Monocyte Derived Dendritic Cells: Sentinels and Translators of Immune Response to *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a versatile opportunistic pathogen causing a wide spectrum of diseases in both humans and animals. My research focused on characterization of the immune responses of monocyte derived dendritic cells (DC) to *S. aureus*. We initially evaluated the potential of circulating monocytes to serve as precursors for DC during *S. aureus* infection. The $CD14^{+}$ monocytes, when stimulated with irradiated (ISA) or live *S. aureus* (LSA), differentiated into $CD11c^{high}$ $CD11b^{high}$ DC (MonoDC) in an autocrine fashion. This was associated with the up-regulation of granulocyte-macrophage colony stimulating factor (GMCSF) and tumor necrosis factor-α; (TNF-α;) gene transcription. We continued our studies to identify the role of TNF-α; in the LSA induced differentiation of monocyte to MonoDC. Blocking TNF-α; reduced the expression of CD11c and increased the expression of CD14 on LSA stimulated monocyte derived MonoDC. Stimulated monocytes were able to secrete monocyte chemotactic protein-1 (MCP-1), a chemokine that recruits monocytes to the site of infection/injury and induces the expression of β_2 integrins on DC. Characterization of the response of DC derived from monocytes using GMCSF and IL-4 revealed that, intact *S. aureus* rather than its purified structural components were efficient in DC activation. In response to ISA or LSA stimulation, DC induced proliferation of T cells collected from the peripheral circulation of cows with a history of *S. aureus* mastitis. Subsequent characterization of the proliferating T cells identified the presence of memory T cells. Finally we identified a unique population of $DEC205^{+}CD8^{\alpha+}$

DC in monocyte derived DC. We further elucidated the role of DC DEC205, a C-type lectin, in *S. aureus* uptake. Blocking of receptor mediated endocytosis resulted in reduced uptake of *S. aureus* by DC. Confocal microscopy confirmed a role for DEC205 in *S. aureus* internalization and delivery to endosomes. DEC205 DC upon stimulation with *S. aureus* displayed enhanced maturation and antigen presentation. In conclusion, monocyte derived DC can uptake *S. aureus* and elicit cell mediated immune responses.

DEDICATION

Dedicated to my husband Gopakumar Moorkanat

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LIST OF ABBREVIATIONS

Antigen Presenting Cells	APC
Carboxy Fluorescein Succinimidyl Ester	CFSE
C-C Chemokine Ligand 2	CCL2
C-C Chemokine Receptor Type 2	CCR2
C-C Chemokine Receptor Type 7	CCR7
Cluster of Differentiation	CD
Cell Mediated Immunity	CMI
C-Type Lectin Receptors	CLRs
Cytotoxic T lymphocyte	CTL
Dendritic Cells	DC
Fluorescent Activated Cell Sorting	FACS
Granulocyte- Macrophage Colony Stimulating factor	GMCSF
Interferon	IFN
Interleukin	IL
Irradiated *S. aureus*	ISA
Langerhans Cell	LC
Lipopolysaccharide	LPS
Lipoteichoic Acid	LTA
Live *S. aureus*	LSA
Macrophage Colony Stimulating factor	MCSF
Mean Fluorescent Intensity	MFI
Methicillin Resistant *S. aureus*	MRSA
Monocyte Chemotactic Protein-1	MCP-1
S. aureus Stimulated Monocyte Derived Dendritic Cell	MonoDC
Macrophage Mannose Receptor	MMR
Nucleotide Oligomerization Domain	NOD
Pathogen Associated Molecular Patterns	PAMPs
Peripheral Blood Mononuclear Cells	PBMC
Pattern Recognition Receptors	PRRs
Peptidoglycan	PGN
Th cells	T helper cells
T Regulatory cells	T Reg cells
Tumor Necrosis Factor - alpha	TNF-α
TNF-α Convertase Enzyme	TACE
Toll-like Receptor	TLR
Vancomycin Resistant *S. aureus*	VRSA

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CHAPTER 1 REVIEW OF LITERATURE

OVERVIEW

Staphylococcus aureus is a major pathogen causing a variety of diseases in both humans and animals [1]. *S. aureus* causes diseases such as superficial skin infections, septic arthritis, osteomyelitis, endocarditis, pneumonia, toxic shock syndrome and septicemia in humans. In dairy cows, it is one of the organisms that cause acute and chronic mastitis [2, 3]. There is an increasing incidence of community acquired *S. aureus* infections in hospitals. Hospital acquired infections are difficult to treat because of the emergence of multidrug resistant strains such as methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) [1, 4]. Emerging virulent strains associated with both severe community-acquired human infections and MRSA mastitis in bovines highlights the occurrence of transfer of strains between humans and animals [5, 6]. This increased threat to both public health and animal populations warrants the need of an animal model system to study host response to *S. aureus* infection.

STAPHYLOCOCCUS AUREUS

S. aureus is a $Gram^+$, nonmotile, nonsporulating, facultative anaerobic coccus (1µm in diameter) usually seen as clusters (bunch of grape) under the microscope and producing hemolysis on blood agar plates. The pathogen is catalase and coagulase positive. Colonies appear as golden yellow due to the presence of a pigment called staphyloxanthin; however, nonpigmented strains are also common.

IMMUNE SYSTEM RECOGNITION

S. aureus colonizes the skin and respiratory tract and any breach in the skin or mucus membrane predisposes the host for an invasive disease [7]. *S. aureus* is considered a classical

extracellular pathogen; however, intracellular infections have been demonstrated [7, 8]. The intracellular persistence in endothelial cells, epithelial cells, and keratinocytes protects *S. aureus* from host immune defenses and antibiotics, adding to the incidence of recurrent and chronic infections [7, 9].

S. aureus is known to produce a variety of toxins and immunomodulatory proteins that possess the ability to evade the innate and adaptive immune mechanisms of the host [10]. Structural components of *S. aureus* include peptidoglycan (PGN), lipoproteins, and lipotechoic acid (LTA) which are often involved in causing septic shock [11] . These pathogen associated molecular patterns (PAMPs) are recognized through host pattern recognition receptors (PRRs), mainly through Toll- like receptor (TLR) 2 [12-14]. *S. aureus* LTA is known to induce the secretion of cytokines and chemokines from monocytes, macrophages and dendritic cells (DC) [15-17]. *S. aureus* PGN also stimulates cytokine and chemokine secretion from innate cells [18, 19]; however, large quantities (10-100µg/ml) are required to induce responses [20].

Previous exposure to *S. aureus* results in less severe subsequent infections and patients with high titer of *S. aureus* specific antibodies are less susceptible to infections (reviewed in [21]). These studies point to the fact that an effective immunological therapy and/or vaccination against *S. aureus* infections in humans and animals is possible [22]. Several studies tested the efficacy of different *S. aureus* vaccines in humans and rodents, but to date, none have provided complete protection [23, 24]. In cows, several studies used toxoid, or bacterin or capsular polysaccharides in vaccines; however, none of the vaccines were found to provide complete protection against *S. aureus* infection [25, 26]. Emerging multidrug resistant strains and the contagious nature of the pathogen in both humans and animals warrant the need of a multicomponent vaccine that provides complete protection against *S. aureus*. As an alternative

approach, DC are being used as biological adjuvants in vaccines to hasten the T cell memory [27].

VERTEBRATE IMMUNE SYSTEM

Living organisms are constantly exposed to various microbes that are present in their environment and need to deal with the invasion of these microbes into the body. A healthy immune system is marked by an ability to distinguish between self and non-self. Immunity can be divided into innate, providing the first line of defense against pathogens, and acquired immunity, often a slow process but specifically mediated through T and B cells [28]. Adaptive immunity displays the remarkable property of memory. Recently, it is considered that the innate immune system also possesses specificity and ability to discriminate between self and foreign antigens [29]. Adaptive immunity is further classified into cell- mediated immunity (CMI) and humoral immunity. CMI is largely mediated by T cells and humoral immunity by B cells. T cells possess a unique antigen binding receptor molecule on cell surface called T cell receptor (TCR). The majority of TCRs recognize antigenic peptides bound to major histocompatibility complex (MHC) derived molecules. T Helper cells (CD4 + cells) recognize processed foreign peptide complexed with MHC class II molecules on the surface of antigen presenting cells (APC) and elicit immune response by secreting cytokines that stimulate CD8 T cells and B cells [30]. Cytotoxic or killer T cells ($CD8^+$ cells) engage with other cells that carry processed foreign peptide complexed with MHC class I molecules on their surface [31]. The CMI also include regulatory T cells (T Reg) which inhibit the production of CD8 T cells once they are not needed, and the memory T cells that are programmed to recognize and respond to a pathogen once it has invaded and been removed. Humoral immunity is mediated through immunoglobulins secreted by the stimulated B cells / plasma cells [32].

Lymphocytes are the core cells of the immune system and play an important role in acquired immunity. They exist as different subsets that differ in their function and protein products. DC, monocytes / macrophages, and B-lymphocytes are the professional APC that express MHC class II receptor, whose function is to present processed antigenic peptides to CD4 T cells.

MONOCYTE DERIVED INFLAMMATORY DC DEVELOPMENT

Monocytes originate from myeloid progenitors, and serve as precursors for tissue macrophages and DC [33]. Circulating monocytes, tissue macrophages, and DC recognize PAMPs from various pathogens through PRRs, such as TLRs, nucleotide oligomerization domains (NODs) and C-type lectin receptors (CLRs) resulting in their activation [34, 35]. On extravasation, monocytes become macrophages or DC depending on stimuli and the cytokines present at the site of infection [36]. Activation of phagocytic cells induces secretion of proinflammatory cytokines, growth factors, and chemokines that recruit additional inflammatory cells to the site of infection resulting in pathogen clearance [37]. Important proinflammatory cytokines include TNF-α, IL-1β and IL-6. The cytokine, TNF-α suppresses expression of the macrophage colony stimulating factor (MCSF) receptor and directs the differentiation of monocytes into DC rather than to macrophages [38]. The growth factor, granulocyte-macrophage colony stimulating factor (GMCSF) in combination with IL-4 strongly up-regulate TNF-α convertase enzyme (TACE) expression and activity in monocytes [39]. TACE is a type 1 transmembrane metalloproteinase that is required for the activation of pro- TNF-α [40]. The function of TACE is to shed ectodomains of membrane-bound proteins such as cytokines, chemokines, growth factors, receptors or adhesion molecules [40, 41]. The chemokine ligand 2 (CCL2) or monocyte chemotactic protein-1(MCP-1) is secreted on stimulation by monocytes and

other innate cells [42] . In response to CCR2-CCL2 interaction, monocytes traffic to the sites of microbial infection [43]. There, monocytes differentiate into macrophages or DC to curtail the infection by phagocytizing and killing the pathogens. Thus, monocytes along with neutrophils form an integral part of innate immune system and play a key role in early containment of infections.

S. aureus expresses leukotoxins and leukocidins that lyse host leukocyte cell membranes [44]. Staphopain B (a staphylococcal cysteine proteinase), in the presence of staphylococcal protein A, may reduce the number of functional phagocytes thereby enabling colonization at infection sites and dissemination of *S. aureus* [45]. Even though phagocytes are recruited to the site of infection, a protective CMI may not be elicited due to the death of neutrophils and monocytes.

Previous research has shown that peripheral blood mononuclear cells (PBMC) stimulated with *S. aureus* superantigens differentiated into a DC phenotype [46]; however, the exact mechanism or the precursor cells that differentiated into DC phenotype was not addressed. Most related studies used *S. aureus* superantigens to study the effect on host immune cells, but, limited information is available on the interaction of whole *S. aureus* with monocytes. The mechanism of differentiation of monocytes to DC in *S. aureus* infection remains elusive. Therefore, we hypothesized that monocytes stimulated with *S. aureus* secrete GMCSF and TNF-α resulting in autocrine stimulation and differentiation into DC.

DENDRITIC CELLS

DC, along with the epithelial cells are considered the sentinels of immune system. DC bridge the innate and adaptive immune system and were initially described in 1973 [47]. They are considered as the professionals of APC because of their unique characteristic of stimulating naïve T

cells [48]. The DC system is formed of large subsets derived from both lymphoid and myeloid progenitors. The exact mechanism of development of DC is not fully known. There are different types of DC subsets which perform specialized immune functions depending upon their location [49]. Migratory DC residing in an immature state at the sites of potential pathogen entries constantly patrol the environment for invading pathogens. Immature DC residing in tissues (eg. Langerhans cells (LC) of the epidermis) or in peripheral blood mature once they encounter an antigen. The mature DC migrates towards the regional lymph node where they present the antigenic peptide through MHC molecules to functional T cells to elicit immune responses [50]. On the other hand, the lymphoid tissue resident DC such as in thymus and spleen do not migrate, and, instead, sample and present antigens in their residence itself [51].

Monocytes ($CD14^+$) and early hematopoetic progenitor cells ($CD34^+$) have the potential to differentiate into DC when cultured with GM-CSF, but the exact regulatory mechanisms of this differentiation is not known [52, 53]. Several studies have shown that mice deficient in MCSF are also deficient in monocytes and skin Langerhans cells. This information points to the fact that migratory DC could be derived from monocytes stimulated with GMCSF and IL-4.

Immature DC constantly patrol for invading pathogens at the common sites of potential pathogen entry [54-56]. The PRR like TLRs and IL-1 receptor family recognize the PAMPs present on the pathogens. Activation through TLRs trigger DC maturation and thereby modulate the adaptive immune responses. This program of maturation of DC brings about the up- regulation of MHC class II [57] and co-stimulatory molecules CD80 and CD86 [58], and expression of CCR7 [59]. The mature DC become more efficient in antigen presentation, while less efficient in phagocytosis [57]. In addition, mature DC have the ability to activate naïve T cells than immature DC. Tissue DC like LC of the skin migrate to the local lymph node during the process of

maturation and present the antigen to the naïve T cells. Since naïve T cells do not traffic to peripheral tissues like skin, migration of DC is very important for the proper immune response. The expression of CCR7 on matured DC facilitates the migration of DC to lymph node [60]. Immature DC activate naïve T cells that regulate different tolerogenic mechanisms including T cell deletion, unresponsiveness and generation of regulatory T cells [61]. Depending upon the stimuli received from the pathogen DC undergo maturation to induce immunity, tolerance or become sessile whereas immature DC induce only tolerance [62].

Naïve T cell activation requires signals from the co-stimulatory molecules and cytokines provided by the mature DC [63]. There are three phases in T cell priming following contact with a DC-MHC II-antigenic peptide complex [64]. Phase one is the contact of T cells with antigen loaded DC and subsequent up-regulation of its activation marker, CD69, depending on the threshold of antigen. Phase two is characterized by further activation of CD69 and onset of IL-2 and interferon-γ (IFN-γ) secretion. In phase three, transient DC- T cell interaction occurs followed by the induction of T cell proliferation.

Bovine monocyte derived DC are characterized by increased expression of MHC class II, CD11c, co-stimulatory molecules CD80 and CD86 and decreased expression of CD14, and CD21 surface markers [65]. The relative expressions of various markers on monocyte and monocyte derived DC are depicted (Table 1.1). CD80 and CD86 are the co-stimulatory molecules present on the antigen presenting cells which interact with the CD28 (stimulatory) and CTLA-4 (inhibitory) receptors of the T cell. The absence of CD80 and CD86 results in lack of co-stimulatory signal delivery to T cells and leads to clonal anergy and lack of proper T cell response [66]. CTLA-4 is the inhibitory molecule present on T cells and interacts with B7 molecules resulting in the down regulation of activated T cells [67]. CTLA-4 inhibits T cell

activation and induces T cell anergy by competitive antagonism of CD28:B7 mediated costimulation; however, the complete absence of CTLA-4 results in unrestricted activation of T cells [68].

The chemokine receptor CCR7 plays a major role in the localization of antigen specific T cells and antigen loaded DC in the lymph nodes [69]. CCR7 is expressed primarily on naïve T cells, central memory T cells, mature DC and mature B cells which frequently move to the secondary lymphoid tissues [70]. Absence of CCR7 results in the absence of a T cell response [71].

DC tightly regulate their ability to induce effector T cells by secreting cytokines. Under the influence of cytokines from DC, CD4 helper T cells (Th cells) polarize to a Th1, Th2, Th17 or a T regulatory (T Reg) type [72]. DC also stimulate CTLs. The T helper cell classification based on cytokine signature, transcription factor and function is shown (Figure 1. 1). Th1 lineage is shaped under the influence of IL-12 and IL-18. The effector cytokines in Th1 response are IFNγ and TNF-β [73]. Th2 lineage is formed when the polarizing cytokine is IL-4. The major cytokines secreted by Th2 cells are IL-4, IL-5 and IL-13 [74]. The IL-17 expressing T cells which are known as Th 17 cells, have been described recently as a third lineage of T helper cells and are formed when naïve T cells are exposed to TGF-β and IL-6 [75, 76]. The Th 17 cells have been identified as important effector cells in many infections characterized by their pro inflammatory function. IL-23 is required for the maintenance of Th17 cells [77]. However, the transcriptional and cytokine mediated regulation of T_H 17 cell differentiation and effector functions is not fully known [75].

DEC205^{+ve}/CD205^{+ve} DC

The DC are professional APC capable of initiating naïve T cell activation. Immature DC express a wide variety of receptors on their surface [54] including phagocytic receptors, CLRs, PRRs and scavenger receptors. Several reports indicate that targeting antigens to DC receptors increases antigen presentation to CD4 and CD8 T cells *in vivo* [78-81]. DEC205/CD205 is a CLR that can function as an endocytic receptor [82]; however, the ligand for this receptor is waiting to be identified. The CLRs are Ca^{++} dependent glycan-binding proteins that internalize their ligands through clathrin coated pits resulting in the delivery of ligands to lysosomes or late MHC-II rich endosomes [83]. Targeting the endocytic receptor DEC205, improves the efficiency of T cell vaccination [79]. Targeting protein antigens to DEC205 increases antigen presentation on MHC I and II [83, 84]. Directing peptide antigen to DEC205 receptors results in an initial increase in CD4 and CD8 T cell proliferation i*n vivo*, followed by a state of tolerance in the absence of DC maturation [84, 85]. However, in the presence of maturation stimuli, targeting protein antigens to DEC205 improves T cell vaccination [84].

DEC205^{+ve} DC can uptake and process protein antigens such as ovalbumin, thereby inducing a strong CD4 and CD8 T cell response [86, 87]; however, the pathogenic ligands for DEC205 or the signaling proteins involved are not yet described [88]. CLRs such as mannose binding lectins have been shown to coordinate TLR2 and TLR6 binding to *S. aureus* [89]. However, there are no reports indicating the role of DEC205 in *S. aureus* uptake by DC. The role of DEC205 in *S. aureus* infection needs to be described. We hypothesized that *S. aureus* uses DEC205 for its internalization, induces DC maturation and facilitate antigen presentation by up regulating MHC expression.

The functional outcomes of DC-T cell interactions are critical in the differentiation of an effective T cell memory and protective immunity. Here we hypothesize that understanding the signals and molecules involved in DC-T cell interactions in response to *Staphylococcus aureus* infection might be useful for the design of a successful vaccine.

Table 1.1. Relative expression of markers on monocytes and DC

Markers	Monocytes	Monocyte derived Mature DC
CD14	+++	+ (Low level of expression)
MHCII	+++	+++ (MFI moderate-high)
CD11c	+	+++ (MFI moderate-high)
CD11b	+++	++ (High on inflammatory DC)
CD205	+	++ (less on monocyte derived DC)
CD80	+	+++ (relatively high on DC)
CD86	++	+++ (High expression)

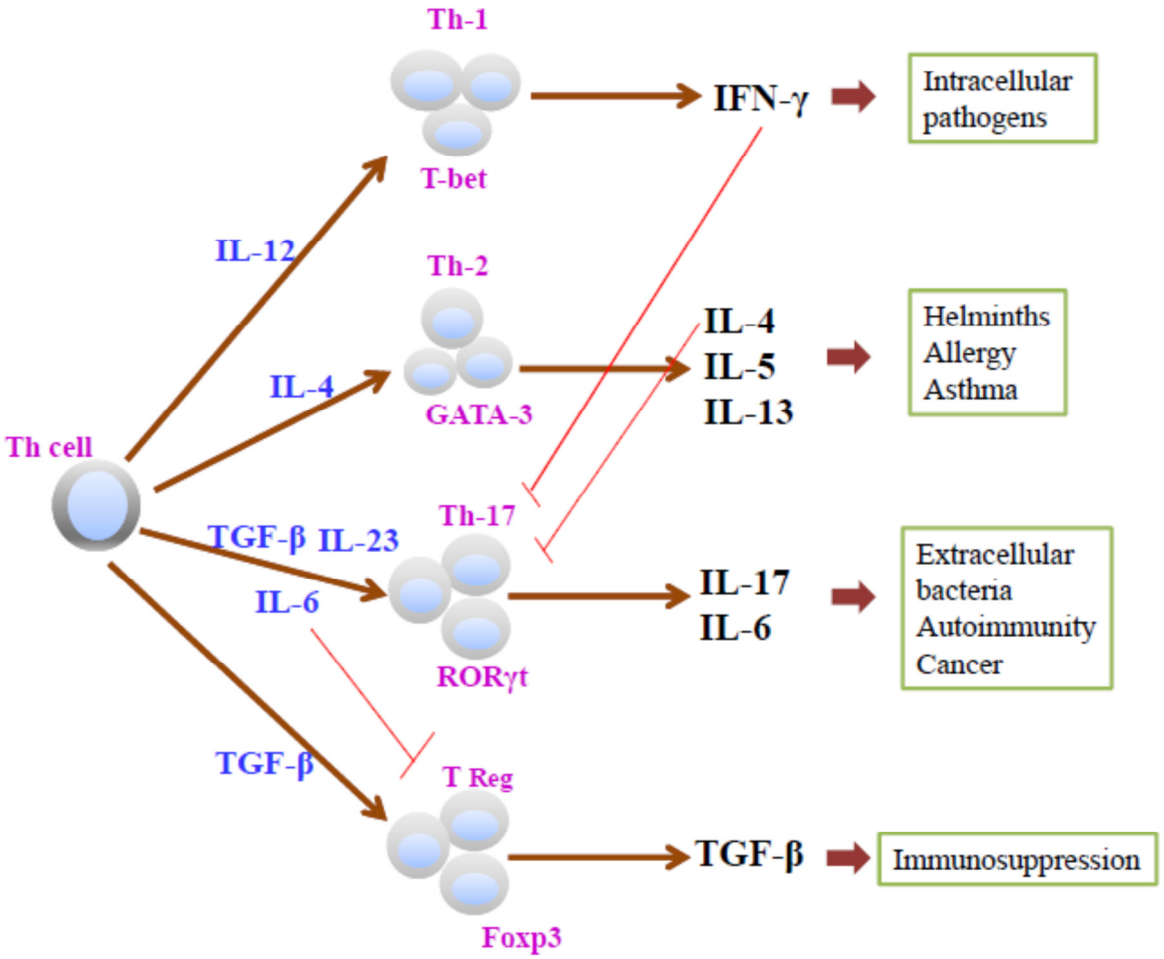

Figure 1.1. T helper cell classification based on their cytokine signature, transcription factor and function. Antigen presenting cells esp. dendritic cells activate T cells and regulate their differentiation into T helper cell type 1 (Th1)/ or Th2/ Th17/TReg cells depending upon the cytokine. The fully-differentiated T cell subtypes secrete specific cytokines that play positive roles in the protecting the host against pathogens (viruses, bacteria, parasites), and negative roles in inducing inflammation or autoimmunity.

