪蛋白单抗的应用研究

获 奖 者：庞明　管雄　左琛香　倪思璐　解雁飞

学　　校：西北农林科技大学

获奖等级：二等奖(创新类)

证书编号：NDC2020CXCY00519

教育部高等学校生物技术、生物工程类专业教学指导委员会

教育部高等学校食品科学与工程类专业教学指导委员会

高等学校国家级实验教学示范中心联席会

《高校生物学教学研究》编辑部

2020 年 8 月 23 日