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CHAPTER 2 *STAPHYLOCOCCUS AUREUS* INDUCES TNF-α AND GMCSF SECRETION BY MONOCYTES AND STIMULATES DENDRITIC CELL DIFFERENTIATION

ABSTRACT

Monocytes originate from myeloid precursors and circulate in the blood. They are considered precursors for tissue macrophages and dendritic cells (DC). We hypothesized that following uptake of *S. aureus*, monocytes secrete granulocyte-macrophage colony stimulating factor (GMCSF) and tumor necrosis factor-α (TNF-α) resulting in autocrine stimulation and subsequent differentiation of these cells into DC (MonoDC). The objectives of this study were to determine the role of monocytes and their secreted cytokines in the process of MonoDC differentiation during *S. aureus* infection. This study used bead purified bovine $CD14^{+}$ peripheral blood monocytes and *S. aureus* strain RN6390B. *S. aureus* uptake by monocytes was assessed by flow cytometry following infection with FITC labeled *S. aureus* at multiplicity of infection (MOI) 10 and 25. As the MOI increased, the uptake of *S. aureus* by monocytes also increased significantly indicated by an increase in the percentage of $FITC^{+ve}$ monocytes. Real time PCR indicated a significant increase in the gene expression of TNF-α and GMCSF after 24 and 48h of incubation. Protein concentrations of TNF-α and GMCSF in the supernatants of stimulated monocytes were confirmed by western blot. ELISA results showed that *S. aureus* stimulation significantly increased chemokine ligand 2 (CCL2) in culture supernatants compared to unstimulated monocytes. To determine differentiation capacity of *S. aureus* stimulated monocytes to MonoDC, monocytes were infected with *S. aureus* (MOI 10) for 2h. Cells were washed extensively and fresh monocyte medium without any exogenous cytokines was added. For confirmation of DC development, monocytes stimulated

with exogenous GMCSF and IL-4, and unstimulated monocytes were used as positive and negative controls, respectively. Phenotypic analysis after 7d of culture indicated a distinct DC morphology similar to positive controls with high level of expression of DC markers CD11b, CD11c, and MHC II and low level expression of monocyte marker CD14 in *S. aureus* stimulated monocytes. Experiments using TNF-α blocking antibody confirmed a role for TNF-α in *S. aureus* stimulated monocyte differentiation into MonoDC. In conclusion, *S. aureus* stimulated monocytes secreted TNF-α and GMCSF in an autocrine fashion and differentiated into MonoDC in a TNF-α dependent manner.

INTRODUCTION

Monocytes originate from myeloid precursors and circulate in the blood. They are considered precursors for tissue macrophages and DC [1]. Monocyte derived DC are routinely used *in vitro* as models for studying the properties of DC. Circulating monocytes, tissue macrophages, and DC become activated upon binding of pathogen-associated molecular patterns (PAMPs) of various pathogens by pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), nucleotide oligomerization domains (NODs) and C-type lectin receptors (CLRs) [2, 3]. Upon extravasation, monocytes become macrophages or DC depending on stimuli and the cytokines present at the site of infection [4]. Activation of monocytes induces secretion of cytokines, chemokines, and growth factors leading to chemotaxis of more inflammatory cells to the site of infection resulting in pathogen clearance [5]. TNF-α directs the differentiation of monocytes to DC instead of macrophages by suppressing macrophage colony stimulating factor (MCSF) receptor expression [6]. Reports indicate that, in monocytes, GMCSF in combination with IL-4 strongly up-regulates TNF-α convertase enzyme (TACE) expression and activity [7]. TACE, a type 1 transmembrane metalloproteinase is the primary protease required for the activation of pro-TNF-α [8]. TACE causes ectodomain shedding of membrane-bound proteins such as cytokines, chemokines, growth factors, receptors or adhesion molecules [8-10]. One important chemokine produced by stimulated monocytes is the CCL2 or monocyte chemotactic protein-1(MCP-1) [11]. In response to chemokine receptor 2 (CCR2)-CCL2 interaction, monocytes traffic to the sites of microbial infection [4]. There, monocytes differentiate into macrophages or DC and curtail the infection by phagocytizing and killing the pathogens. Therefore, monocytes, along with neutrophils, form an essential part of the innate immune system.

Peripheral blood mononuclear cells (PBMC) stimulated with *S. aureus* superantigens differentiate into MonoDC [12]; however, the exact mechanism or the precursor cells that differentiate into DC phenotype remains unknown. Most studies use *S. aureus* superantigens to study the effect of this pathogen on host immune cells, therefore, limited information is available about the interaction of whole *S. aureus* and monocytes. The mechanism of differentiation of monocytes to DC in *S. aureus* infection remains elusive. We hypothesized that monocytes stimulated with *S. aureus* secrete GMCSF and TNF-α, which induces differentiation into MonoDC in an autocrine manner. The objectives of this study were to determine the role of monocytes and their secreted cytokines in the process of MonoDC differentiation during *S. aureus* infection.

MATERIALS AND METHODS

Animals

Four Holstein Friesian dairy cows from the Virginia Tech Dairy Research Facility were used for this study. All the animals were free from any visible signs of disease at the time of blood collection. As per the history, these animals were never reported to have *S. aureus* mastitis during their lifetime. The animal experiments complied with the ethical and animal experiment regulations of Virginia Tech IACUC.

Propagation and fluorescent labeling of *S. aureus*

Single colonies of RN6390B strain of *S. aureus* were cultured in tryptic soy broth for 4h with rigorous shaking (12400 Incubator Shaker, New Brunswick Scientific CO, Inc.). The cultures were washed three times with Hank's balanced salt solution (HBSS) (Invitrogen, NY, USA), and pelleted at 1500xg for 10 min. Cultures were then serially diluted and drop plated to get the actual colony counts per mL. Bacteria were irradiated in a Model 109 research cobalt irradiator (JL, Shepherd and Asscocites, San Fernando, CA) for 3h. Before use, the irradiated *S. aureus* (ISA) were washed twice with HBSS and diluted to 10^9 CFU/mL with RPMI 1640 medium.

Fluorescent labeling of *S. aureus* was performed with some modifications of the procedure described earlier [13]. Briefly, 10^9 CFU /mL of RN6390B strain *S. aureus* in carbonate bicarbonate buffer were incubated with 100 µg of fluorescein isothiocyanate (FITC) (46425; Thermo scientific, IL, USA) isomer I /mL for 2h in the dark at 37ºC. The cultures were then washed three times with HBSS and analyzed for uniformity of staining by fluorescent microscopy. Labeled cultures were suspended in HBSS and stored at -20ºC until use.

Culturing of monocytes and monocyte derived DC

PBMC were isolated from healthy cows. Briefly, PBMC were isolated from 250 mL of blood drawn from the jugular vein into 250mL K_3-EDTA-vacuum bottles and enriched by discontinuous density gradient centrifugation with the procedure described earlier [14]. Briefly, 10mL of the buffy coat were collected and suspended in 20 mL of 1x HBSS. The suspended buffy coat was layered over Ficoll-Paque ^{TM} plus (GE Healthcare Biosciences AB, Uppsala, Sweden) and centrifuged at 330xg for 45 min at 25ºC. The mononuclear cell layer was removed and washed three times using HBSS. Cell viability and number were determined by Trypan blue exclusion test. The PBMC were incubated with anti-human CD14 microbeads (Miltenyi Biotec, CA, USA) (10uL/10^7 PBMC) for 20 minutes on ice. A positive selection of $CD14^+$ cells was performed by magnetic cell sorting according to manufacturer's instructions. The purity of the cells (> 98%) was assessed by flow cytometry and cell viability was assessed (> 99%) by Trypan blue exclusion.

Purified $CD14^+$ monocytes were infected with ISA or live *S. aureus* (LSA) (MOI 10) for 2h. After 2h of incubation, the cells were washed extensively and fresh monocyte medium with gentamicin was added. On every third d, half of the medium was replaced by fresh media. The cells were cultured for 7-8d and analyzed by flow cytometry for the expression of various DC markers. Unstimulated monocytes and monocytes cultured with recombinant bovine (rb)-GMCSF and rb-IL-4 for 7d were used as negative and positive controls, respectively.

For DC differentiation, CD14 bead purified peripheral blood monocytes were cultured in plastic petri dishes for 6-7d in Rosewell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, NY, USA) containing 10% FBS (Hyclone Labs, UT, USA), 10mM HEPES, 4mM L-glutamine (Invitrogen, New York, USA), $5x10^{-5}$ M 2-betamercaptoethanol (Sigma-Aldrich,

MO, USA), rb GMCSF (200ng/mL) and rb IL-4 (100ng/mL) as described earlier [15]. Media and cytokines were replenished on every third d.

Flow cytometric analysis for surface markers

Fresh monocytes, ISA or LSA stimulated 7d MonoDC and unstimulated DC were stained with anti-bovine MCA1651G (Abd serotec, Raleigh, NC, USA) (IgG2b PE) for DEC205, MM61A (IgG1Texas red) for CD14, TH16B (IgG2a FITC) for MHCII, H58A (IgG2a FITC) for MHCI, MM10A (IgG2b PE) for CD11b, BAQ153A (IgM APC) for CD11c, MUC76A (IgG2a FITC) for CD11a, and BAQ15A (IgM APC) for CD21 (VMRD, Pullman, WA, USA) and assessed by flow cytometry. Briefly, the cell suspensions were stained with primary antibodies followed by incubation for 1h on ice. After three washings, fluorochrome-conjugated isotype specific secondary antibodies (Invitrogen/Caltag lab, New York, USA) were added and incubated for another 30 min on ice followed by three washings. Percentages of cells and mean fluorescence intensity (MFI) were determined using FacsCalibur flow cytometer (BD biosciences, San Jose, CA, USA) and analyzed using Flowjo software v. 7.6.1 (Tree star Inc., Ashland, OR).

Endocytosis Assay

For endocytosis assay, fresh monocytes (2×10^6) were infected with FITC labeled *S. aureus* with different MOI 10, 25, 50 and 100 in antibiotic free media and incubated for 2h at 37ºC. After 2h of incubation, the cells were washed and treated with 100µg lysostaphin (Sigma-Aldrich, MO, USA), for 7 min. Cells were then fixed with 1% formaldehyde and analyzed by flow cytometry for $FITC^{+ve}$ cells and MFI.

Blocking of TNF-α with polyclonal antibody

Monocytes ($2x10^6$) were stimulated with LSA for 2h. After 2h of incubation, cells were washed three times and extracellular bacteria were lysed with lysostaphin. Monocyte medium with rabbit anti-bovine polyclonal TNF-α antibody (5µg/mL) (AbD Serotec) or isotype control was added to the culture. Monocytes stimulated with exogenous GMCSF and IL-4 were used as positive controls. The cultures were replenished with fresh monocyte media every 3d and cultured for 7d. Cells were collected and analyzed for DC cell surface markers by flow cytometry.

Real-Time PCR

Total RNA was extracted from unstimulated, and 24 and 48h ISA and LSA stimulated monocytes using Qiagen's RNeasy Mini Kit with DNase (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. cDNA was prepared from 1 µg RNA using Superscript II Reverse Transcriptase and oligo dT primers (Invitrogen, Carlsbad, California, USA). Bovine specific primers / probes were designed using Primer Express 3 software (Applied Biosystems, USA) as described earlier [16]. The designed primers and probes were purchased from IDT (Integrated DNA Technologies, Inc, USA) (GMCSF forward: 5´ GACACTGATGCTGTGATGAATGAC 3´, reverse: 5´ CAGGCCGTTCTTGTACAGCTT3´, probe- TCCCAGGAACCAACGTGCCTGC; TNF-α- forward: 5´TCTCCTTCCTCCTGGTTGCA3´, reverse: 5´ GTTTGAACCAGAGGGCTGTTG 3´, probe- 5´ CCCAGAGGGAAGAGCAGTCCCCA3´) and we confirmed their specificity to detect a DNA standard for each gene derived from bovine monocytes / DC cDNA by running a standard curve. Real-time (RT)-PCR using primers and Taqman probes (Applied Biosystems, USA) was used to determine the levels of transcription of GMCSF and TNF-α. The RT-PCR reactions were

conducted using the Taqman Universal master mix (Applied Biosystems, USA) and analyzed using ABI Prism 7300 Real-Time PCR System (Applied Biosystems, USA). CT Values were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control and expressed as fold change as calculated by the $2^{-(\Delta\Delta CT)}$ method. Briefly, ΔCT = CT of the target subtracted from the CT of GAPDH, and ΔΔCT=ΔCT of samples for target subtracted from the ΔCT of corresponding control samples. Statistical analysis was performed on ΔCT values and expressed as fold change with respect to unstimulated monocytes.

ELISA for Bovine CCL2

Protein levels of CCL2 in cell culture supernatants from unstimulated or stimulated monocytes was quantified using Bovine CCL2 (MCP-1) VetSet ELISA Development Kit according to manufacturer's protocol (Kingfisher Biotech Inc., St. Paul, MN, USA). Values are expressed in ng/mL of supernatant.

Western blot

Bradford assay was performed to assess the total protein in samples to ensure equal protein loading [17]. The proteins were separated by Novex Midi Gel (Invitrogen Co, CA, USA) electrophoresis at 100V for 90 min and transferred on to a Immobilon-FL PVDF membrane at 60mA for 90 min. The resulting membrane was blocked with Starting Block^{TM} blocking buffer (Thermo Scientific, IL, USA) for 1h. The membrane was incubated with the primary antibody (TNF-α; polyclonal Q06599, AHP852Z, AbD Seortec Raleigh, NC, USA; and GMCSF; GM-CSF 20.1, VMRD, Pullman, WA, USA) at 4ºC overnight in blocking buffer. Immunoreactive proteins were visualized by incubation with goat anti-mouse or anti-rabbit IgG secondary antibody (Bio-Rad) for 1h. The signals were detected with an ODYSSEY Infrared Imaging

System (LI-COR, Lincoln, NE, USA) and the gray value of protein bands quantified with Photoshop (Adobe, San Jose, CA).

Statistical analysis

Statistical analysis was performed by one-way ANOVA, using Graph Pad Prism 5.0 (San Diego, CA, USA). To compare treatment means, Tukey's test was performed. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Monocytes exhibit efficient phagocytic capacity

Compared to neutrophils and macrophages, monocytes show a lower phagocytic response [18]. Phagocytosis related cell responses result in the generation and release of inflammatory mediators. Our first aim was to assess the ability of monocytes to uptake *S. aureus*. Flow cytometric analysis of monocytes infected with FITC labeled *S. aureus* demonstrated that as the MOI increased, the uptake of *S. aureus* by monocytes also increased ($P = 0.02$) indicated by an increase in the percentage of $FITC^{+ve}$ positive monocytes (Figure 2.1A). The number of bacteria per cell was also increased when the MOI was increased from 10 to 25, represented as MFI (Figure 2.1B). Though *S. aureus* uptake increased from MOI 10 to 25, the number of viable cells at MOI 25 was reduced as compared to MOI 10 (data not shown). Within 2h of stimulation, monocytes infected with MOI 50 and 100 were dead and disintegrated suggesting the ability of *S. aureus* toxins to kill leukocytes. Results indicate that monocytes can survive and phagocytize *S. aureus* to elicit an immune response when the level of infection is MOI 10 or lower.

S. aureus stimulated monocytes differentiate in to an inflammatory DC phenotype

During our experiments we observed that cultured monocytes, when stimulated with ISA or LSA were able to differentiate into MonoDC after 7d despite a lack of exogenously added GMCSF or IL-4. After 7d of culture, the MonoDC had similar phenotypic characteristics of the DC differentiated from monocytes with the addition of GMCSF and IL-4. The expression of different markers on 7d monocytes, ISA and LSA stimulated MonoDC are shown (Figure 2.2). The GMCSF and IL-4 differentiated DC (positive control) had a distinct morphology with greater intensity of expression of MHC class II, CD11c, CD11b and CD11a; however, with a

lower expression of CD14 and lack of CD21 expression compared to 7d old unstimulated monocytes.

Increased CCL2 levels in supernatants of monocytes stimulated with ISA or LSA

Stimulated monocytes secrete MCP-1/CCL2 to recruit additional monocytes, memory T cells, and DC to sites of injury and infection. Secreted MCP-1/CCL2 has the ability to induce the expression of β2 integrins on leukocytes [19]. We investigated the presence of MCP-1/CCL2 in 24h cell culture supernatants of monocytes stimulated with ISA or LSA. ELISA results show that both ISA and LSA stimulation significantly increased ($P < 0.0001$) CCL2 in supernatants with respect to unstimulated monocytes (Figure 2.3). Results indicate that *S. aureus* stimulated monocytes have the ability to secrete MCP-1/CCL2, a chemokine, which can attract more monocytes to the site of infection.

GMCSF and TNF-α mRNA and protein levels increase in monocytes stimulated with ISA or LSA.

We analyzed the changes in gene expression and protein levels of GMCSF and TNF-α in *S. aureus* stimulated monocytes. Monocytes stimulated with ISA or LSA up regulated the GMCSF and TNF-α expression after 24 and 48h. ISA or LSA stimulation significantly increased the gene expression of GMCSF (Figure 2.4A) by monocytes at 24h ($P < 0.001$) and 48h ($P < 0.01$) compared to unstimulated cells. Similarly, monocyte transcription of TNF-α mRNA (Figure 2.4B) also increased with ISA or LSA stimulation compared to unstimulated monocytes. Significant increase in TNF-α mRNA induction in ISA or LSA stimulated monocytes was noted at 24h ($P < 0.001$) relative to control. At 48h, although ISA and LSA stimulations increased TNF-α mRNA transcription compared to unstimulated, only ISA stimulation increased TNF-α mRNA ($P < 0.05$) significantly.

To confirm whether the increase in mRNA expression was translated to protein, we conducted Western blot analysis for TNF-α and GMCSF. TNF-α and GMCSF were detected in the supernatants of monocytes stimulated with ISA or LSA (Figure 2.5). Results confirmed that the mRNA expression translated to TNF-α and GMCSF protein in *S. aureus* stimulated monocytes.

TNF-α expression is required for increased expression of CD11c on LSA differentiated MonoDC.

TNF-α is synthesized as a membrane-anchored pro-TNF-α that is released by TACE [9]. To identify a role for TNF-α in the differentiation of LSA stimulated monocytes into MonoDC, we conducted a blocking assay. Flow cytometry analysis revealed that when anti-bovine polyclonal TNF-α antibody was used to block the secreted TNF-α, LSA stimulated 7d MonoDC expressed greater intensity of expression of CD14, the monocyte/macrophage marker and lower intensity of expression of CD11c compared to isotype control or LSA differentiated MonoDC (without any blocking antibody) (Figure 2. 6). Monocytes stimulated with exogenous GMCSF and IL-4 differentiated into DC with greater intensity of expression of CD11c and lower intensity of expression of CD14 compared to unstimulated DC. These results confirm the role of monocyte-derived TNF-α in *S. aureus* induced differentiation of MonoDC.

DISCUSSION

In this study, we report that stimulation with ISA or LSA triggers monocyte differentiation into MonoDC. Several studies have shown that monocytes can be differentiated to DC under the influence of GMCSF and IL-4 in 5-7d [15, 20-23]. Our data confirm that bovine MonoDC possess a higher intensity of expression of CD11b along with other surface markers. This $CD11c^{high}$ $CD11b^{high}$ MonoDC could be a subset of inflammatory DC having a specific function in immune response against *S. aureus* [24]. The $CD11b^{high}$ $CD11c^{high}$ monocyte derived DC represent an inflammatory DC phenotype with high levels of MHC II expression [25]. Several studies have demonstrated that in mice, *in vivo* differentiation of monocytes to DC occurs upon antigen stimulation [26, 27]. It is known that dermal monocyte derived DC are more efficient in contributing to T cell mediated immunity compared to DC differentiated from monocytes in lymph nodes [28]. In a mouse model, $CD11b^{high}$ DC derived from monocytes were recruited to the peripheral non lymphoid tissue in response to inflammation induced memory CD8 T cell activation [29, 30]. Previous studies have shown that monocyte derived DC exert microbicidal activity against *Listeria monocytogenes* [31], induce Th1 response by producing IL-12 [26] and cross prime antigen specific CD8 T cells [32]. Hence, we presume that $CD11b^{high}$ $CD11c^{high}$ DC differentiated from circulating bovine monocytes in response to *S. aureus* stimulation would be efficient in mounting an effective immune response to *S. aureus* infection.

Our preliminary studies showed that there was no difference in the expression levels of surface markers between adhered out monocytes and monocytes isolated using CD14 magnetic beads. In this study, monocytes displayed efficient phagocytosis of *S. aureus*; however, at higher MOI, monocytes were dead and disintegrated, signifying the capacity of *S. aureus* toxins to kill leukocytes. The necrosis of monocytes occurring at and above MOI 25 in this study mimics a

natural acute infection. This could be a reason for not developing a protective immune response during or after *S. aureus* infection. We postulate that recruited monocytes might be undergoing necrosis with the ultimate effect that monocyte mediated DC differentiation is physically inhibited at the site of infection. We assume that monocyte phagocytic responses are of great importance in the initial containment and in the context of initiation of an immune response to *S. aureus* infection.

Previous studies show that irradiation of *S. aureus* reduces production of toxins [33]. Therefore, we used ISA to minimize the effect of toxins on monocytes and eliminate the role of secreted toxins in monocyte differentiation to MonoDC. Our results indicate that phagocytosis-associated monocyte responses resulted in the generation and release of inflammatory mediators such as GMCSF and TNF-α. Our results are consistent with a previous study that reported induction of TNF-α and GMCSF gene expression in monocytes when stimulated with *S. aureus* superantigens [12]. We speculate that upon ISA or LSA stimulation, monocytes secreted GMCSF eventually up-regulated the expression and activity of TACE resulting in the shedding of MCSF membrane receptor thereby driving their differentiation to MonoDC [8, 10]. Increased expression of TACE might have resulted in the cleavage of pro-TNF-α [9]. Blocking experiments confirmed the role of monocyte-derived TNF-α in the differentiation of monocytes to MonoDC. Previous research indicates that TNF-α suppresses MCSF receptor expression on monocytes facilitating DC differentiation [6].

TNF-α is a potent activator of CCL2. MCP-1/CCL2 is specifically involved in the chemotaxis of monocytes, CD4 and γδ T cells [34] and acts as an activator for monocytes [35]. Our results indicate that monocytes can secrete CCL2 upon *S. aureus* stimulation. Secreted CCL2 has the ability to recruit more immune cells to the site of *S. aureus* infection. Raised

levels of CCL2 in *S. aureus* infection may lead to greater monocyte mediated immunity. Previous research has shown that CCL2 can up-regulate the expression of β2 integrins and elicit cytokine secretion by peripheral blood monocytes [19]. In the present study, elevated levels of CCL2 may have up-regulated the expression of β2 integrins, CD11a, CD11b and CD11c in MonoDC.

Recruitment of immune cells such as monocytes and DC is a prerequisite for initiation of specific immune responses. Immuno-adjuvant effect of chemokines could be used at the site of vaccination to enhance recruitment of inflammatory cells, especially, monocytes and immature DC [36]. The potential of monocytes to recruit additional cells, enhance their differentiation into DC and the ability of these inflammatory DC to elicit a protective long lasting immune response could be exploited for formulating *S. aureus* vaccines.

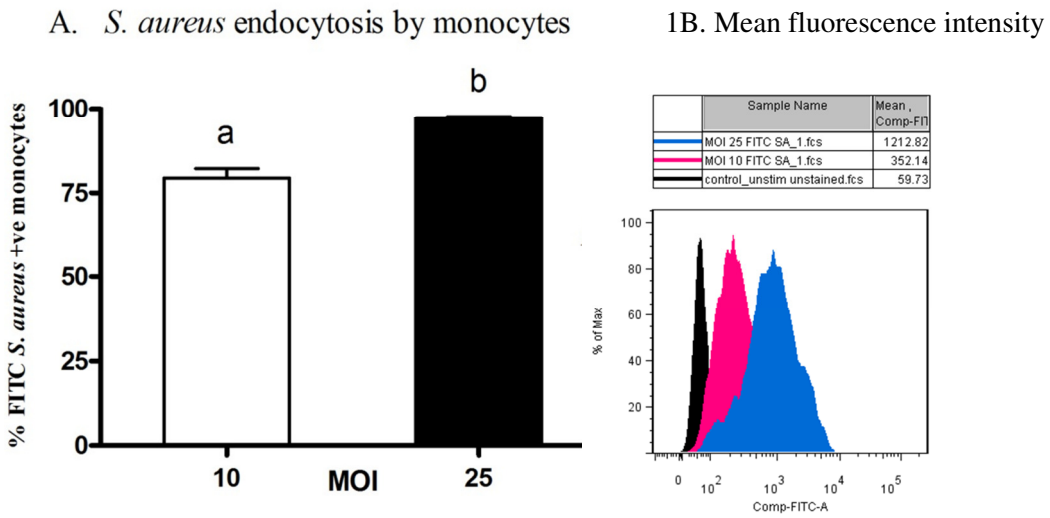

Figure 2.1. *S. aureus* endocytosis by monocytes. $2x10^6$ monocytes (n=3) were infected with FITC labeled *S. aureus* at different MOI for 2h, treated with lysostaphin, fixed and analyzed by flow cytometry. **(A)** Increasing MOI resulted in increased uptake of *S. aureus* by monocytes (P = 0.003). MOIs with different letters significantly differ from each other. Data represent mean $\pm$ SE. **(B)** Histograms showing difference in mean fluorescence intensity of monocytes infected with $FITC^{+ve}$ *S. aureus* at MOI 25 (blue, right), MOI 10 (pink, middle), and uninfected cells (black, left).

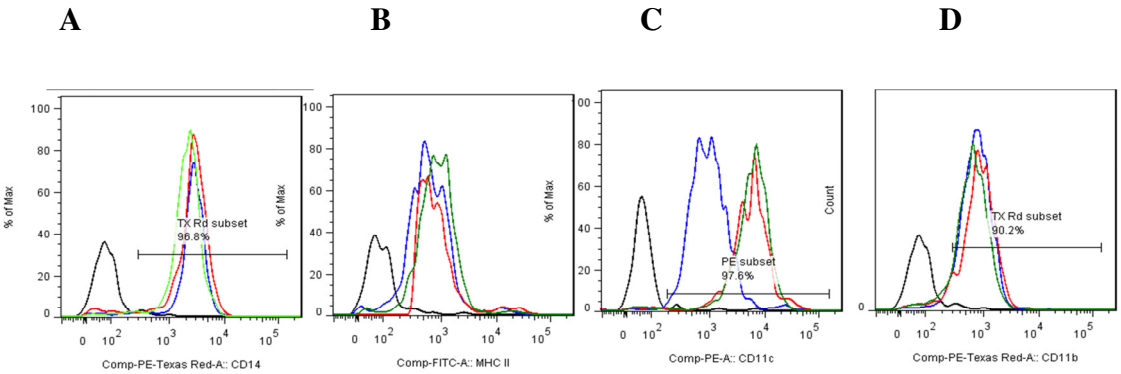

Figure 2.2. *S. aureus* stimulated monocyte differentiation into DC. Monocytes were stimulated (MOI 10) with ISA and LSA for 2h, cultured for 7d in without any exogenous cytokines and analyzed by flow cytometry for surface markers. Black histograms represent unstained, blue histogram denotes 7d cultured monocytes, red and green histogram represents LSA and ISA stimulated MonoDC, respectively immunostained for CD14(A), MHC II (B), CD11c (C) and CD11b (D). *S. aureus* stimulated monocytes shows higher intensity of expression of CD11c, the DC marker compared to unstimulated monocytes. Data representative of 4 independent experiments.

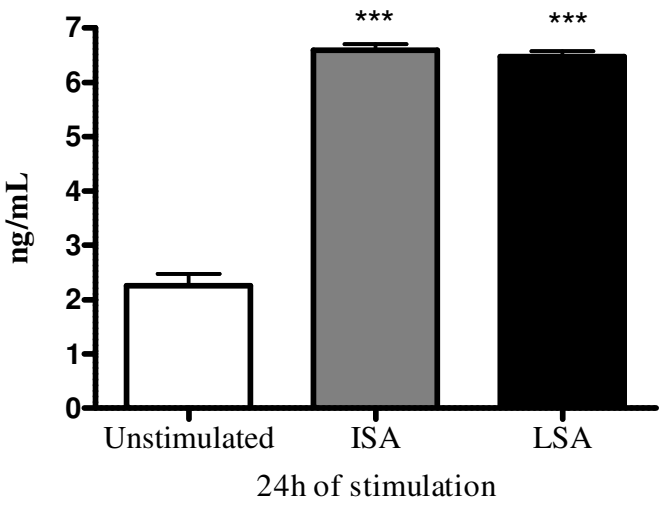

Figure 2.3. CCL2 levels in supernatants of unstimulated, ISA, and LSA loaded monocytes. Monocytes were loaded with ISA and LSA (MOI 10) for 2h. After 2h, cells were washed three times, fresh monocyte media with gentamicin was added and cells were cultured for 24h. CCL2 levels were measured by ELISA in cell culture supernatants. Both ISA and LSA stimulation of monocytes increased (***$P < 0.001$) the levels of CCL2 in supernatants compared to unstimulated monocytes. Results shown represent the mean $\pm$ SE of data from cells of four cows.

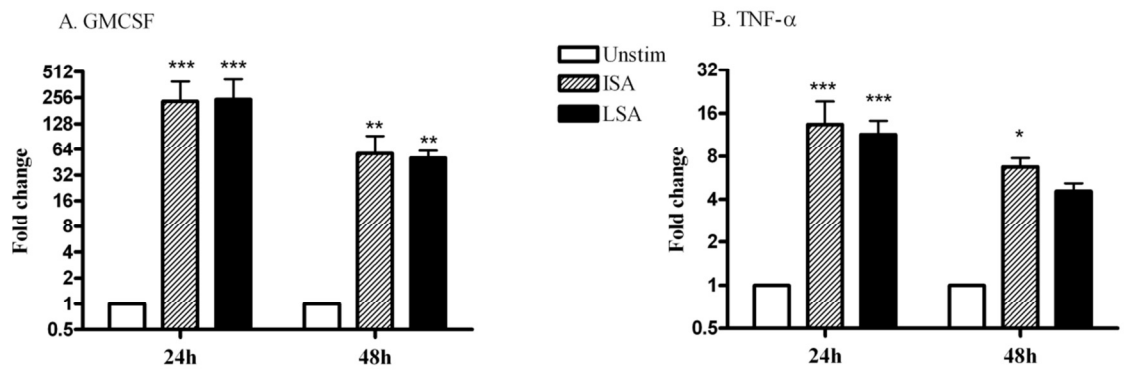

Figure 2.4. GMCSF and TNF-α mRNA transcription in monocytes stimulated with ISA or LSA at 24 and 48h after stimulation. Monocytes were loaded with ISA and LSA (MOI10) for 2h. After 2h, cells were washed three times and fresh monocyte media with gentamicin was added. Total RNA was isolated after 24 and 48h of *S. aureus* loading and mRNA expression was determined by real time PCR. All the results were normalized using GAPDH. Results are expressed as fold change from unstimulated monocytes calculated using the ddCT method. Both ISA and LSA stimulation increased GMCSF **(A)** and TNF-α **(B)** mRNA induction in monocytes at 24h (***$P < 0.001$) compared to unstimulated monocytes. Both ISA and LSA stimulation increased GMCSF mRNA induction in monocytes (**$P < 0.01$) at 48h with respect to control. However, only ISA treatment induced TNF-α mRNA (*$P < 0.05$) at 48h compared to unstimulated monocytes. Results shown represent the mean $\pm$ SE of data from three cows.

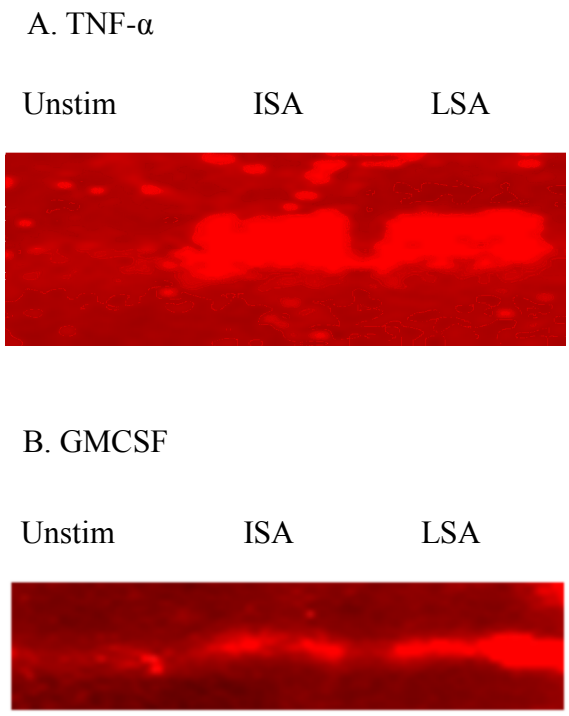

Figure 2.5. Immunoblots of TNF-α and GMCSF in the supernatants of unstimulated, ISA and LSA stimulated monocytes. Monocytes were stimulated with media, ISA or LSA for 2h. After 2h of incubation, cells were washed and added media with gentamicin without any cytokines. Supernatants collected at 48h of stimulation were immunoblotted for TNF-α and GMCSF. Increased secretion of **(A)** TNF-α and **(B)** GMCSF in the supernatants of ISA, and LSA stimulated monocytes compared to unstimulated. Data representative of three independent experiments.

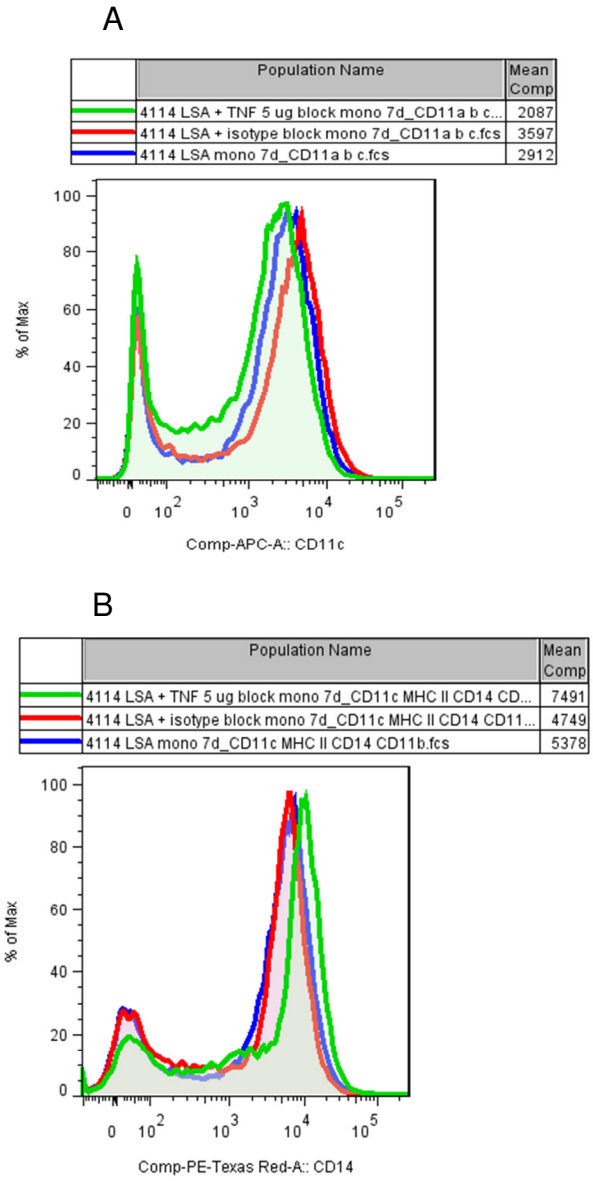

Figure 2.6. Blocking of TNF-á reduces the expression of CD11c on LSA stimulated MonoDC. Blue, red and green histograms represent LSA stimulated 7d monocytes, LSA stimulated monocytes cultured for 7d in the presence of isotype control and TNF-α antibody, respectively. Blocking with TNF-α antibody reduced the expression of CD11c **(A)** and showed greater intensity of expression of CD14 **(B)** in LSA stimulated monocytes compared to controls. Data representative of three independent experiments.

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CHAPTER 3 *STAPHYLOCOCCUS AUREUS* AND ITS STRUCTURAL COMPONENTS: RECOGNITION AND RESPONSE BY MONOCYTE DERIVED DENDRITIC CELLS

ABSTRACT

Staphylococcus aureus is considered to be a classical extracellular pathogen causing a variety of diseases in both humans and animals. However, *S. aureus* can also cause intracellular infections, which may involve specific receptor mediated recognition and subsequent host immune response. The objective of this study was to investigate the recognition and response of bovine monocyte derived dendritic cells (DC) to *S. aureus* and its different structural components, such as lipotechoic acid (LTA), and peptidoglycan (PGN). Peripheral blood monocytes were cultured for 7d in medium supplemented with recombinant bovine granulocyte-monocyte stimulating factor colony (rb-GMCSF) and recombinant bovine interleukin (rb-IL)-4. DC were infected with *S. aureus* at different multiplicity of infection (MOI) (10, 25, 50 and 100) and uptake of *S. aureus* was assessed by flow cytometry. Results indicate that as MOI increased from 10 to 100, the uptake increased significantly. In order to test the viability of DC loaded with *S. aureus*, Annexin V and propidium iodide (PI) staining was done at 6 and 24h. There was no significant difference in the percentage of live DC compared to control DC at 6h and 24h. Blocking of endocytic pathways involved in *S. aureus* uptake confirmed endocytosis through caveolar and clathrin coated pits and also by pinocytosis. DC were stimulated with irradiated *S. aureus* (ISA), live *S. aureus* (LSA), LTA, PGN, or LPS, for 3, 6, 12, 24 and 48h . The expression kinetics of Toll like receptors (TLRs) 2, 4, and 6, nucleotide oligomerization domain (NOD) 2, CD80, CD86, and tumor necrosis factor (TNF)-α, IL-1β, IL-6, transforming growth factor

(TGF)-β, IL-12, IL-23 and IL-27 mRNA were analyzed by quantitative real-time PCR (RT-PCR). Stimulation with LSA, ISA, LTA, PGN or lipopolysaccharide (LPS) induced various TLRs and cytokine genes. In order to assess the activation and antigen presentation by DC, the surface expression of CD11b, CD11c, CD14, MHC I & II were assessed by flow cytometry after 24 or 48h of stimulation. The surface expression of CD11b, MHC I, and MHC II increased when stimulated with *S. aureus*. Finally, LSA or ISA loaded DC triggered CD4, CD8 and γδ T cell proliferation. In conclusion, DC recognize *S. aureus* and its structural components and initiate innate and adaptive immune responses.

INTRODUCTION

Staphylococcus aureus is an important pathogen causing a variety of diseases in both humans and animals. *S. aureus* colonizes the skin and the respiratory tract, therefore any breach in the skin or mucus membrane predisposes the host for an invasive disease [1]. This pathogen causes diseases such as superficial skin infections, septic arthritis, osteomyelitis, endocarditis, pneumonia, toxic shock syndrome and septicemia in humans. *S. aureus* is considered a classical extracellular pathogen; however, intracellular infections have also been demonstrated [1, 2]. The intracellular persistence protects *S. aureus* from host immune defenses and antibiotics, and adds to the incidence of recurrent and chronic infections [3]. *S. aureus* produces a variety of leukocidins and immunomodulatory proteins , which enhance the ability of this bacteria to evade the innate and adaptive immune mechanisms of the host (reviewed, [4]. Even though several *S. aureus* vaccines have been developed using bacterins, capsular polysaccharides and superantigens, none of them offer a specific or long term protection. Recently, we have shown that DC are able to induce memory T cell proliferation (Chapter 4). Elucidating the response of DC to *S. aureus* and its various pathogen associated molecular patterns (PAMPs) may contribute to the use of DC as potential adjuvant in *S. aureus* vaccines.

The DC are considered the sentinels of immune system possessing the unique characteristic of stimulating naïve T cells. They bridge the innate and adaptive immune system. The DC differentiated from infiltrated monocytes at the site of infection or inflammation [5] can play an important role in the induction and regulation of immune response to *S. aureus.* The innate immune system recognizes microorganisms via germ line encoded evolutionarily conserved pattern recognition receptors (PRRs) such as TLRs [6, 7]. Although TLRs are expressed on various immune cells, antigen presenting cells (APC) such as DC and macrophages

highly such receptors. It has been suggested that TLRs recognize pathogens at either the cell surface or lysosome/endosome membranes; however, they are not used for the detection of intracellular pathogens that have invaded the cytosol [8]. Engagement of the TLRs with PAMPs results in dimerization of TLR and conformational changes so as to recruit adaptor molecules such as MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF). The adaptor molecules, MyD88 and TRIF activate distinct signaling pathways that direct the production of proinflammatory cytokines and type I interferons (IFNs), respectively [8]. On the other hand, cytosolic PRRs stimulate the cleavage and activation of caspase-1, which in turn cleaves and activates pro-IL-1β and pro-IL-18 [9, 10]. Thus, the immune system initiates response depending upon the recognition of PAMPs by membrane or cytosolic PRRs.

TLR2 plays a major role in the recognition of *S. aureus* and is involved in the recognition of a variety of *S. aureus* structural components, including LTA, lipoproteins, and PGN [11-13]. Research has shown that LTA in Gram positive bacteria activates the cells via the TLR2/TLR6 heterodimer [14-16]. Maturation of DC induced by LTA was modest with a moderate increase in CD80, CD86, TNF-α and IL-12p40 in human monocyte derived DC [17]. A synergistic effect on DC maturation has been noted when LTA and PGN were given together [17] and both LTA and PGN induced DC maturation via TLR2 [18]. In murine macrophages *S. aureus* utilizes TLR2 as a mean of facilitating intracellular infection characterized by diminished superoxide production after TLR2 activation [19]. TLR1 and TLR6 were shown to complex with TLR2 in macrophages and facilitate recognition of PGN [14, 15]. Intracellular PGN can be recognized by the intracellular PRR nucleotide-binding oligomerization domain containing 2 (NOD2) [20].

Since *S. aureus* can also cause intracellular infections [19], there is a need to explore the role of cytoplasmic PRRs that could recognize the pathogen. The NOD proteins which contain

N-terminal caspase recruitment domains (CARD) are implicated in the recognition of bacterial components. Binding of ligands to NOD1 and NOD2 causes their oligomerization and subsequent NF-kB activation resulting in downstream signaling. NOD2 recognizes muramyl dipeptide, the catabolism product of PGN [21]. Expression of IL23p19 is co-regulated with IL-1b secretion through NOD2 dependent signaling in human DC stimulated with PGN [22]. Reports from *S. aureus* activated macrophages indicate that intracellular sensing by NOD2 was essential for cytokine production in the absence of TLR2 [23]. Data available on the NOD2 expression in bovine monocyte derived DC in response to *S. aureus* are scanty or deficient. We proposed that bovine monocyte derived DC recognize *S. aureus* and its purified structural components and initiate cytokine response and induce T cell proliferation. The objectives of the current study were to characterize (a) the PRRs responsible for the recognition of *S. aureus,* and (b) the cytokine response and induction of T cell proliferation by bovine monocyte derived DC.

MATERIALS AND METHODS

Animals

Holstein Friesian dairy cows from the Virginia Tech Dairy Research Facility were used for this study. All the animals were free from any visible signs of illness at the time of blood collection. The animal experiments complied with the ethical and animal experiment regulations of Virginia Tech IACUC.

Propagation, irradiation and fluorescent labeling of *S. aureus*

Single colonies of RN6390B strain of *S. aureus* were cultured in tryptic soy broth for 4h with rigorous shaking (12400 Incubator Shaker, New Brunswick Scientific CO, Inc.). The cultures were washed three times with Hank's balanced salt solution (HBSS) (Invitrogen, NY, USA), and pelleted at 1500xg for 10 min. Cultures were then serially diluted and drop plated to get the actual colony counts per mL. Bacteria were irradiated in a Model 109 research cobalt irradiator (JL, Shepherd and Asscocites, San Fernando, CA) for 3h. Before use, the ISA were washed twice with HBSS and diluted to 10^9 CFU/mL with RPMI 1640 medium.

Fluorescent labeling of *S. aureus* was performed with some modifications of the procedure described earlier. Briefly, 10^9 CFU /mL of RN6390B strain *S. aureus* in carbonate bicarbonate buffer were incubated with 100µg of fluorescein isothiocyanate (46425; Thermo scientific, IL, USA) isomer I /ml for 2h in dark at 37ºC. The cultures were then washed three times with HBSS and analyzed for uniformity of staining by fluorescent microscopy. Labeled cultures were suspended in HBSS and stored at -20ºC until use.

Culturing of monocytes and monocyte derived DC

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood. Briefly, PBMC were isolated from 250 mL of blood drawn from the jugular vein into 250mL K_3-EDTA-

vacuum bottles and enriched by discontinuous density gradient centrifugation with the procedure described earlier [24]. Briefly, 10mL of the buffy coat were collected and resuspended in 20mL of 1x Hanks balanced Salt Solution (HBSS). The suspended buffy coat was layered over Ficoll-Paque ^{TM} plus (GE Healthcare Biosciences AB, Uppsala, Sweden) and centrifuged at 330xg for 45 min at 25ºC. Mononuclear cell layer was removed and washed three times using HBSS. Cell viability and number were determined by Trypan blue exclusion test. The PBMC were incubated with anti-human CD14 microbeads (Miltenyi Biotec, CA, USA) (10µL/10^7 PBMC) for 20 min on ice. A positive selection of $CD14^+$ cells was performed by magnetic cell sorting according to manufacturer's instructions. The purity of the cells (> 98%) was assessed by flow cytometry and cell viability was assessed (> 99%) by Trypan blue exclusion.

Purified $CD14^+$ monocytes were infected with live *S. aureus* (LSA) or irradiated *S. aureus* (ISA) ISA or LSA (MOI 10) for 2h. After 2h of incubation, the cells were washed extensively and fresh monocyte medium with gentamicin was added. Every 3d, half of the medium was replaced by fresh media. The cells were cultured for 7-8d and analyzed by flow cytometry for the expression of various DC markers. Unstimulated monocytes and monocytes cultured with rb-GMCSF and rb-IL-4 for 7d were used as negative and positive controls, respectively.

For DC differentiation, CD14 bead purified peripheral blood monocytes were cultured in plastic petri dishes for 6-7d in RPMI-1640 medium (Invitrogen, NY, USA) containing 10% FBS (Hyclone Labs, UT, USA), 10mM HEPES, 4mM L-glutamine (Invitrogen, New York, USA), $5x10^{-5}$ M 2-betamercaptoethanol (Sigma-Aldrich, MO, USA), rb-GMCSF (200ng/mL) and rb-IL-4 (100ng/mL) as described earlier [25]. Media and cytokines were replenished on every 3d.

Flow cytometric analysis of surface markers

Fresh monocytes, ISA or LSA stimulated 7d old monocytes and unstimulated DC were stained with anti-bovine MCA1651G (Abd serotec, Raleigh, NC, USA) (IgG2b PE) for DEC205, MM61A (IgG1Texas red) for CD14, TH16B (IgG2a FITC) for MHCII, H58A (IgG2a FITC) for MHCI, MM10A (IgG2b PE) for CD11b, BAQ153A (IgM APC) for CD11c, MUC76A (IgG2a FITC) for CD11a, and BAQ15A (IgM APC) for CD21 (VMRD, Pullman, WA, USA) and assessed by flow cytometry. Briefly, the cell suspensions were stained with primary antibodies followed by incubation for 1h on ice. After three washings, fluorochrome-conjugated isotype specific secondary antibodies (Invitrogen/Caltag lab, New York, USA) were added and incubated for another 30 min on ice followed by three washings. Apoptosis and necrosis were assessed by staining the cells with annexin V, Pacific Blue™ conjugate (Invitrogen, New York, USA) and PI (Sigma-Aldrich, MO, USA), respectively. Percentages of cells and mean fluorescence intensity (MFI) were determined using FacsCalibur flow cytometer (BD biosciences, San Jose, CA, USA) and analyzed using Flowjo software v .7.6.1 (Tree star Inc., Ashland, OR).

Endocytosis and Endocytosis Inhibition Assay

For endocytosis assay, DC (10^6) were infected with FITC labeled *S. aureus* with different MOI 10, 25, 50 and 100 in antibiotic free media and incubated for 3h at 37ºC. After 3h of incubation, the cells were washed and treated with 100µg lysostaphin (Sigma-Aldrich, MO, USA), for 7 min. Cells were then fixed with 1% formaldehyde and analyzed by flow cytometry for $FITC^{+ve}$ positive cells and MFI.

In order to elucidate the various endocytic pathways involved in the uptake of *S. aureus* by DC, DC were pretreated for 30 min with media (1% FBS) containing one of the following

inhibitors; chlorpromazine (10 µg/mL) (Sigma-Aldrich, MO, USA), filipin (5 µg/mL) (Sigma-Aldrich, MO, USA), or sucrose (450 mM) to block different endocytic pathways. *S. aureus* labeled with FITC were added to the cells at MOI 50 for 1h. After 1h, cells were washed twice with HBSS, treated with lysostaphin 100µg (Sigma-Aldrich, MO, USA) and fixed with 1% formaldehyde. The cells were analyzed by flow cytometry for $FITC^{+ve}$ DC.

DC Activation

The DC were stimulated with whole LSA (MOI, 50) for 3h, ISA (MOI, 50) or different bacterial membrane components such as LTA (InvivoGen, San Diego, CA, USA) (1µg/mL), PGN (InvivoGen, San Diego, CA, USA) (5µg/mL) or LPS (Sigma-Aldrich, MO, USA) (0.5µg/ml) for 3, 6, 12, 24 and 48h. The LSA infection was terminated after 3h by adding gentamicin to the media. The expression levels of different PRRS, co-stimulatory molecules and various cytokine genes were assessed by RT-PCR at 3, 6, 12, 24 and 48h of stimulation. The MFI of expression of MHCI, MHCII, CD11b, CD11c, and CD14 were determined by FACS after 24h of stimulation.

Real-Time RT-PCR

Total RNA was extracted from 3, 6, 12, 24 and 48h ISA, LSA, PGN, and LTA stimulated DC using Qiagen's RNeasy Mini Kit with DNase (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. Unstimulated and LPS treated DC were used as negative and positive controls, respectively. The cDNA was prepared from 1µg RNA using Superscript II Reverse Transcriptase and oligo dT primers (Invitrogen, Carlsbad, California, USA). Bovine specific primers / probes were designed using Primer Express 3 software (Applied Biosystems, USA) as described earlier [26]. The designed primers and probes were purchased from IDT (Integrated DNA Technologies, Inc, USA) (Table 3.1). We confirmed their specificity to detect a cDNA

standard for each gene derived from bovine monocytes / DC cDNA by running a standard curve. Real-time RT-PCR using primers and Taqman probes (Applied Biosystems, USA) were used to determine the level of transcription of GMCSF and TNF-α. The RT-PCR reactions were conducted using the Taqman Universal master mix (Applied Biosystems, USA) and analyzed using ABI Prism 7300 Real-Time PCR System (Applied Biosystems, USA). The CT values were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control and expressed as fold change as calculated by the $2^{-(\Delta\Delta CT)}$ method. Briefly, ΔCT = CT of the target subtracted from the CT of GAPDH, and ΔΔCT=ΔCT of samples for target subtracted from the ΔCT of corresponding control samples. Statistical analysis was performed on log transformed values of $2^{-(\Delta\Delta CT)}$ values and expressed as fold change with respect to unstimulated DC.

Statistical analysis.

Statistical analysis was performed by one-way ANOVA, or two-way using Graph Pad Prism 5.0 (San Diego, CA, USA). To compare treatment means, Tukey's test was performed. A *P* value of < 0.05 was considered statistically significant.

RESULTS

S. aureus uptake by DC reaches maximum at MOI 50

Immature DC have high endocytic capacity. To assess the endocytosis of *S. aureus* by bovine DC; DC were loaded with different MOI of FITC labeled *S. aureus* for 3h. Flow cytometry analysis showed that as MOI was increased from 10 to 100, the uptake also increased significantly ($P < 0.0001$) (Figure 3.1a and 1b). *S. aureus* uptake by DC increased ($P < 0.05$) as we increased MOI from 10 to 25. Numerically, MOI 100 showed the maximum uptake, however, there was no significant difference between MOI 50 and 100 ($P > 0.05$). *S. aureus* uptake by DC at MOI 50 and 100 showed significant differences from MOI 10 ($P < 0.001$) and MOI 25 ($P < 0.05$). Results indicate that DC are efficient in endocytosing *S. aureus.*

Clathrin-mediated, caveolar and fluid phase dependent endocytosis are involved in the internalization of *S. aureus* by DC

We investigated the effects of inhibitors of clathrin-mediated (chlorpromazine), caveolar dependent (filipin) and fluid phase dependent endocytosis (sucrose) on internalization of FITC labeled *S. aureus* by DC (Figure 3.2). Treatment with different inhibitors significantly decreased the internalization of *S. aureus* compared to control ($P < 0.001$). Simultaneous treatment with three inhibitors interfering with three different pathways of internalization did not differ ($P > 0.05$) from the effect of chlorpromazine or sucrose alone. However, compared to chlorpromazine and sucrose, filipin treatment inhibited the internalization of *S. aureus* less significantly ($P <0.01$). Results suggest that although three pathways are involved in the internalization of *S. aureus,* clathrin-mediated and fluid phase endocytosis play major roles in *S. aureus* uptake by DC.

ISA/LSA loading fails to induce apoptosis in DC

Apoptosis / programmed cell death is a physiologic mechanism that maintain homeostasis of body tissues. DC were loaded with ISA and LSA (MOI 50) and analyzed for apoptosis and necrosis after 6 and 24h by flow cytometry using Annexin V and PI, respectively. The percentage of live DC following loading with ISA or LSA were 81.2 and 78.0, respectively at 24h compared to 90.6 in control (Figure 3.3). Similarly, at 6h, the live DC percentages in control, ISA and LSA treatments were 79.9, 76.8, and, 75.3, respectively (Figure 3.4A). There was no significant difference in the percentage of live DC between ISA and LSA loaded DC ($P > 0.05$) at 6 (Figure 3.4) and 24h.

Relative expression kinetics of mRNA of PRRs, cytokines and co-stimulatory molecules in stimulated DC

We tested the mRNA expression pattern of PRRs, cytokines and co-stimulatory molecules in DC at 3, 6, 12, 24 and 48h of stimulation with media, ISA, LSA, PGN, LTA or LPS. It is noted that there was no effect on NOD2 expression by any of the treatments. TLR2 gene expression increased at 3, 6, 12, 24 and 48h for all treatments compared to unstimulated DC; however, PGN and LTA were less efficient in induction (Figure 3.5). TNF-α gene expression was increased significantly by ISA, LSA, and *S. aureus* structural components compared to unstimulated DC ($P < 0.001$). IL-1β expression was increased at all time points for all treatments ($P = 0.0003$) except by PGN at 48h. Similarly, IL-6 expression was increased at all time points for all treatments except by PGN at early time points ($P = 0.005$). It is also noted that TGF-β expression was increased significantly at 12 and 48 h for all treatments ($P= 0.009$). Expression of the Th1 inducing cytokine IL-12p40 mRNA expression was significantly up-regulated by ISA, LSA and LTA ($P = 0.008$). A significant increase in IL-23p19 expression was

induced by ISA and LSA at all-time points *(P* = 0.0001). The expression of IL-27 was up-regulated by all treatments at different time points; however, the effect was more pronounced for ISA. Transcription of the costimulatory molecule CD80 was significantly increased by all treatments; whereas this was not the case for CD86. Results suggest recognition of *S. aureus* and its components through TLR2. In addition, *S. aureus* and its structural components were able to up-regulate the expression of cytokines and co-stimulatory molecules suggesting activation of DC.

Surface receptor expression of CD11b, MHCI, MHC II and CD14 increases in LSA stimulated DC

Here we assessed the ability of *S. aureus* or its PAMPs to induce DC activation, antigen presentation and maturation. DC were stimulated with LSA, PGN, or LTA for 3h and assessed by flow cytometry for MFI of expression of β2 integrins (CD11b and CD11c), MHC I and II, and CD14 (Figure 3.6). Unstimulated and LPS stimulated DC were used as negative and positive controls, respectively. Stimulation of the DC with *S. aureus* for 24h resulted in greater intensity of expression of CD11b, CD14 and MHC class I and II compared to unstimulated DC. CD11b expression increased significantly with LSA after 24h compared to PGN (*P* = 0.01) and LTA (*P* = 0.03); however, there was no difference between PGN and LTA stimulated DC. CD11c surface expression did not change in any of the stimulated DC. MHC I surface expression increased significantly when DC were stimulated with LSA compared to PGN (*P* = 0.02) and LTA (*P* = 0.03), while no difference was noted between PGN and LTA. The MHC II expression increased with LSA stimulation compared to PGN (*P* = 0.02), however there was no difference in the expression levels of MHC II between LSA and LTA or PGN and LTA. CD14 surface expression

in LSA stimulated DC was significantly up-regulated compared to PGN ($P = 0.02$) and LTA ($P = 0.02$).

Proliferative response of T cells to *S. aureus* loaded DC

We further assessed the ability of DC to induce *S. aureus* specific lymphocyte proliferation. The CSFE labeled lymphocytes were co-cultured with either ISA or LSA loaded DC for 4d. Flow cytometric analysis showed both ISA and LSA loaded DC induce CD4, CD8 and γδ T cells compared to the controls (Figure 3.7). The LSA, ISA, DC and ConA stimulated lymphocytes were our controls. Unstimulated DC induced proliferation of 2.0, 4.8, and 1.1 % of CD4, CD8, and γδ T cells, respectively. Any proliferation induced by ISA or LSA stimulated CD4, CD8, and γδ T cells was gated out when we analyzed for proliferating ($CFSE^{low}$) CD4, CD8 and γδ T cells. Twelve and 9.3 % CD4 cells and 8.3 and 20.4 % CD8 T cells proliferated in response to LSA or ISA loaded DC, respectively. Similarly, 3.5 and 5.3 % γδ T cells proliferated in response to ISA or LSA loaded DC, correspondingly.

DISCUSSION

This study details for the first time, the response of bovine monocyte derived DC to *S. aureus* and its structural components. Previous studies used *S. aureus* superantigens or its structural components alone to study the effect on host immune cells, but, limited information is available on the interaction of *S. aureus* to bovine monocyte derived DC. Monocyte derived DC are widely used as a model for studying functional capabilities of inflammatory DC *in vivo*. Our data confirm that bovine monocyte derived DC possess a high intensity of expression of CD11b along with other characteristic DC markers. This $CD11c^{high}$ $CD11b^{high}$ DC could be a subset of inflammatory DC having a specific function in immune response, consistent with previous research [27]. Our preliminary studies showed that there was no difference in the expression levels of surface markers between adhered out monocytes and monocytes isolated using CD14 magnetic beads.

Immature DC constantly sample the environment for invading pathogens at the common sites of potential pathogen entry. Previous research showed the function of an array of surface receptors present in immature DC in antigen uptake and their activation [8]. Presumably, in our study, these surface receptors might have played a role in *S. aureus* uptake by immature DC. We sought to determine the specific endocytic pathways involved in the entry of *S. aureus* into bovine immature DC. As demonstrated through the use of specific inhibitors of endocytic pathways, all three pathways are involved in the internalization of *S. aureus,* but clathrin-mediated and fluid phase endocytosis play major roles in uptake of *S. aureus* by DC.

It has been postulated that in general, pathogens adopt two strategies to overcome the host immune system: first, interfering directly with PRRs of antigen presenting cells; or second, triggering apoptosis of proximal antigen presenting cells. Apoptosis / programmed cell death is a

physiologic mechanism that maintain homeostasis of body tissues. It has been reported that viable DC can uptake apoptotic DC resulting in the induction of tolerance by promoting regulatory T cells [28]. We hypothesized that if *S. aureus* loaded DC undergo apoptosis/necrosis at a higher rate than unstimulated DC; this might contribute to the defective immune responses. The ISA was used to minimize the effect of leukocidins and other toxins on DC. Instead, we observed that bovine monocyte derived DC are resistant to *S. aureus* induced cell death compared to monocytes (Chapter 2). It appears that bovine DC are capable of mounting an effective immune response against *S. aureus*

The results suggest that whole *S. aureus* is required to induce antigen presentation compared to its structural components alone. The PRRs like TLRs and IL-1 receptor family recognize the PAMPs present on the pathogens or endogenous danger signal like TNF-α. It has been reported that *S. aureus* and its structural components are recognized through TLR2 [11-13]. *S. aureus* carries PGN and LTA as its structural components of cell wall [29]. DC maturation induced by LTA was modest with a moderate increase in CD80, CD86, and TNF-α and IL-12p40 in human monocyte derived DC [17]. Previous studies have shown synergistic effect on DC maturation when LTA and PGN were given together [17] and induced maturation via TLR2 [18]. Consistent with earlier studies, we found that TLR2 gene expression along with increased cytokine gene expression in response to *S. aureus*, LTA and PGN. Previous research has reported that LTA activated cellular responses through TLR2, facilitated by LPS binding protein (LBP) and CD14; however, independent of TLR4 and MD-2 [30]. Synergistic effect of LTA and PGN may attribute to the up-regulation of CD14 in LSA treated DC. LSA and ISA rather than *S. aureus* structural components appear to be more effective in activating immature bovine DC. This program of maturation is characterized by the up regulation of MHC molecules and CD80.

The mature DC become more efficient in antigen presentation, while less efficient in phagocytosis [31]. DC after antigen acquisition migrate to the local lymph node during the process of maturation and present the antigen to the naïve T cells [32]. Since naïve T cells do not traffic to peripheral tissues, migration of DC to lymph nodes is very important for the activation of naïve T cells. Effective activation induces clonal expansion and differentiates into effector and memory T cells. Depending upon the stimuli received by DC from the pathogen it can be matured to induce immunity, tolerance or even become sessile whereas immature DC induces only tolerance. In humans, studies have shown that monocyte derived DC induce a superior memory T cell proliferation because of their ability to produce IL-12p70 and IL-23. In a mice model, $CD11b^{high}$ DC derived from monocytes recruited to the peripheral non lymphoid tissue in response to inflammation induced memory activation of CD8 T cells [33, 34].

In this study, the greater expression of integrins, co-stimulatory and MHC molecules consequent to *S. aureus* uptake indicates an ability of bovine monocyte derived DC for maturation, antigen presentation and migration. The greater expressions of integrins suggest the ability of matured DC to migrate towards regional lymph nodes to elicit an immune response. The β2-integrins CD11b and CD11c are up regulated on leukocytes during inflammation, contributing to their migration and subsequent functional responses in various pathophysiological conditions [35, 36]. The up-regulation of MHC I in stimulated DC might be an indication of the cytosolic route of antigen processing or a cross presentation [37]. In this study, we didn't conduct any experiments to differentiate cross presentation from other two classical antigen processing pathways. Greater intensity of expression of MHC II indicates the endocytic processing of *S. aureus* as with any classical extracellular pathogen. The up-regulation of IL-12p40 and IL-27 genes suggests the role of mature DC in shaping a Th1 response against

S. aureus. Similarly, *S. aureus* and its structural components modify the transcription of Th17 polarizing cytokine genes in bovine DC. The cytokines, IL-6, TGF-β, and IL-23 induce Th17 cell differentiation. In this study, we have noted that stimulated DC up-regulate IL-6 gene expression along with other cytokines. IL-6 is one of the DC-derived factors that act in concert with TGF-β to differentiate Th17 cells [38].

Subsequent to the uptake of antigen and maturation of DC, the optimal interaction occurs between DC and naïve CD4, CD8 and γδ T cells in an antigen specific way. In the present study, we observed that DC induced proliferation of CD4, CD8 and γδ T cell subsets. It is known that $CD11b^{high}$ DC derived from monocytes recruited to the peripheral non-lymphoid tissues in response to inflammation induce memory CD8 T cell activation [5, 33]. In conclusion, bovine monocyte derived DC induced T cell proliferation in response to *S. aureus.*

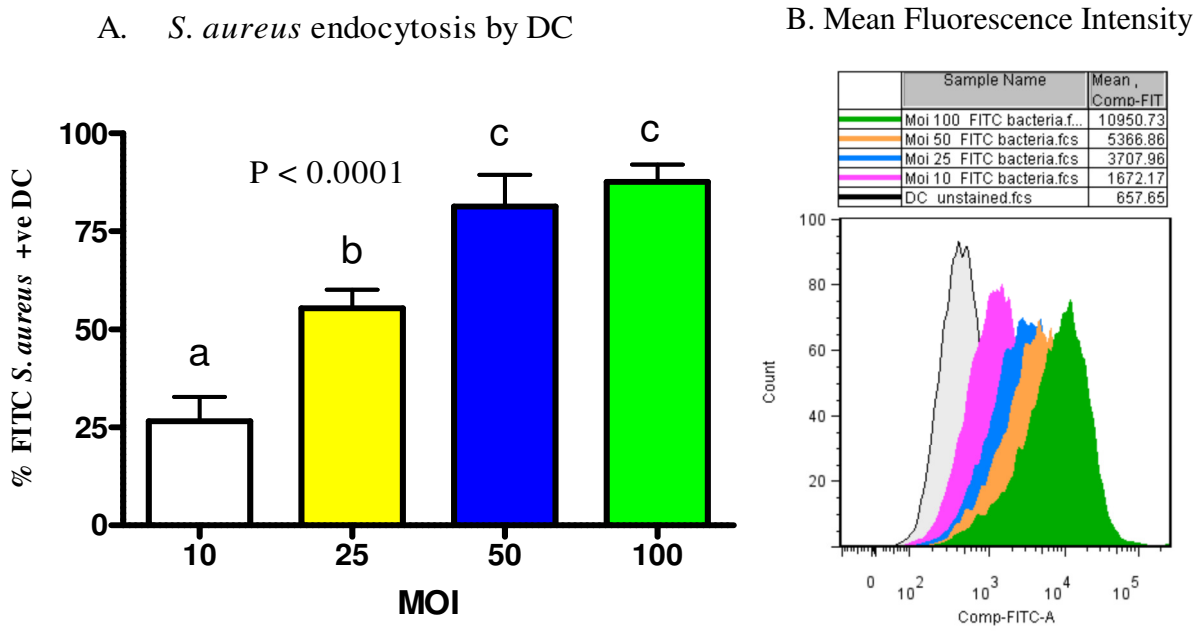


Figure 3.1. *S. aureus* endocytosis by DC. A.1x10^6 DC (n=4) were loaded with FITC labeled *S. aureus* at different MOI for 3h and the cells were collected after treating with lysostaphin, fixed and analyzed by flow cyometry. **(A)** Increasing MOI resulted in increased uptake of *S. aureus* by DC ($P < 0.0001$). MOIs with different letters significantly differ from each other. Data represent mean $\pm$ SE. **(B)** Histograms showing mean fluorescence intensity of DC loaded with FITC labeled *S. aureus* at different MOI; black (unstained DC), pink (MOI 10), blue (MOI 25), orange (MOI 50) and green (MOI 100).

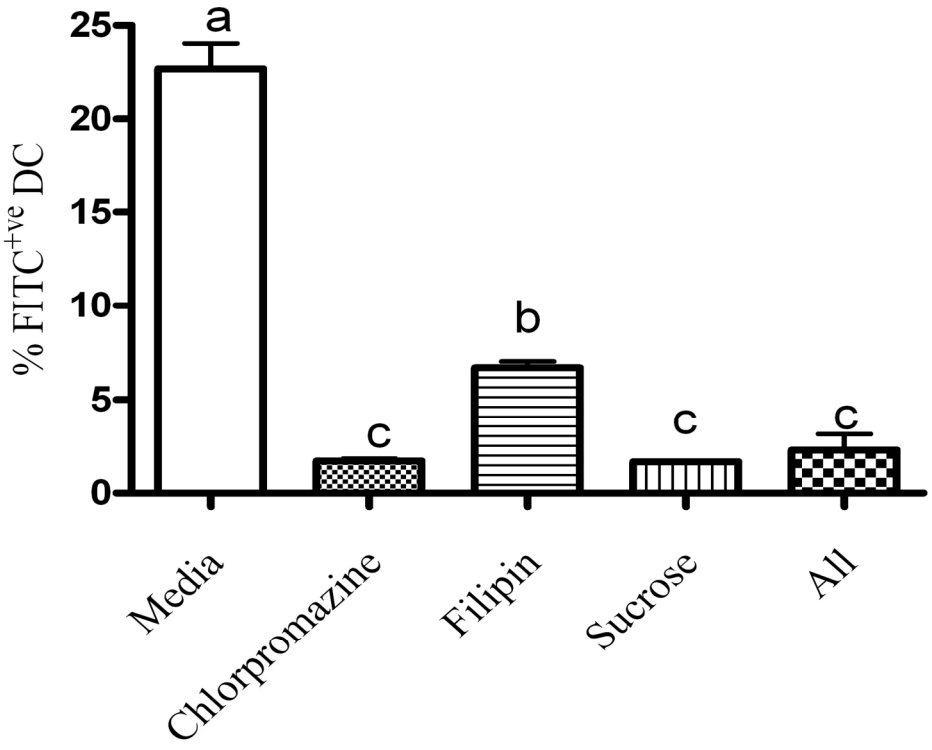
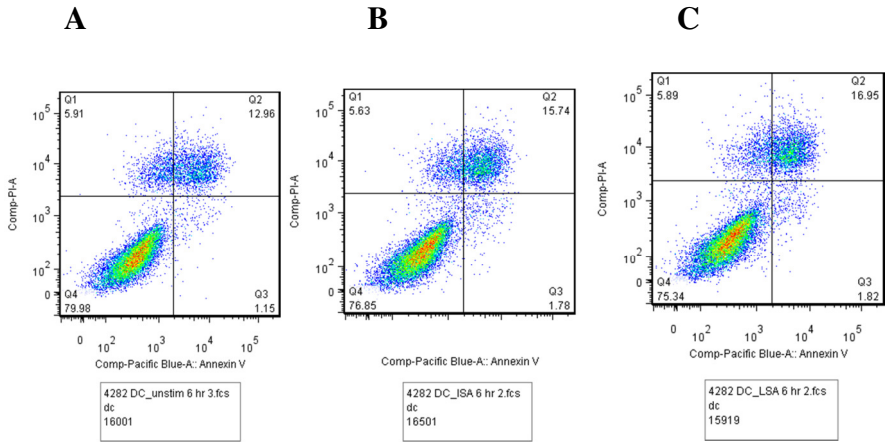


Figure 3.2. Effects of inhibitors on internalization of *S. aureus* by DC. Cells were pretreated with media only, chlorpromazine, or filipin or sucrose. Subsequently, FITC labeled *S. aureus* were added and after 1h and cells were treated with lysostaphin. Cells were fixed and analyzed by flow cytometry. One way ANOVA was carried out to determine the statistical significance of the data and Tukey's test to compare treatment means. Treatment with different letters significantly differs from each other. Data represent mean $\pm$ SE. Data representative of three individual experiments.



D. Viability of DC

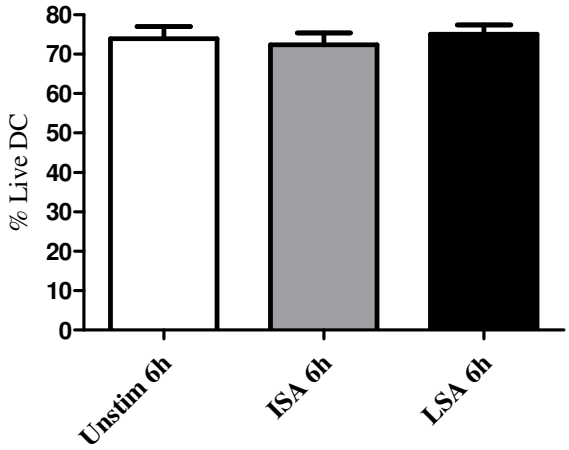


Figure 3.3. Apoptosis and necrosis of DC after 6 h of ISA and LSA loading. DC (n=3) were loaded with ISA or LSA (MOI 50) for 3h and infection was terminated by adding gentamicin after 3h. DC were collected and stained for Annexin V and PI after 6 h of initial stimulation and analyzed by flow cytometry. There was no difference between unstimulated DC (A), ISA (B) and (C) LSA loaded cells. X-axis represents Annexin V and Y-axis represents PI in the dot plots. Flow cytometry plots are representative of three individual experiments. (D) Mean $\pm$ SE of % live DC.

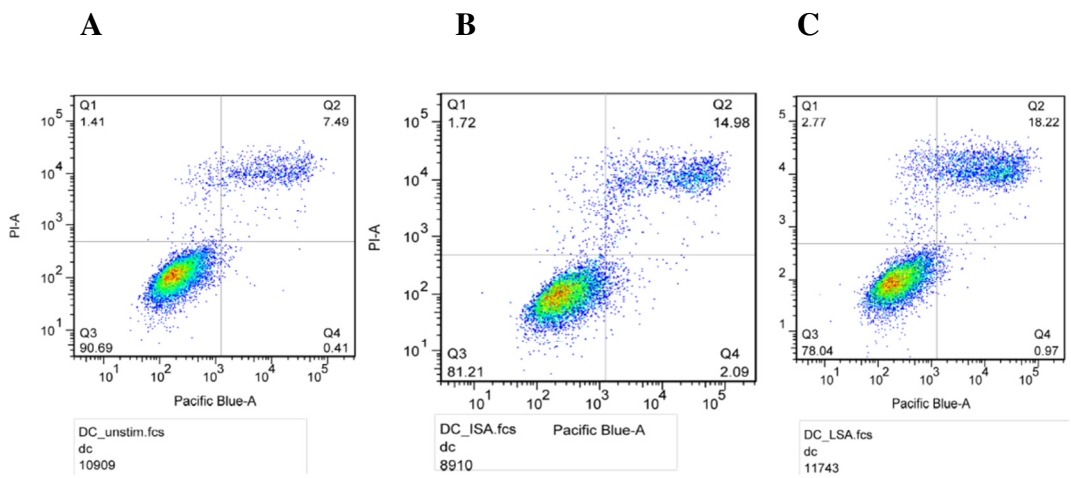
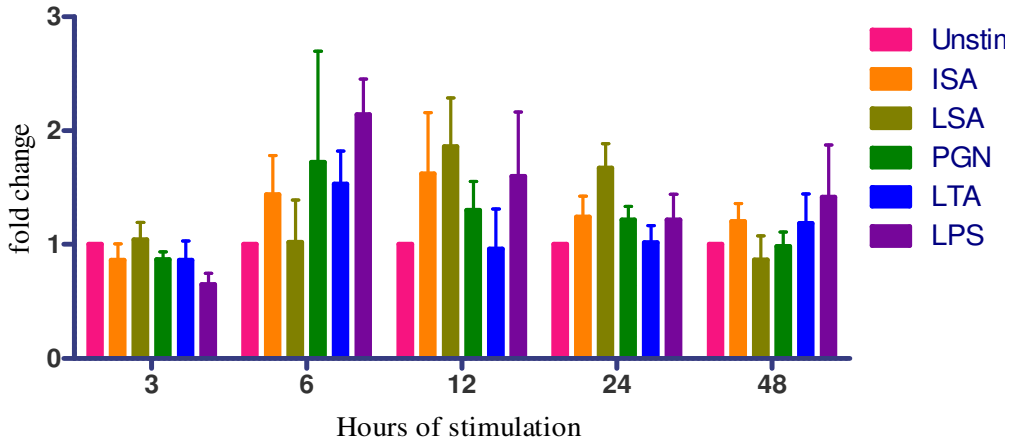
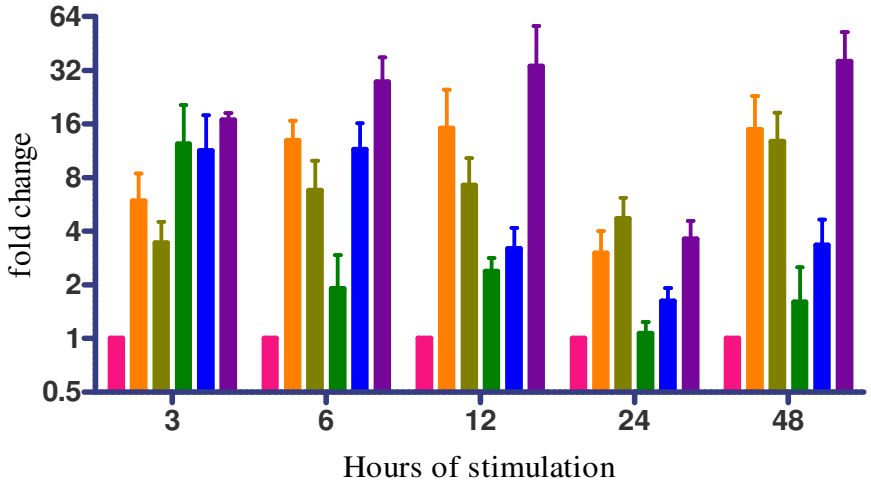


Figure 3. 4. Apoptosis and necrosis of DC after 24 h of ISA and LSA loading. DC were loaded with ISA or LSA (MOI 50) for 3 hours. After 3h, DC were washed twice with HBSS and added fresh media with gentamicin. DC were collected and stained for Annexin V and PI after 24h of initial stimulation and analyzed by flow cytometry. There was no difference between ISA and LSA loaded DC. Dot plots represent apoptosis and necrosis in unstimulated (A), ISA (B) and (C) LSA loaded DC. X-axis represents Annexin V and Y-axis represents PI in the dot plots. Flow cytometry plots are representative of three individual experiments.

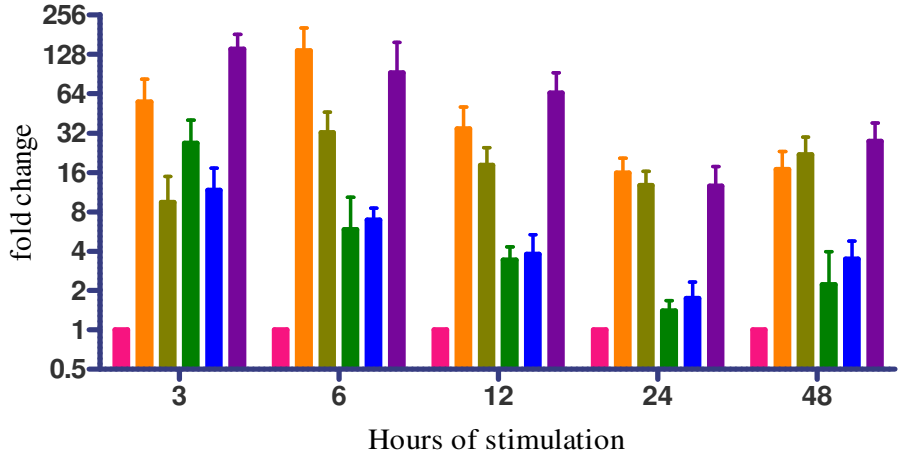
A. NOD2



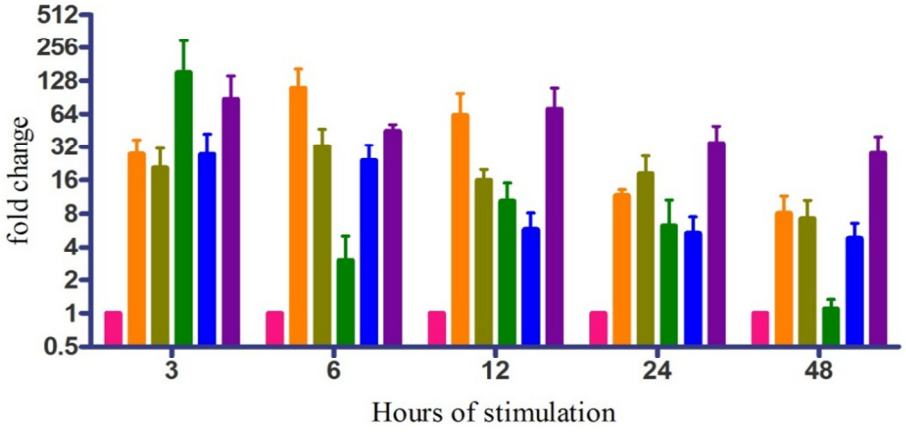
B. TLR2



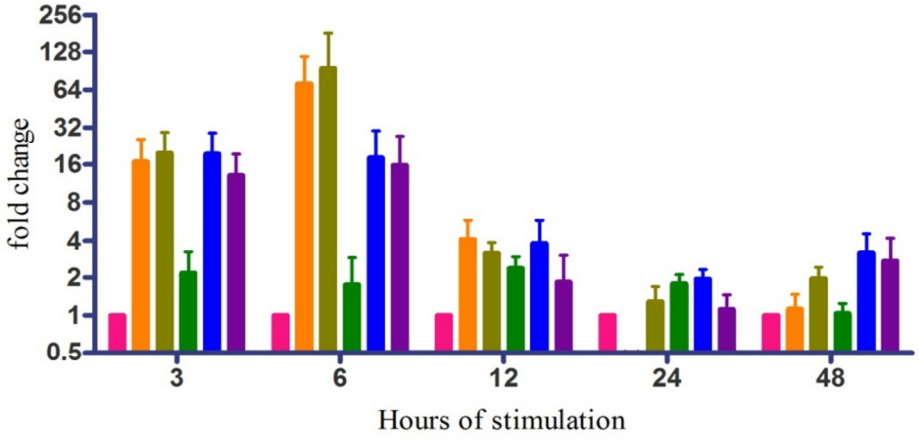
C. TNF-α



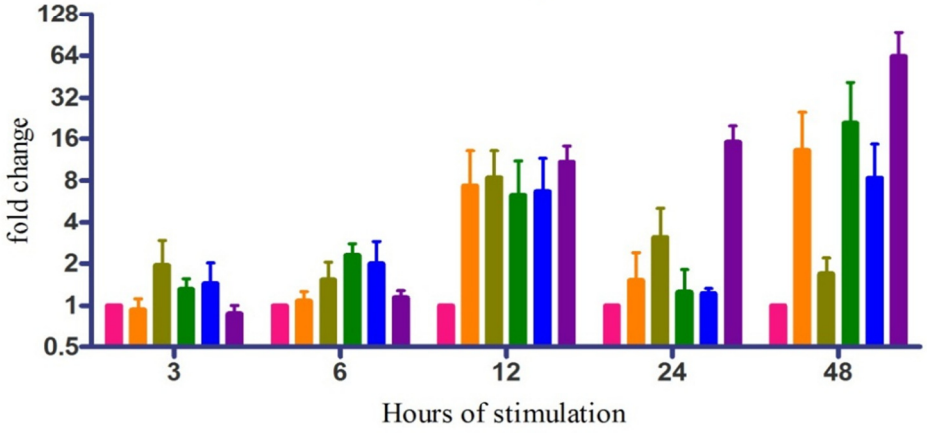
D. IL-1β



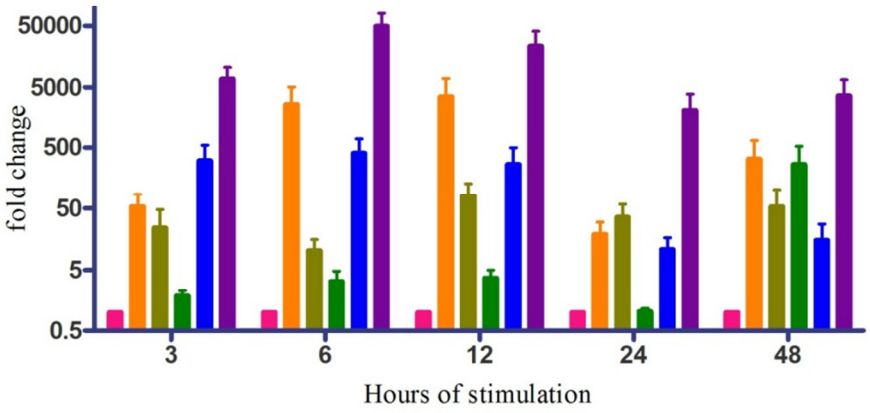
E. IL-6



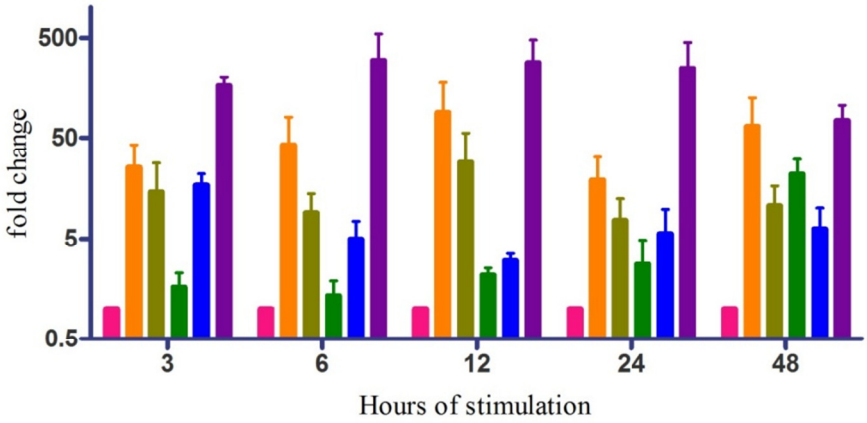
F. TGF-β



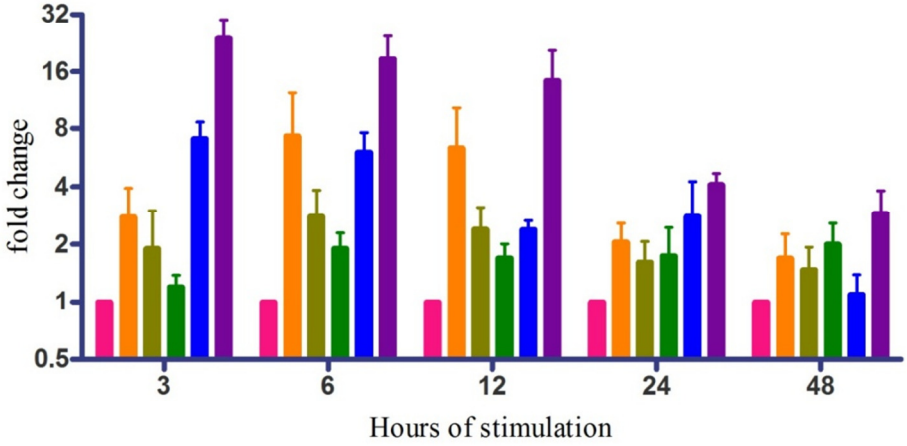
G. IL-12p40



H. IL-23p19



I. IL-27



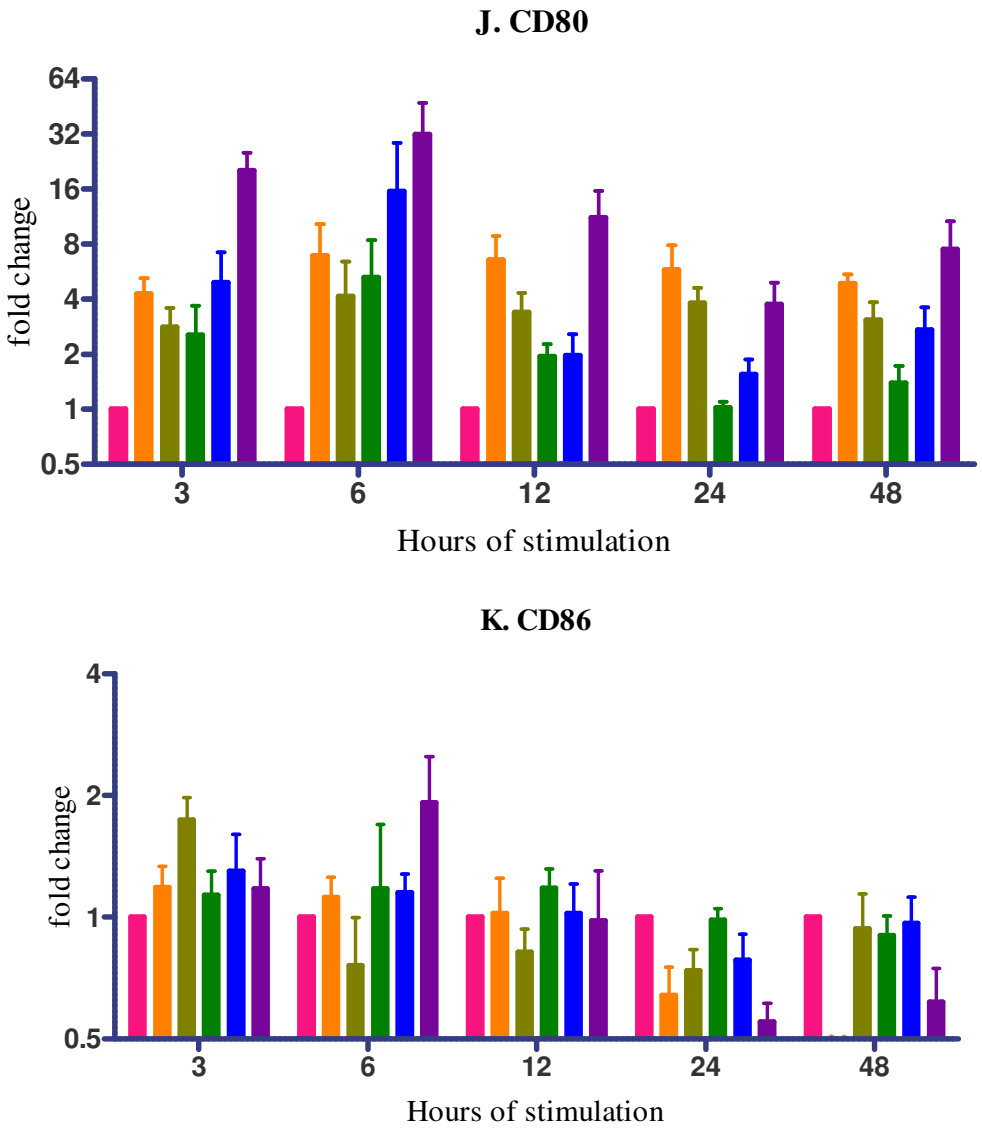


Figure 3.5. mRNA induction of (A) NOD2, (B) TLR2, (C) TNF-α, (D) IL-1β, (E) IL-6, (F) TGF-β, (G) IL-12p40, (H) IL-23p19, (I) IL-27, (J) CD80, and (K) CD86 in DC stimulated with ISA, LSA, PGN, LTA ,LPS or unstimulated. DC were stimulated with ISA and LSA (MOI50), PGN (5mg/mL), LTA (1mg/mL) and LPS (0.5mg/mL) for 3h. After 3h, infection was stopped by adding fresh media with gentamicin and incubated for another 3, 6, 12, 24 or 48h. Total RNA was collected at3, 6, 12, 24 or 48h of initial stimulation and mRNA expression was determined by real time PCR. All the results were normalized with the GAPDH. All results are expressed as fold change from unstimulated DC calculated using the ddCT method. The expression levels are mean $\pm$ SE of five independent experiments.

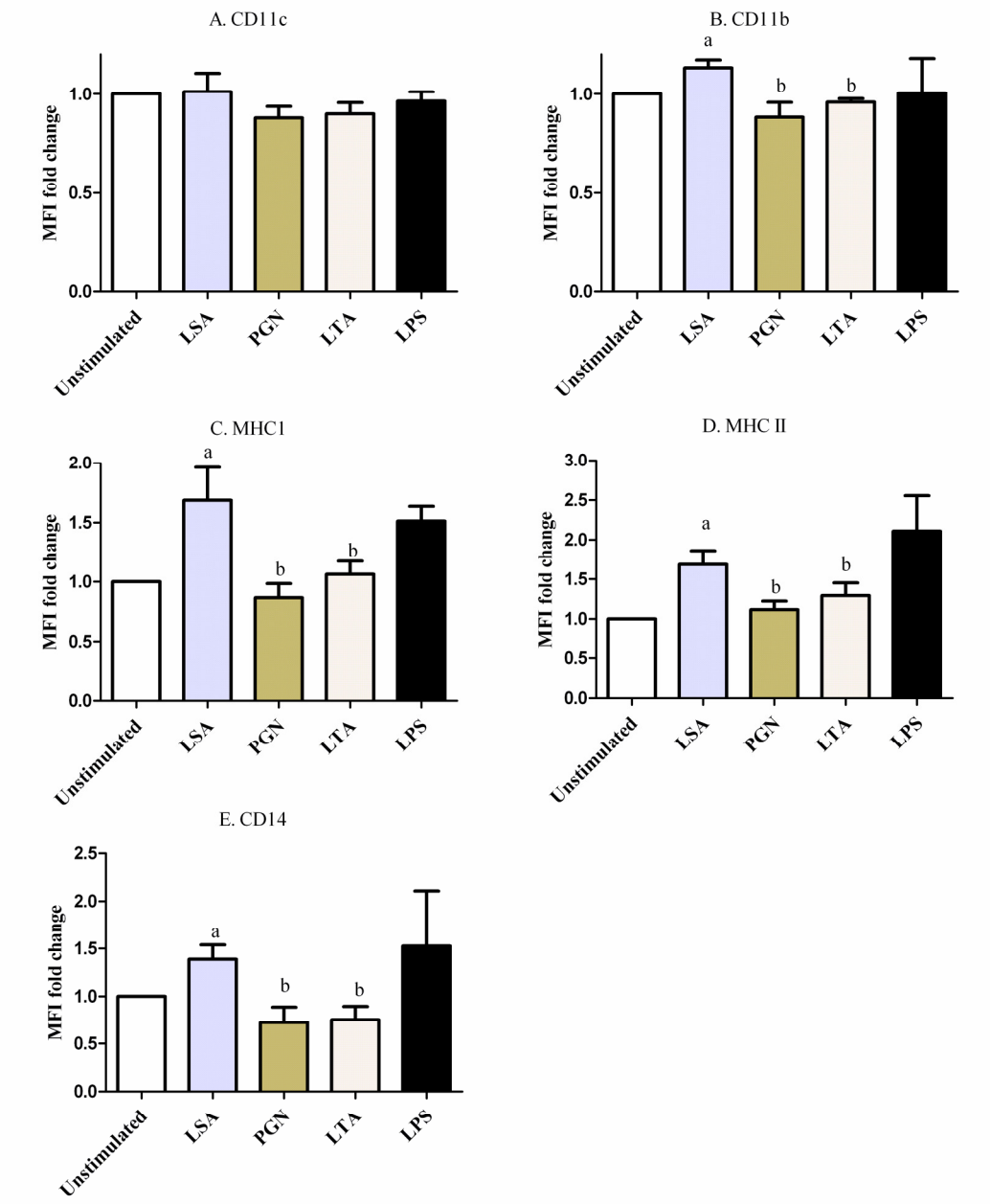


Figure 3.6. Surface intensity expression of markers in stimulated DC. DC (n=4) were stimulated with LSA, PGN, and LTA or with media for 3h and surface intensity of expression of (A) CD11c, (B) CD11b, (C) MHC I, (D) MHC II and (E) CD14 analyzed after 24h of stimulation by flow cytometry. Media stimulated and LPS stimulated DC represents negative and positive controls, respectively. Values are expressed as fold change with respect to unstimulated DC. Student's t test was performed to determine statistical significance between LSA, PGN and LTA. Treatments with different letters significantly differ from each other.

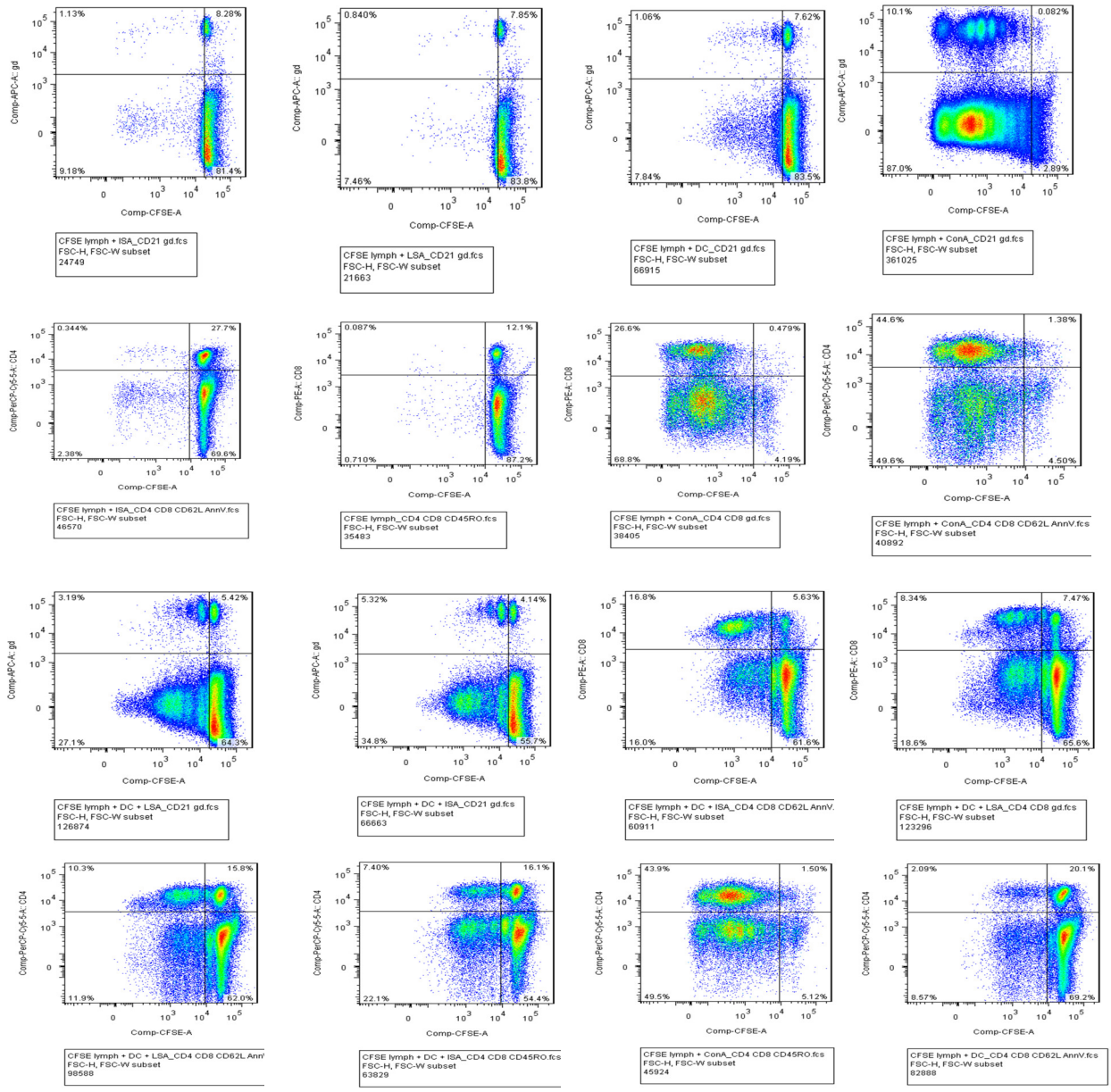


Figure 3.7. Flow cytometry plots showing proliferation of CFSE labeled lymphocytes (CD4, CD8 and γδ) stimulated with ISA or LSA loaded DC, unstimulated DC, ConA, ISA and LSA. Addition of CFSE labeled lymphocytes to DC loaded with ISA or LSA resulted in CD4, CD8 and γδ T cell proliferation after 5d. X-axis represents CFSE and Y-axis represents γδ APC, or CD8APC or CD4 PerCP Cy5.5 CD4 in the dot plots. Data representative of four experiments.

Table 3.1. Primer and probe sequences from 5’ to 3’ end

Gene	Forward primer	Reverse primer	Probe
GAPDH	5′ACCCCTTCATTGACCTTCA3′	5′GATCTCGCTCCTGGAAGATG3′	5′TTCCAGTATGATTCCACCCACGGCA3′
TNF-α	5′TCTCCTTCCTCCTGGTTGCA3′	5′GTTTGAACCAGAGGGCTGTTG3′	5′CCCAGAGGGAAGAGCAGTCCCCA3′
IL-6	5′CCAGAGAAAACCGAAGCTCTCA3′	5′CTCATCATTCTTCTCACATATCTCCTTT3′	5′AGCGCATGGTCGACAAAATCTCTGC3′
IL-1	5′CACCTCTCTCTCAATCAGAAGTCCTT3′	5′CGAGGTATCCAGGGACATAAACTTA3′	5′CAAGCTATGAGCCACTTCGTGAGGACCA3′
TGF-β	5′CGAGCTGTATCAGATTCTCAAATCC3′	5′TTTCACGACTTTGCTGTCAATGT3′	5′TTAACATCTCCAACCCAGCG3′
TLR 4	5′TGCGTACAGGTTGTTCCTAACATT3′	5′TAGTTAAAGCTCAGGTCCAGCATCT3′	5′AAAATCCCCGACAACATCCCCATATCAA3′
TLR1	5′GATGCCGAGAGCCTTCAAGA3′	5′ACACGTCCAAAATAAAATGGAATTC3′	5′AGTCTGCACATTGTTTTCCCCACAGGA3′
NOD2	5′CACGGATCAGGAGCGTCACT3′	5′CACCTTGCGGGCATTCTT3′	5′CCGCCCCCACGTCAGTCCAG3′
CD80	5′GGTGCTCACTGGTCTTTTTTACTTC3′	5′AGTGGATGTGTTGTAATCACAGGATAG3′	5′TTCAGGCATCACCCCAAAGAGTGTGA3′
CD86	5′TCTCTGTTTCCACTGTTCCTTTTTC3′	5′TGGCAGTTCTCCAGTCTCGTT3′	5′TGCTGCTTCCTTGAAAAGTCATGCCTTC3′

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CHAPTER 4 IDENTIFICATION OF MEMORY T CELLS TO *STAPHYLOCOCCUS AUREUS:* A STEP CLOSER TO VACCINE?

ABSTRACT

Staphylococcus aureus is a versatile pathogen causing a variety of diseases in both humans and animals. Increase in nosocomial infection and chronic mammary gland infection by antibiotic resistant strains warrants the need for an effective vaccine. This study assessed the presence of *S. aureus* specific memory T cells in previously infected cows using $CD14^{+}$ monocyte derived dendritic cells (DC). *S. aureus* loaded DC showed increased cell surface expression of MHC I class & II and cytokine gene induction, suggesting DC activation and antigen presentation. Initial screening of infected cows for memory T cells showed a significant increase in lymphocyte proliferation compared to control animals. Further characterization of proliferating $CFSE^{low}$ CD4 and CD8 T cells from infected animals revealed the presence of memory markers, CD45RO and CD62L. This is the first study to show the presence of *S. aureus* specific CD8 memory T cells in the peripheral circulation of any species. Future experiments will identify the *S. aureus* antigens that initiate T cell clonal expansion.

INTRODUCTION

Staphylococcus aureus is a major pathogen causing a wide spectrum of diseases in both humans and animals. Diseases range from superficial skin infections to severe invasive clinical illnesses such as septic arthritis, osteomyelitis, endocarditis, pneumonia, toxic shock syndrome and septicemia. *S. aureus* colonizes normal skin and respiratory tracts and any breach in the skin or mucous membrane predisposes the host to invasive disease [1]. Community associated and nosocomial *S. aureus* infections have appeared as serious health threats due to the emergence of multidrug resistant strains [2]. A recent epidemiological study has identified methicillin resistant *S. aureus* (MRSA) as a major public health problem in the United States [3]. In dairy animals, *S. aureus* is one of the organisms that causes acute and chronic mastitis [4]. Emerging virulent strains associated with both severe community-acquired human infections and MRSA mastitis in bovines emphasizes the occurrence of transfer of strains between humans and animals [5, 6]. This increased threat to both public health and animal populations warrants the need of an animal model system to study host response to *S. aureus* infection. Being a natural host, bovines provide an excellent animal model for the study of host immune mechanisms involved in *S. aureus* infection. *S. aureus* is considered a classical extracellular pathogen; however, intracellular infections have also been demonstrated [1, 7]. The intracellular persistence in endothelial cells, epithelial cells, and keratinocytes protects *S. aureus* from host immune defenses and antibiotics, adding to the incidence of recurrent and chronic infections [8].

Previous exposure to *S. aureus* results in less severe subsequent infections and patients with high titer of *S. aureus* specific antibodies are less susceptible to infections (reviewed in [9]). These studies point to the fact that an effective immunological therapy and/or vaccination might be possible to *S. aureus* infections in humans and animals [10]. Several studies tested the

efficacy of different *S. aureus* vaccines in humans and rodents, but to date none have provided complete protection [11, 12]. In cows, several studies used toxoid, or bacterin or capsular polysaccharides in vaccines; however, none of the vaccines found to provide a complete protection against *S. aureus* infection in cows [13, 14]. Emergence of multidrug resistant strains in both humans and animals, and contagious nature of the pathogen warrant the need for a multicomponent vaccine to confer complete protection to *S. aureus*.

The current vaccines available against *S. aureus* are designed to elicit antibody response. The intracellular persistence of *S. aureus* necessitates the need of a cell mediated immune response to clear the pathogen. Once challenged, it is presumed that antigen specific memory T cells persist in the individual after the resolution of initial immune response. Memory $CD8^+$ T cells may form a key cellular element of vaccine induced protection. To date, there is no information on the presence of memory CD8 T cells in vivo against *S. aureus*. In fact, to our knowledge there have been no studies to confirm the presence of any *S. aureus* specific memory based cellular immune. Although a recent study demonstrated decreased central memory T cells in circulation of children with invasive *S. aureus* infections, the antigen specificity of these memory T cells has not been addressed [15]. Variability in the prevalence and expression of virulence factors by *S. aureus* necessitates a multicomponent vaccine or combination technologies using novel adjuvants such as DC in the development of a vaccine. Effective activation and antigen presentation by DC induces clonal expansion and differentiation of T cells into effector and central memory T cells. The properties of development and maintenance of a central memory T cell pool and subsequent activation of these cells to effector phenotype during challenge could be harnessed in a properly designed vaccine.

Identification of memory T cells and their subsets will aid in the formulation of an efficient vaccine that would provide complete protection against *S. aureus* infection. The present study uses *S. aureus* loaded monocyte derived DC to stimulate autologous lymphocytes *ex vivo*. The objective of our study was to determine the presence of *S. aureus* specific memory T cells in the peripheral circulation of naturally infected dairy cows and to analyze their proliferation upon restimulation.

MATERIALS AND METHODS

Propagation of *S. aureus*

S. aureus strains used in our study are shown in Table 4.1. *S. aureus* isolates were cultured from the infected quarter milk of cows at the Virginia Tech Dairy Center, analyzed using standard biochemical tests, and saved at -80ºC for future use [16]. Frozen isolates of *S. aureus* were streaked on EBA plates and incubated overnight at 37ºC. Single colonies were cultured in tryptic soy broth at 37ºC for 4h with rigorous shaking. The incubation was restricted to 4h to minimize toxin production. Bacteria were washed twice with PBS at 4ºC and CFU/mL determined. Bacteria were irradiated in a Model 109 research cobalt irradiator (JL, Shepherd and Asscocites, San Fernando, CA) for 3h. Before use, the irradiated *S. aureus* (ISA) were washed twice with PBS and diluted to 10^8 CFU/mL with RPMI 1640 medium.

Animals

Dairy cows from the Virginia Tech dairy research facility were used for this study. All the animals were free from any visible signs of disease at the time of blood collection. The animal experiments complied with the ethical and animal experiment regulations of Virginia Tech IACUC. Cows previously diagnosed with *S. aureus* clinical mastitis and recovered (infected; n=5) were used as the infected group. Control cows (control; n=5) were chosen based on milk culturing and lack of a previous record of *S. aureus* mastitis.

Generation of bovine Monocyte derived DC

Peripheral blood mononuclear cells (PBMC) were isolated from infected and control cows. Briefly, PBMC were isolated from 250mL of blood drawn from the jugular vein into 250mL K_3-EDTA-vacuum bottles and enriched by discontinuous density gradient centrifugation with the procedure described earlier [17]. Briefly, 10mL of the buffy coat were collected and resuspended in 20mL of 1x Hanks balanced Salt Solution (HBSS) (Invitrogen, NY, USA). The

suspended buffy coat was layered over Ficoll-Paque ^{TM} plus (GE Healthcare Biosciences AB, Uppsala, Sweden) and centrifuged at 330xg for 45 min at 25ºC. Mononuclear cell layer was removed and washed three times using HBSS. Cell viability and number were determined by trypan blue exclusion test. The PBMC were incubated with antihuman CD14 microbeads (Miltenyi Biotec, CA, USA) (10uL/10^7 PBMC) for 20 min on ice. A positive selection of $CD14^+$ cells were performed by magnetic cell sorting according to manufacturer's instructions. The purity of the cells (always > 98%) was assessed by flow cytometry and cell viability was assessed (always > 99%) by trypan blue exclusion.

For DC differentiation, $CD14^+$ cells were cultured in plastic petri dishes for 6-7d in RPMI-1640 medium (Invitrogen, NY, USA) containing 10% FBS (Hyclone Labs, UT, USA), 10mM HEPES, 4mM L-glutamine (Invitrogen, New York, USA), $5x10^{-5}$ M 2-betamercaptoethanol (Sigma-Aldrich, MO, USA), recombinant bovine granulocyte monocyte colony stimulating factor (200ng/mL) and recombinant bovine interleukin-4 (100ng/mL) [18]. Media and cytokines were replenished every third d. Seven d old DC, macrophages and fresh monocytes were stained with anti-bovine MM61A (IgG1Texas red) for CD14, TH16B (IgG2a FITC) for MHCII, H58A (IgG2a FITC) for MHCI, MM10A (IgG2b PE) for CD11b, BAQ153A (IgM APC) for CD11c, MUC76A (IgG2a FITC) for CD11a, and BAQ15A (IgM APC) for CD21 (VMRD, Pullman, WA, USA) and assessed by flow cytometry.

Flow cytometric analysis

To ascertain the cell surface markers in monocytes, DC, live *S. aureus* (LSA) or ISA stimulated DC, and lymphocytes, 1 x 10^6 cell suspensions were stained with primary antibodies followed by incubation for 1h on ice. After three washings, fluorochrome-conjugated isotype specific secondary antibodies (Invitrogen/Caltag lab, USA) were added and incubated for

another 30 min on ice followed by three washings. Percentages of cells and mean fluorescence intensity (MFI) were determined using FacsCalibur flow cytometer (BD biosciences, San Jose, CA, USA) and analyzed using Flowjo software v. 7.6.1 (Tree star Inc., Ashland, OR).

Real-Time PCR

Total RNA was extracted from the unstimulated and stimulated DC using Qiagen's RNeasy Mini Kit with DNase (Qiagen, Valencia, CA, USA). cDNA was prepared from 1 µg RNA using Superscript II Reverse Transcriptase and oligo dT primers (Invitrogen, Carlsbad, California, USA). Real-time (RT)-PCR using bovine specific primers and Taqman probes (ABI, USA) was used to determine the level of transcription of TLR2, and cytokines (IL-12, IL-23 and IL-27). The Real-time RT-PCR reactions were conducted using the Taqman Universal master mix (Applied Biosystems, USA) and analyzed using ABI Prism 7300 Real-Time PCR System (Applied Biosystems, USA). CT Values were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control and expressed as fold change as calculated by the $2^{-(\Delta\Delta CT)}$ method. Briefly, ΔCT = CT of the target subtracted from the CT of GAPDH, and ΔΔCT=ΔCT of samples for target subtracted from the ΔCT of corresponding control samples. Statistical analysis was performed on ΔCT values and expressed as fold change with respect to unstimulated DC.

Proliferation Assay

For an initial screening of DC induced *S. aureus* specific memory lymphocyte proliferation, peripheral blood monocytes ($CD14^+$) were isolated from infected cows (n= 5) and control cows (n = 5). In order to assess the ability of DC to induce lymphocyte proliferation, DC (10^4) were loaded with bovine *S. aureus* isolates (multiplicity of infection (MOI), 5 or 10). The infection was terminated after 4h by adding gentamicin containing media after washing twice

and DC were incubated overnight. Day old cultures of autologous lymphocytes (1:10) were added to respective wells 24h after loading of DC. All experiments were done in quadruplicate. Concanavalin A stimulated and unstimulated lymphocytes were used as positive and negative controls, respectively. Lymphocyte proliferation was measured using Cell Titer solution (Promega, San Luis Obispo, CA USA) according to manufacturer's instruction after 5d.

Characterization of memory T cells

Autologous lymphocytes from the infected cows were labeled with CellTrace™ carboxyfluorescein diacetate succinimidyl (CFSE) ester Cell Proliferation Kit (Molecular probes, OR, USA) at a final concentration of 2.5µM for visualizing the proliferating cells as previously described [19]. CFSE labeled lymphocytes were co-cultured with ISA or LSA loaded DC for 5d. On day 5, the lymphocytes were collected and immunostained for T cell markers with bovine specific antibodies including IL-A11 (IgG2a Percp CY 5) for CD4, BAQ 111A (IgM PE) for CD8 and CACTB32A (IgG1Pe-Texas red) for WC1 N3γδ T cells. The lymphocytes were analyzed for apoptosis by staining with Annexin V. The proliferating CD4 and CD8 T cells were immunostained for memory markers, IL-A116 (IgG3Alexa 594) for CD45RO and BAQ92A (IgG1Alexa 594) for CD62L and analyzed by flow cytometry.

Statistical Analysis

The ΔCT values of RT-PCR were analyzed by two-way ANOVA using Graph Pad Prism 5.0 (San Diego, CA, USA). Proliferation data were analyzed using Student's *t* test. Significance was declared at $P < 0.05$.

RESULTS

S. aureus isolates

It has been documented that *S. aureus* produces a variety of secreted toxins during stationary phase of growth [20]. *S. aureus* isolates were profiled to determine genotypes and toxin production potential. In order to control for secreted toxin production, we cultured the bacteria only to their log phase of growth and washed ISA twice before loading of DC. *S. aureus* expresses various enterotoxins and staphylococcal protein A (Spa) that subverts the host immune responses. All *S. aureus* isolates used in this study expressed superantigens, enterotoxins A, B, C and D (Table 4.1). The majority of the isolates expressed Spa type 102 followed by 105. Isolate 4102 as identified as *S. chromogenes* and therefore used only as a control for memory development and T cell responses.

Generation of DC

The DC differentiated with GMCSF and IL-4 had greater intensity of expression of CD11a, CD11b, CD11c, and MHC class II compared to 7d monocytes. DC had a lower intensity of expression of the surface molecule CD14, which is a definitive marker for monocytes and macrophages. The distinct phenotype and expression patterns of surface molecules on following culture with cytokine cocktail confirmed the generation of DC from peripheral blood derived $CD14^+$ monocytes (data not shown).

Up-regulation of pattern recognition receptors and cytokine gene expression in activated DC

DC recognizes pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). Downstream signaling of PRRs leads to the translocation of NFκ-B to the nucleus and induces transcription of cytokine genes. Loading DC with LSA or ISA

induced up regulation of TLR2, IL-12, IL-27, and IL-23 gene expression. To assess the ability of DC to induce the transcription of cytokine genes, real time RT PCR was conducted after 24 or 48h of continuous stimulation. To mimic the *in vivo* state of infection, we incubated the cells with LSA or ISA for 24 or 48h. Stimulation of monocyte derived DC with LSA or ISA for 24 or 48h results in the induction of (Figure 4.1) (A) TLR2, (B) cytokines IL-12 (C), IL-27, and (D) IL-23 gene expression compared to unstimulated DC. Stimulation of DC with ISA or LSA increased the transcription of TLR2 ($P < 0.0001$), IL-12 ($P = 0.0002$), IL-27 ($P = 0.0003$), and IL-23 ($P < 0.0001$) mRNA compared to control. The typical pattern of cytokine induction in DC is suggestive of a Th1 or Th17 response.

MHC I and II molecules up-regulated in *S. aureus* loaded DC

To assess the ability of immature DC to mature and present antigen after loading with ISA or LSA, the expression of surface MHC molecules were measured after 24h. Greater MFI of expression of MHC II (Figure 4.2A & B) and MHC I (Figure 4.2C & D) molecules were observed after 24h of stimulation of DC with either LSA or ISA indicative of DC maturation and antigen presentation. The ability of *S. aureus* loaded DC to up regulate the MHC molecules along with transcription of adaptive immunity triggering cytokines are suggestive of the capacity of DC to prime CD4 and CD8 T cells.

Proliferative response of T cells

We next determined the ability of DC to induce efficient antigen specific lymphocyte proliferation in cows previously infected with *S. aureus* compared with control cows. In the initial screening experiments (Figure 4.3), greater proliferative response ($P < 0.01$) was observed in infected cows compared to control cows suggesting the presence of *S. aureus* specific memory lymphocyte proliferation. To delineate the proliferating lymphocytes in the infected cows,

lymphocytes were labeled with CFSE and co-cultured with either ISA or LSA loaded DC for 4d. Flow cytometric results revealed (Figure 4.4) a greater percentage of proliferating CD4, CD8, and γδ T cells compared to the controls. CFSE labeled lymphocytes were also cultured with unstimulated DC or with ISA or LSA to account for proliferation. Unstimulated DC induced proliferation of 6.1, 15.9, and 12.5 % of CD4, CD8, and γδ T cells, respectively. We gated out any proliferation induced by ISA or LSA stimulated CD4, CD8, and γδ T cells when we analyzed for proliferating CD4, CD8 and γδ T cells. As a result, 24.2 and 24.7% CD4 cells and 80.0 and 60.5% CD8 T cells proliferated in response to LSA or ISA loaded DC, respectively. Similarly, 54.2 and 35.65 % γδ T cells proliferated in response to ISA or LSA loaded DC, correspondingly.

Characterization of memory T cells

We further characterized the proliferating $CFSE^{low}$ T cells by immunostaining with memory markers; CD45RO and CD62L. $CD45RO^{high}$ and $CD62L^{low}$ expressing CD4 and CD8 were distinguished as memory phenotypes (Figure 4. 5). Accordingly, 27.5 and 29.5% of proliferating CD4 expressed CD45RO and 17.0 and 22.6% of CD4 expressed CD62L in response to DC loaded with ISA and LSA, respectively. Similarly, 62.1 and 62.1 % of proliferating CD8 expressed CD45RO and 62.0 and 43.1% of CD8 expressed CD62L in response to DC loaded with ISA or LSA. Presence of $CD45RO^{high}$ and $CD62L^{low}$ expressing T cells among proliferating CD4 and CD8 T cells confirms an effector memory phenotype in previously infected cows.

DISCUSSION

DC are professional antigen presenting cells capable of driving naïve T cell responses; however, little or no information is available on the ability of DC to induce *S. aureus* specific memory T cells, if present. The current study assessed the ability of DC to induce *S. aureus* specific T cell proliferation *ex vivo* and characterized the T cell subsets. This study demonstrates for the first time, the presence of *S. aureus* specific memory T cells in peripheral circulation of dairy cows. Phenotypic marker expression and morphology of DC in this study are consistent with other studies that have shown that monocytes can be differentiated to DC under the influence of GMCSF and IL-4 [18, 21-24]. Our preliminary studies showed that there was no difference in the expression levels of surface markers between adhered out monocytes and monocytes isolated using CD14 magnetic beads. Monocyte derived DC are widely used as a model for studying functional capabilities of inflammatory DC *in vivo*. In humans, they induce a superior memory T cell proliferation because of their ability to produce IL-12p70 and IL-23 [25]. The up-regulation of MHC I in DC might be an indication of a cytosolic route of antigen processing or a cross presentation. In this particular study, we did not conduct any experiments to differentiate cross presentation from the other two classical antigen processing pathways. Greater intensity of expression of MHC class II indicated the endocytic processing of *S. aureus* as with any classical extracellular pathogen. The up-regulation of TLR2 indicates the engagement of specific ligands of *S. aureus* on DC. Up-regulation of IL-12, IL-27, IL-23, and MHC I along with CD8 T cell proliferation are suggestive of a cell mediated response to counteract the ability of *S. aureus* to cause intracellular infections. It is yet to be investigated whether an intracellular infection occurs in association with an extracellular infection or as a way to undermine the host immune response. The typical pattern of cytokine induction and MHC up-

regulation usually trigger a T cell mediated response, esp Th1 or Th17 type is consistent with previous research [26]. The PAMPs of different pathogens stimulate DC to produce cytokines IL-6, TGF-β and IL-23 that are required for induction of IL-17 producing cells [27, 28]. The mechanism of IL-17 mediated immune response against *S. aureus* infection is not yet characterized.

In the initial screening experiments, we found significant increase in lymphocyte proliferation in previously infected cows compared to naïve cows. Greater proliferations of lymphocytes from cows previously infected with *S. aureus* as compared with naïve cows suggest the presence of an antigen specific lymphocyte population. We acknowledge that the naïve cows are free of clinical *S. aureus* mastitis; however, naïve cows may have been previously exposed to *S. aureus.* Lack of proliferation of lymphocytes indicates no memory response in naïve cows. To further characterize the proliferating lymphocytes, we assessed the response to ISA and LSA. Increased proliferation of CD4, and CD8 in response to *S. aureus* loaded DC indicated the presence of a T cell mediated memory response in cows previously exposed to *S. aureus* mastitis. A similar study has shown that DC induce efficient proliferation of T lymphocytes *in vitro* from calves vaccinated with BCG [21]. In addition, increased expression of MHC molecules and up regulation of cytokine genes suggest the antigen presentation through both MHC II and MHC I, thus driving CD4 and CD8 T cell proliferation, respectively. Antigen specific memory T cells to various pathogens have been reported [29, 30]; but, limited information is available on *S. aureus* specific memory T cells. A recent study demonstrated decreased central memory T cells in circulation of children with invasive *S. aureus* infections; however, the antigen specificity of the memory T cells has not been addressed [15]. The results of our research provide additional information about the existence of *S. aureus* specific memory

T cells in the peripheral circulation of naturally infected dairy cows. The presence of $CD45RO^{high}$ and $CD62L^{low}$ T cells among proliferating lymphocytes confirms an effector memory phenotype in previously infected cows. Several studies have reported that the bovine memory CD4 and CD8 T cells express CD45RO and CD62L [29, 30]. Challenge induced CD8 T cells falls in either central or effector memory T cell group and they differ in their functional ability and phenotypic marker expressions [31]. Whether a CD8 mediated immune response is itself sufficient to prevent *S. aureus* infection has yet to be determined.

The γδ T cells represent an important T cell population in bovines, and are present in skin and mucosal surfaces [32]. Presumably, these cells play an important role in inducing immune response to infection. γδ T cells are responsible for recognition of a wide variety of antigens including toxins, phophosantigens and lipid antigens [33]. These cells play a role in the early containment of pathogens in the initial infection site or exert an immunomodulatory effect in excessive inflammation [34]. In this experiment, γδ T cells proliferated significantly in response to LSA or ISA. It has been shown that DC are more efficient in stimulating antigen specific γδ T cell proliferation compared to monocytes [35]. In this study, memory phenotyping was not performed because memory markers for γδ T cells in bovines have yet to be developed. Various studies have reported that vaccinations and infections can primeWC1 γδ T cells *in vivo* [36-38]. Research has established that memory CD8 T cells to BCG vaccine reside in the αβ T cells and not in the γδ T cells [39]. The protective role of γδ T cells in *S. aureus* infection in bovines and humans remains to be investigated.

Even though the presence of memory T cells in the peripheral circulation is evident in many infections, the migration of these cells to peripheral tissues such as skin, intestines and lungs are limited [40]. Migration of memory T cells depend on the expression of different

molecules such as CD45RO, CCR7and CD62L or specific combination of these molecules [41]. This could be a reason why even though memory T cells are present in peripheral circulation of previously infected cows, the migratory capacity of these memory cells to the mammary gland might be impaired resulting in the occurrence of chronic infections. Another reason could be the capability of S. *aureus* to ward of the immune response by forming abscesses. Future studies will be directed towards the identification of *S. aureus* antigens responsible for T cell memory and to increase the migration potential of central memory T cells to the site of infection. In conclusion, memory T cells are present in the peripheral circulation of cows previously infected with *S. aureus* and are able to proliferate on a second stimulation.

Table 4.1. Profile of *S. aureus* isolates used in this study

Isolate	Suffix	spp	spa	cassettes	Enterotoxin genes
3693	3_44	aureus	402	tmk	C
4028	3_9	aureus	102	zb	C
4003	3_62	aureus	102	zb	C
4078	6_81	aureus	102	zb	C
4183	6_13	aureus	88	ujgfmbbbbpb	C, A
4131	7_68	aureus	105	u.new.gfmbbbpb	
3783	6_57	aureus	102	zb	C
4077	1_21	aureus			
3937	6_14	aureus	105	ujgfmbbbpb	
4102	1_11	chromogenes			
4065	9_33	aureus	92	ujgfmbbpb	C, A
4170	9_24	aureus	309	tjmbmdm	B, D
4338	9_35	aureus	105	ujgfmbbbpb	C

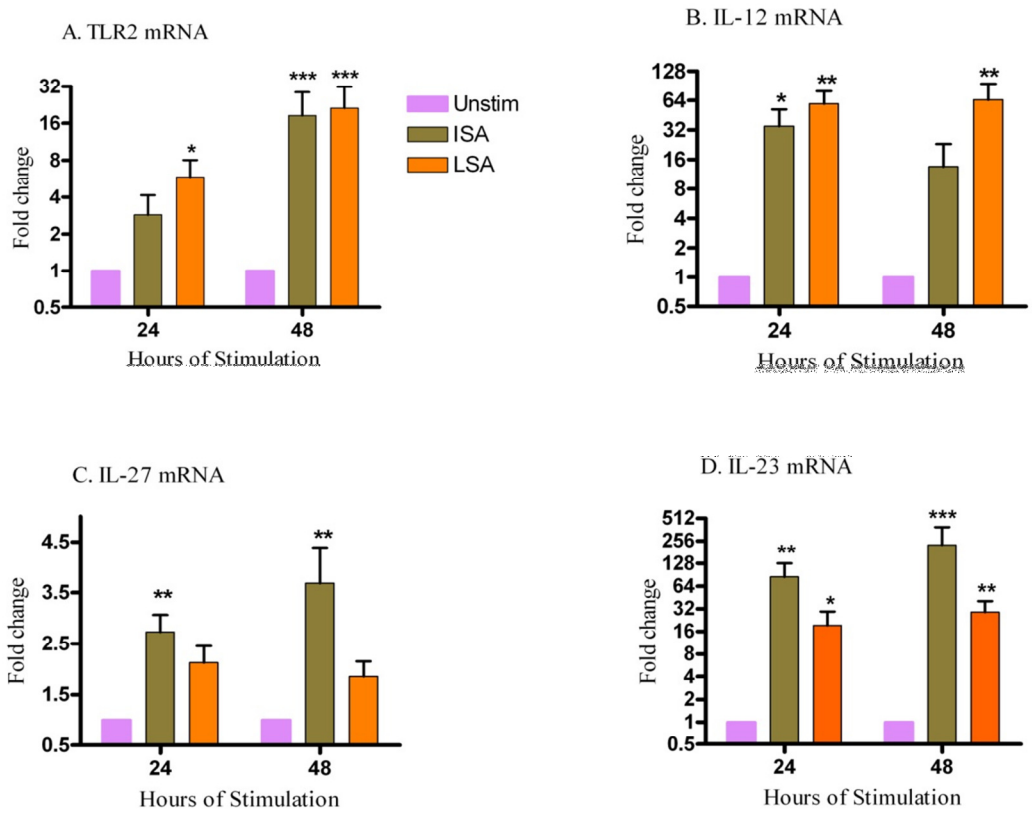

Figure 4.1. Transcription of TLR2 and cytokine genes at 24 and 48 h of *S. aureus* stimulation. Stimulation of monocyte derived DC with LSA or ISA resulted in the induction of (a), TLR2 and cytokines (b) IL-12 (c), IL-27 and, (d) IL-23 gene expression. Data represent mean ± SE from six independent experiments. The stars above the bars indicate significant differences (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$) compared to unstimulated cells.

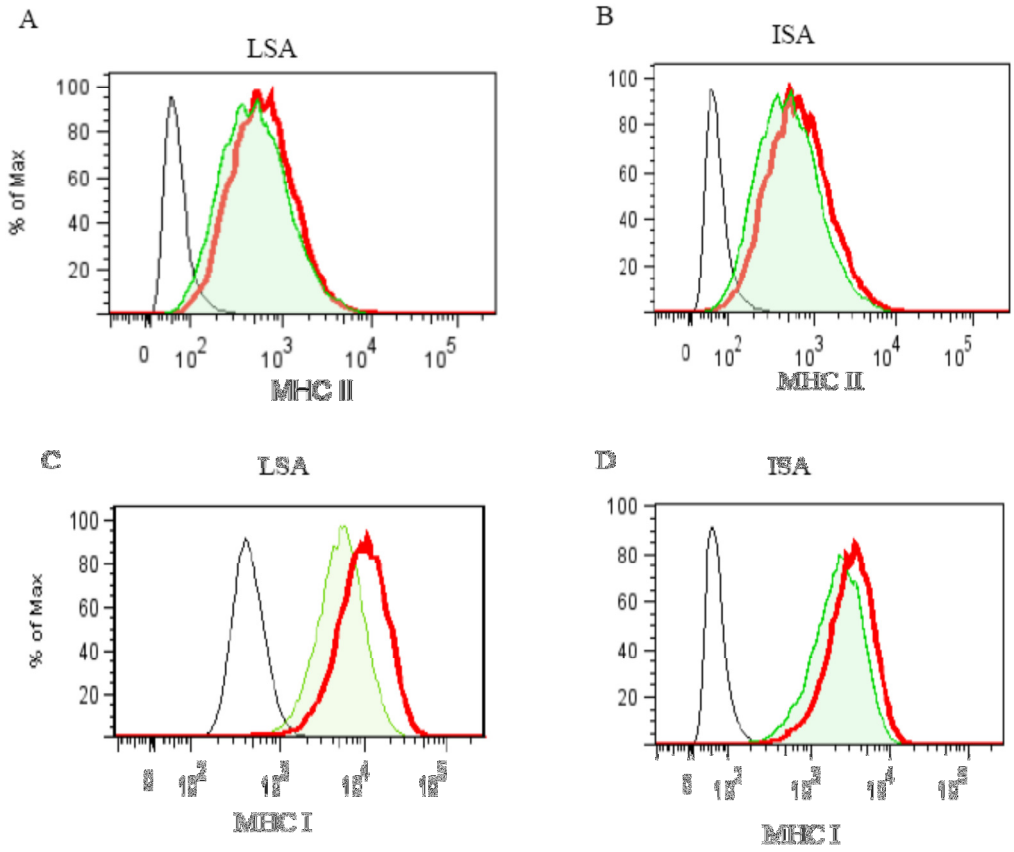

Figure 4. 2. Increased intensity of expression of MHC in *S. aureus* loaded DC. DC loaded with LSA and ISA showing increased intensity of expression of MHC II (A&B) and MHC 1(C & D) proteins after 24 hours of initial stimulation. Black unfilled histograms represent isotype controls; green filled histogram for unstimulated DC and red unfilled histogram for LSA or ISA loaded DC. Data representative of three independent experiments.

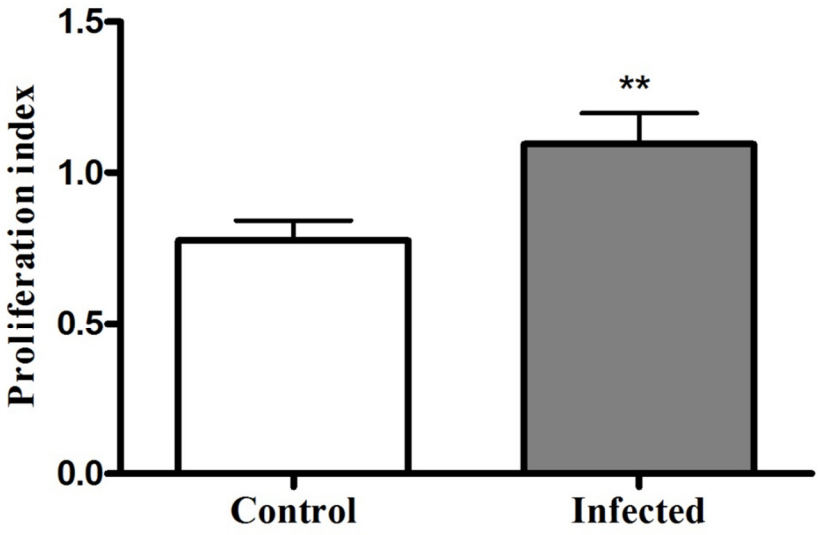

Figure 4. 3. LSA loaded DC induced lymphocyte proliferation. Bovine DC (n=5) were stimulated with MOI 5 for 4 hours and the infection terminated with gentamicin. 24 hours later autologous lymphocytes were added to the DC cultures with appropriate controls. Values represent the OD values of the controls subtracted from OD values of LSA loaded DC cultured with lymphocytes for each cow. Data represent mean $\pm$ SE. Significance was $P = 0.01$.

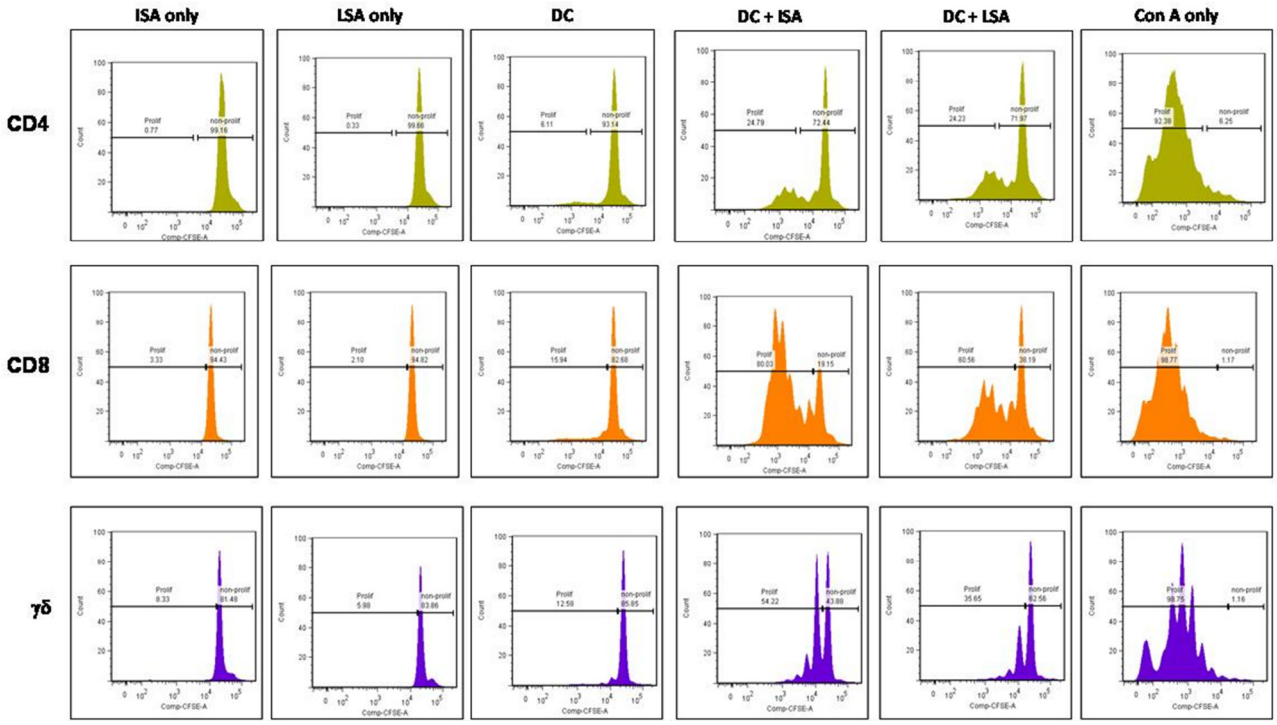

Figure 4. 4. Proliferation of T cell subsets in response to *S. aureus* loaded DC. Lymphocytes were loaded with CFSE and stimulated with ISA, LSA, DC, or DC loaded with ISA or LSA, or ConA for 5d. Flow cytometry data presented is percentage of CFSE positive cells within each population. Gate on the right indicates non-proliferating cells; gate on the left indicates proliferating population. Data representative of five independent experiments.

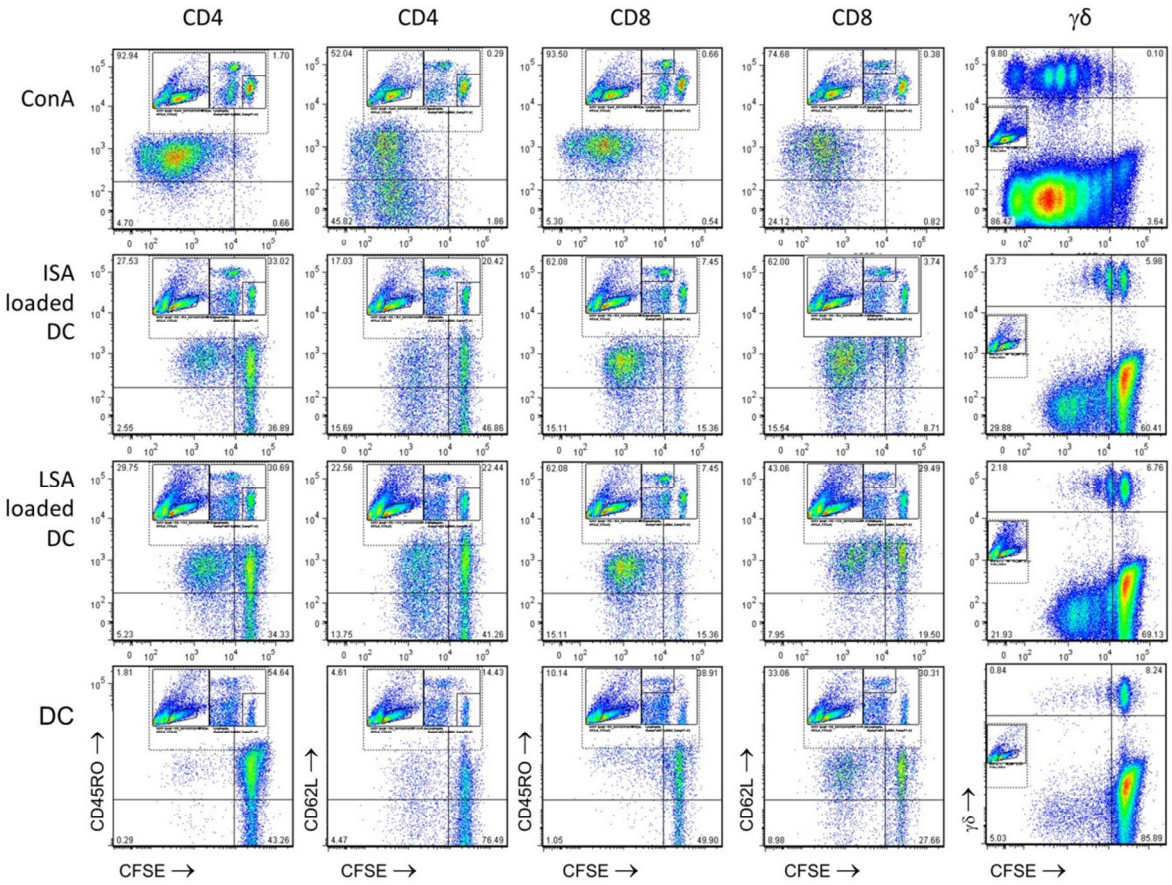

Figure 4. 5. Presence of memory T cells among T cell subsets proliferating in response to *S. aureus* loaded monocyte derived DC. Lymphocytes were labeled with CFSE and stimulated for 5 d with ConA, monocyte derived DC loaded with ISA, LSA or media. Dot plots represent cells in the CD4 or CD8 gate labeled with CFSE (X-axis) and expressing the memory markers CD45RO or CD62L (Y-axis). WC1γδ data is presented from cells in the lymphocyte gate. Insets represent source gates for each population. Data representative of five independent experiments.

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CHAPTER 5 A NOVEL ROLE FOR DEC205 IN THE INTERNALIZATION OF *STAPHYLOCOCCUS AUREUS* BY DENDRITIC CELLS

ABSTRACT

Dendritic cells (DC) recognize pathogen-associated molecular patterns (PAMPs) from various pathogens through pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), nucleotide oligomerization domains (NODs) and C-type lectin receptors (CLRs) leading to their activation. CLRs are Ca^{++} dependent carbohydrate -binding proteins. DEC205 is an endocytic CLR expressed on DC that mediates endocytosis. Although various studies have targeted proteins to DEC205 receptor, the pathogenic ligands for this receptor have not yet been identified. Hence, we propose that DEC205 plays an important role in the endocytosis of *S. aureus*. The goal of this study is to investigate whether DC use DEC205 for internalization of *S. aureus* and facilitate antigen presentation by up regulating MHC expression. Blocking of receptor mediated endocytosis using chlorpromazine confirmed uptake of *S. aureus* by DC through receptor mediated endocytosis. After 3h of loading of DC with *S. aureus,* significant down-regulation of surface DEC205 suggests DEC205 internalization. This pattern continued till 24h of loading and the receptor recycle back to the surface by 48h. Confocal microscopy confirms the co-localization of CD205 and *S. aureus*. To assess antigen presentation and activation of DEC205 DC, DC were loaded with irradiated (ISA) or live (LSA) *S. aureus* and analyzed by flow cytometry, real-time RT-PCR and Western blot for antigen presentation, cytokine genes and cytokines, respectively. Results show that DC up-regulate both MHC I and MHC II indicating antigen presentation. The induction of CD80 and CCR7 and various cytokine mRNA confirms DC activation. In conclusion,

S. aureus uses DEC205 for its internalization resulting in DC activation and antigen presentation. The findings of this study will aid in targeting DC endocytic receptors for *S. aureus* vaccine formulation.

INTRODUCTION

Staphylococcus aureus is a versatile pathogen causing a wide spectrum of diseases in both humans and animals [1]. Some of these infections may be fatal. A significant number of clinical cases of *S. aureus* infections are caused by methicillin resistant strains (MRSA)[2]. Nosocomial and community associated MRSA infections have emerged as a serious health threats around the globe [3-5] . Notably, it has recently been estimated that MRSA causes more deaths annually in the USA than AIDS [6]. Such drug resistant *S. aureus* strains have been transmitted from humans to animals and vice versa, potentially an emerging zoonosis [7].

The multidrug resistance and contagious nature of the pathogen has made the treatment of *S. aureus* infections often very difficult. Unfortunately, given the high costs and lack of success in antimicrobial discovery, not many pharmaceutical companies are investing in the development of new antibiotics. In addition, overuse of new antibiotics may eventually results in the spread of resistant strains [8]. Horizontal transfer of genes encoding for antibiotic resistance and or virulence factors is responsible for multidrug-resistant *S. aureus* infections and current epidemiology [9, 10]. So far there is no working vaccine or effective passive immunization available for the treatment of severe *Staphylococcal* infection, despite considerable research efforts. *S. aureus* possesses the ability to evade the innate and adaptive immune mechanisms of the host [11, 12]. As a consequence of problems related to antibiotic resistance and past failures of immunotherapy, currently researchers are intensively investigating alternative therapeutic

options, especially the development of vaccines using DC and therapeutic antibodies against S. *aureus* infections.

DC are professional antigen presenting cells capable of initiating naïve T cell activation. Immature DC express a wide variety of receptors on their surface [13] including phagocytic receptors, lectins, pattern recognition receptors (PRRs) and scavenger receptors. Several reports indicate that targeting antigens to DC receptors increases antigen presentation to CD4 and CD8 T cells *in vivo* [14-17]. Antigen presentation on MHC I and II is increased when protein antigens are targeted to DC DEC205 [18-20]. DEC205/CD205 is a CLR that can function as an endocytic receptor [21]; however, the ligand for this receptor is waiting to be identified. The CLRs are Ca^{++} dependent glycan-binding proteins that internalize their ligands through clathrin coated pits resulting in the delivery of ligands to lysosomes or late MHC II rich endosomes [18]. *In vivo* targeting of antigen to DEC205 receptors results in an initial increase in CD4 and CD8 T cell proliferation, followed by a state of tolerance in the absence of DC maturation [19, 22]. However, the presence of maturation stimuli or targeting protein antigens to DEC205 in matured DC improves T cell vaccination [15]. DEC205 has been shown to uptake and process protein antigens such as ovalbumin, thereby inducing a strong CD4 and CD8 T cell response [23, 24]; however, the pathogenic ligands for DEC205 or the signaling proteins involved are not yet described [25].

S. aureus is a classical extracellular pathogen that has a variety of mechanisms to subvert innate and adaptive immunity [26]. CLRs such as mannose binding lectins have been shown to coordinate Toll like receptor (TLR) 2 and TLR6 binding to *S. aureus* [27]. However, there are no reports so far indicating the role of DEC205 in *S. aureus* uptake by DC. Our preliminary data suggest that DEC205 plays a role in the uptake of *S. aureus* by DC. Hence, the role of DEC205

in *S. aureus* infection needs to be described. We hypothesized that DC use DEC205 for *S. aureus* internalization, induce DC maturation and facilitate antigen presentation by up-regulating MHC expression. Our objectives were to elucidate the role of DEC205 in *S. aureus* uptake and to evaluate antigen presentation and activation of DC upon *S. aureus* stimulation.

MATERIALS AND METHODS

Animals

Four dairy cows from the Virginia Tech Dairy Research Facility were used for this study. As per the records, all the animals were free from any visible signs of disease at the time of blood collection. When screened for the level of expression of DEC205 in monocyte derived DC, we observed that there is high variability (5-94%) between animals. Hence, we used monocyte derived DC ($DEC205^{high}$) from three cows (>80% of DC express DEC205) to perform experiments. As per the history, these animals were never reported to have *S. aureus* mastitis during their lifetime. The animal experiments complied with the ethical and animal experiment regulations of Virginia Tech IACUC.

Propagation, irradiation and fluorescent labeling of *S. aureus*

Single colonies of RN6390B strain of *S. aureus* were cultured in tryptic soy broth for 4h with rigorous shaking. The cultures were washed three times with Hanks balanced Salt Solution (HBSS) (Invitrogen, NY, USA) HBSS and pelleted at 1500xg for 10 min. Cultures were then serially diluted and drop plated to get the actual colony counts per mL. Bacteria were irradiated in a Model 109 research cobalt irradiator (JL, Shepherd and Asscocites, San Fernando, CA) for 3h. Before use, the irradiated *S. aureus* (ISA) were washed twice with HBSS and diluted to 10^9 CFU/mL with Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, NY, USA).

Fluorescent labeling of *S. aureus* was performed with some modifications of the procedure described earlier [28]. Briefly, 10^9 CFU /mL of RN6390B strain *S. aureus* in carbonate bicarbonate buffer were incubated with 50µg of Alexa 555 succinidymyl ester (A20009; Molecular Probes, Invitrogen, USA) or 100 µg of fluorescein isothiocyanate (FITC) (46425; Thermo scientific, IL, USA) isomer I /ml for 2h in dark at 37ºC. The cultures were then

washed three times with HBSS and analyzed for uniformity of staining by fluorescent microscopy. Labeled cultures were suspended in HBSS and stored at -20ºC until use.

Culturing of monocyte derived DC

Peripheral blood mononuclear cells (PBMC) were isolated from infected and control cows. Briefly, PBMC were isolated from 250 mL of blood drawn from the jugular vein into 250mL K_3-EDTA-vacuum bottles and enriched by discontinuous density gradient centrifugation with the procedure described earlier [29]. Briefly, 10 mL of the buffy coat were collected and suspended in 20 mL of 1x HBSS. The suspended buffy coat was layered over Ficoll-Paque ™ plus (GE Healthcare Biosciences AB, Uppsala, Sweden) and centrifuged at 330xg for 45 min at 25ºC. Mononuclear cell layer was removed and washed three times using HBSS. Cell viability and number was determined by trypan blue exclusion test. The PBMC were incubated with anti-human CD14 microbeads (Miltenyi Biotec, CA, USA) (10µL/10^7 PBMC) for 20 min on ice. A positive selection of $CD14^+$ cells were performed by magnetic cell sorting according to manufacturer's instructions. The purity of the cells (> 98%) was assessed by flow cytometry and cell viability was assessed (> 99%) by Trypan blue exclusion. For DC differentiation, CD14 bead purified peripheral blood monocytes were cultured in plastic petri dishes for 6-7d in RPMI-1640 medium (Invitrogen, NY, USA) containing 10% FBS (Hyclone Labs, UT, USA), 10mM HEPES, 4mM L-glutamine (Invitrogen, New York, USA), $5x10^{-5}$ M 2-betamercaptoethanol (Sigma-Aldrich, MO, USA), recombinant bovine granulocyte monocyte colony stimulating factor (rb-GMCSF) (200ng/mL) and interleukin (IL)-4 (100ng/mL) as described earlier [30]. Media and cytokines were replenished on every 3d.

Flow cytometric analysis for surface markers

Seven day old DC were stained with anti-bovine MCA1651G (AbD Serotec, Raleigh, NC, USA) (IgG2b PE) for DEC205, MM61A (IgG1Texas red) for CD14, TH16B (IgG2a FITC) for MHCII, H58A (IgG2a FITC) for MHCI, MM10A (IgG2b PE) for CD11b, BAQ153A (IgM APC) for CD11c, MUC76A (IgG2a FITC) for CD11a, and BAQ15A (IgM APC) for CD21 (VMRD, Pullman, WA, USA) and assessed by flow cytometry. Briefly, 1 x 10^6 cell suspensions of unstimulated, LSA or ISA stimulated DC were stained with primary antibodies followed by incubation for 1h on ice. After three washings, fluorochrome-conjugated isotype specific secondary antibodies (Invitrogen/Caltag lab, New York, USA) were added and incubated for another 30 min on ice followed by three washings. Percentages of cells and mean fluorescence intensity (MFI) were determined using FacsCalibur flow cytometer (BD biosciences, San Jose, CA, USA) and analyzed using Flowjo software v 7. 6. 1 (Tree star Inc., Ashland, OR). The presence of $DEC205^+$ DC was assessed by flow cytometry for each experiment and was always greater than 80%.

Endocytosis and endocytosis inhibition assay

For endocytosis assay, FITC labeled *S. aureus* (MOI 50) were added to 7d old DC cultures in antibiotic free media and incubated for 3h at 37ºC. For endocytosis inhibition assay, immature DC were pretreated with 1% serum medium containing 10µg/mL of chlorpromazine (Sigma-Aldrich, MO, USA) or medium alone for 30 min at 37ºC and subsequently incubated with FITC labeled *S. aureus* (MOI 50) for an additional 3h. Cells were then washed twice with HBSS and treated with lysostaphin for 7 min. Cells were immediately fixed and analyzed in a flow cytometer for $FITC^{+ve}$ DC.

Live confocal laser scanning microscopy

DC (10^4) were cultured in 36mm glass bottom dishes (Mat Tek Corporation, Ashland, MA, USA). DC were incubated with anti-bovine DEC205 primary antibody (MCA1651G; Abd serotec, Raleigh, NC, USA) for 30 min at 37°C. Alexa 647-conjugated IgG2b Ab (Invitrogen/Caltag lab, New York, USA) was used to visualize the staining of primary Ab for another 30 min. Cells were then incubated with Alexa 555 labeled *S. aureus* (MOI 50) for 3h. Cells were washed three times and treated with lysostaphin followed by washing and staining for another 30 min each with 250nM Oregon green tubulin (T34075; Molecular Probes, Invitrogen, USA) and 1µM LysoTracker blue DND-22 (L-7525; Molecular Probes, Invitrogen, USA). Cells were washed and added RPMI media without phenol red to the DC culture. Cells were visualized at a magnification of 40x or 100x with a Zeiss confocal microscope (LSM510 META microscope) and analyzed using ZEN 2009 software (Carl Zeiss Microimaging, Germany). For co-localization after 15 min, we used FITC labeled *S. aureus* and Alexa 594 labeled secondary antibody to visualize DEC205. Cells were visualized at a magnification of 62x with a LSM510 META microscope (Zeiss).

Real-time RT-PCR

Total RNA was extracted from the unstimulated, 6h ISA and LSA stimulated DC using Qiagen's RNeasy Mini Kit with DNase (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. cDNA was prepared from 1µg RNA using Superscript II Reverse Transcriptase and oligo dT primers (Invitrogen, Carlsbad, California, USA). Bovine specific primers / probes were designed using Primer Express 3 software (Applied Biosystems, USA) as described earlier [31]. Real-time RT-PCR using bovine specific primers and Taqman probes (Applied Biosystems, USA) were used to determine the level of transcription of cytokines (IL-12, IL-23 and IL-27) and maturation markers (CD80, CD86 and CCR7). The RT-PCR reactions

were conducted using the Taqman Universal master mix (Applied Biosystems, USA) and analyzed using ABI Prism 7300 Real-Time PCR System (Applied Biosystems, USA). The CT Values were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control and expressed as fold change as calculated by the $2^{-(\Delta\Delta CT)}$ method. Briefly, ΔCT = CT of the target subtracted from the CT of GAPDH, and $\Delta\Delta CT = \Delta CT$ of samples for target subtracted from the ΔCT of corresponding control samples. Statistical analysis was performed on ΔCT values and expressed as fold change with respect to unstimulated DC.

Western blot

Bradford assay was performed to assess the total protein in samples to ensure equal protein loading [32]. The proteins were separated by Novex Midi Gel (Invitrogen Co, CA, USA) electrophoresis at 100V for 90 min and transferred on to a Immobilon-FL PVDF membrane at 60mA for 90 mins. The resulting membrane was blocked with Starting Block^{TM} blocking buffer (Thermo Scientific, IL, USA) for 1h. The membrane was incubated with the primary antibody (IL12p40; clone CC301 MCA1782EL, AbD Seortec Raleigh, NC, USA) at 4ºC overnight in blocking buffer. Immunoreactive proteins were visualized by incubation with goat anti-mouse IgG secondary antibody (Bio-Rad). The signals were detected with an ODYSSEY Infrared Imaging System (LI-COR, Lincoln, NE, USA) and the gray value of protein bands quantified with Photoshop (Adobe, San Jose, CA).

RESULTS

Existence of a $CD205^{+}CD8\alpha^{+}$ subset in bovine monocyte derived DC

During the experiments, we noticed that monocyte derived DC from certain animals express very high levels of DEC205. To analyze the presence of a $DEC205^{+}CD8\alpha^{+}$ subpopulation as in mouse, DC were immune-stained for the presence of CD8α along with DEC205. Flow cytometry results indicate that 14% of DC express both DEC205 and CD8α (Figure 5.1).

DEC205 expression decreases after 3h of *S. aureus* stimulation

We found from our preliminary experiments that maximum uptake of *S. aureus* occurs by 3h in DC (Chapter 3). Since DEC205 is an endocytic receptor, we considered the possibility that its surface expression changes following *S. aureus* uptake. After 3h of incubation with *S. aureus,* extracellular bacteria were lysed with lysostaphin treatment followed by staining the DC for DEC205 expression. Here, we noticed that after 3h of *S. aureus* uptake by DC, the percentages of DC expressing DEC205 decreased (Figure 5. 2A) compared to unstimulated DC ($P = 0.005$). Upon *S. aureus* uptake by DC, the MFI of expression of DEC205 also decreased (Figure 5.2B) from 3948 to 1320 suggesting receptor internalization along with *S. aureus*.

Blocking of endocytic receptors with chlorpromazine reduces *S. aureus* uptake

To confirm whether the internalization of *S. aureus* was through clathrin coated pits, DC were treated with 10µg/mL of chlorpromazine (Sigma- Aldrich, USA) 30 min prior to *S. aureus* loading. DC cultures were then incubated with FITC labeled *S. aureus* for 3h followed by lysostaphin treatment. After 3h, flow cytometry analysis showed that chlorpromazine treatment decreased *S. aureus* uptake from 81.1 to 26.2 % (Figure 5.3) compared to the untreated DC confirming a role for uptake by receptor mediated endocytosis. We were able to establish the same results when we repeated the experiment with DC from 3 cows.

Recycling of DEC205 receptors occurs in *S. aureus* stimulated DC

In order to assess the recycling of DEC205 back to the cell surface on *S. aureus* stimulated DC, DC were stained for DEC205 after 24 and 48h of stimulation. We found that the surface expression levels were significantly lower in ISA ($P = 0.05$) and LSA ($P = 0.01$) stimulated DC compared to the unstimulated controls (Figure 5.4) suggesting receptor internalization along with *S. aureus*. However, *S. aureus* loaded DC up-regulated surface expression of DEC205 at 48h of stimulation signifying recycling or denovo synthesis of this receptor. There was no significant difference ($P = 0.99$) in surface DEC205 expression in stimulated DC compared to unstimulated DC at 48h after stimulation.

DEC205 and *S. aureus* co-localization occurs in DC

To verify the internalization of *S. aureus* and DEC205, we looked for any co-localization (*S. aureus*- DEC205) and receptor internalization following addition of *S. aureus* to DC cultures at 15 min and 3h, respectively. In order to demostrate DEC205 mediated endocytosis of FITC labeled *S. aureus*, DC were visualized at 62x magnification in a confocal microscope after probing DC with anti-DEC 205 antibody at 15 min of *S. aureus* addition. Colocalization was observed at 15 min at the surface of DC (Figure 5. 5A). To visualize ligand-receptor internalization and delivery to the lysosomal compartments, anti-DEC205 probed DC were stained with lysotracker blue after 3h incubation with *S. aureus*. Tubulin was stained using Oregon green. DC were analyzed under 40x or 100x objective of a confocal microscope. After 3h, the majority of the DEC205 was found to be intracellular with relatively small amounts of expression at the cell surface (Figure 5.5B). It is of note that in Figure 5.5C, $DEC205^{+}$ DC show abundant bacterial uptake compared to $DEC205^{low/-}$ DC. This reinforces our previous result that surface DEC 205 plays an important role in *S. aureus* internalization.

Stimulation of DEC205$^+$ DC with *S. aureus* results in antigen presentation and DC activation

The final question we asked was whether *S. aureus* stimulation induces antigen presentation with DC activation. We observed up regulation of both MHC I and MHC II surface expression on DC 24h after stimulation with ISA or LSA (Figure 5.6). The results indicate that MHC class II molecules are redistributed from the intracellular components to the cell surface upon maturation.

We tested the mRNA expression of TLR2, cytokines and co-stimulatory molecules in DEC205 DC at 6h of stimulation with media, ISA, LSA, or LPS. The cytokine responses at 6h of stimulation were assessed by real-time RT-PCR. DC activation was confirmed by increased gene expression of cytokines, and costimulatory molecules such as CD80 and CCR7 at 6h of stimulation with ISA and LSA (Figure 5.7). Stimulation with ISA, LSA or LPS significantly increased ($P < 0.05$) gene expression of TLR2 in DC compared to control. TNF-α gene expression was increased ($P < 0.001$) for all treatments compared to unstimulated DC at 6h. Similarly, IL-1β expression was increased for ISA ($P < 0.01$), LSA ($P < 0.001$), and LPS ($P < 0.01$) treatments. Compared to control, IL-6 gene expression was increased ($P < 0.01$) for ISA and LSA; however, LPS was less efficient in induction at 6h ($P > 0.05$). Gene expression of IFN-γ was up-regulated in LPS stimulated DC ($P < 0.0001$) whereas ISA and LSA treatments were not significant. A significant increase ($P < 0.01$) in IL-23p19 expression was induced by ISA, LSA, and LPS at 6h. The Th1 response inducing cytokine IL-12p40 mRNA expression was significantly up-regulated by ISA, LSA and LPS ($P < 0.0001$). Similarly, the gene expression of IL-27 was induced ($P < 0.001$) by all treatments at 6h. The costimulatory molecule CD80 transcription was significantly increased ($P < 0.01$) by all treatments. The CCR7 mRNA expression was significantly induced in LSA ($P < 0.01$) and LPS ($P < 0.001$); however, no effect was noticed with ISA stimulation. Results suggest that

recognition of *S. aureus* and signaling through TLR2 results in the gene expression of cytokine and co-stimulatory molecules. The data indicates and activation and antigen presentation in DEC205 DC.

IL-12p40 and TNF-α protein expression increases in stimulated DC

Stimulation of DEC205^{+ve} DC with ISA, LSA or LPS substantially increased the level of IL-12p40 (Figure 5.8A) and TNF-α (Figure 5.8B) in 24h supernatants suggesting their ability to polarize T cells to Th1 or Th17 pathway. IL-12p40 forms the shared subunit for both IL-12 and IL-23. The protein could be either IL-12 or IL-23 as indicated by the mRNA induction of both genes occurring at 6h of stimulation. These results imply that a cell mediated immune response can be elicited by DEC205 DC to *S. aureus*.

DISCUSSION

In the current study our data suggest that DC use DEC205 for *S. aureus* internalization. Other surface CLRs or TLRs may also play a role in the uptake of *S. aureus*. We could find only less than 5% $DEC205^+$ monocytes in the peripheral circulation of the animals used in this study. These monocytes gained DEC205 expression when they were cultured with GMCSF and IL-4 for DC differentiation. Our preliminary studies showed that there was no difference in the expression levels of surface markers between adhered out monocytes and monocytes isolated using CD14 magnetic beads. When screened for the level of expression of DEC205 in DC in twelve dairy cows, we observed that there is high variability (5-94%) between animals. This is also the first study that reports the presence of $DEC205^+CD8\alpha^+$ population in bovine monocyte derived DC. This population has the unique ability to cross present antigens to CD8 T cells [33-35]. CD8α is believed to facilitate the presentation of uptaken antigen to CD8 T cells via MHC I [36-38]. Presumably, $DEC205^+CD8\alpha^+$ population might have a role in the induction of immune response to *S. aureus*. Apart from the specific endocytic pathway inhibition assays, we used animals with very low and high level of natural DEC205 expression in monocyte derived DC for *S. aureus* uptake studies (data not shown). DC from $DEC205^{high}$ animals showed increased uptake of *S. aureus* compared to DC from $DEC205^{low}$ animals suggesting the role of DEC205 in *S. aureus* uptake.

DEC205 is an endocytic CLR present in DC and our data are consistent with other studies which show the endocytic ability of this receptor. DEC205 receptors mediate adsorptive uptake and possess cytosolic domains with clathrin-coated pit localization [21]. Blocking of receptor mediated endocytosis via clathrin coated pits using chlorpromazine resulted in significant reduction of *S. aureus* uptake by DC. Down-regulation of DEC205 surface expression

as well as increased expression of DEC205 along with FITC labeled *S. aureus* in the intracellular compartment 3h after addition of *S. aureus* was confirmed by flow cytometry and confocal microscopy. These data strongly suggest that DEC205 has an important role in the internalization of *S. aureus*. We have shown that *S. aureus* loaded DC recycle DEC205 to the surface at 48h of stimulation and the data are consistent with earlier studies [39]. As a result of recycling, there will be a substantial enhancement in the efficiency with which peptides are saved and presented as MHC–peptide complexes [18].

DEC205 mediated delivery of *S. aureus* to the endolysosomal compartments along with MHC I and II up-regulation suggest DC's ability to cross present antigens. Cross-presentation is the processing of extracellularly derived non-replicating internalized antigen onto MHC I for recognition by $CD8^+$ T cells. Studies report that an endolysosomal compartment present in DC facilitates the acquisition of exogenously derived peptides for cross presentation. In DC, cross presentation occurs when DC acquires exogenous antigens by phagocytosis and transfer to cytosol for proteosomal degradation. Following degradation, loading of processed peptides to MHC I occurs in endoplasmic reticulum and transported to the cell surface for presenting antigens to CD8 T cell (reviewed in [40]).

DC activation is characterized by the induction of cytokines and costimulatory molecules upon recognition of pathogenic stimuli. DC activation induces the expression of chemokine receptors such as CCR7 enabling them to migrate to the draining lymph nodes to elicit T cell response [41]. CD80 gene induction suggests DC's costimulatory ability on maturation. In addition, mRNA induction and protein expression of cytokines such as IL-12, IL-23 and IL-27 indicating DC ability to polarize T cells to a Th1 or Th17 response. As suggested by earlier studies, IL-12 might have induced the up-regulation of IFNγ genes [42]. In our study, we

detected increased expression of TNF-α which may have contributed to effective maturation of antigen loaded DC [43].

Previous research has shown that ligand binding / targeting to DEC205 alone does not induce DC activation [19]; however, in our study, stimulating DC with ISA or LSA resulted in DC activation. Signaling through PRRs such as TLRs might have induced DC activation in our study. Specifically, we showed that *S. aureus* stimulation of DEC205 DC induces gene expression of proinflammatory and adaptive immunity eliciting cytokines, and co-stimulatory molecules along with antigen presentation on MHC class I and II. Altogether, our results are the first to suggest that DEC205 DC were able to uptake, process, mature and present *S. aureus* antigens on MHC efficiently. Additional blocking experiments are needed to confirm the role of DEC205 in *S. aureus* internalization. This experimental approach will provide evidence that DEC205 is essential for optimal immune response of DC to *S. aureus.*

The functional outcomes of DC-T cell interactions in response to target antigens are critical in the differentiation of an effective T cell memory and protective immunity. Characterization of the signals and molecules involved in DC-T cell interactions in response to *S. aureus* infection might be useful for the design of a successful vaccine. Targeting the DEC205 endocytic receptor on DC for antigen delivery would hopefully generate long-lived memory T cells that confer lasting immunity against *S. aureus* with appropriate specificity.

Our results are suggestive of *S. aureus* as a ligand for DEC205 receptor and an inducer of antigen presentation and DC activation. In this study, we were able to identify a unique $DEC205^{+}CD8^{+}$ population in bovine monocyte derived DC. Using DEC205 for *S. aureus* targeted delivery, the ability of DC for antigen presentation and activation could be enhanced. This could be harnessed for developing a T cell based vaccine for *S. aureus*.

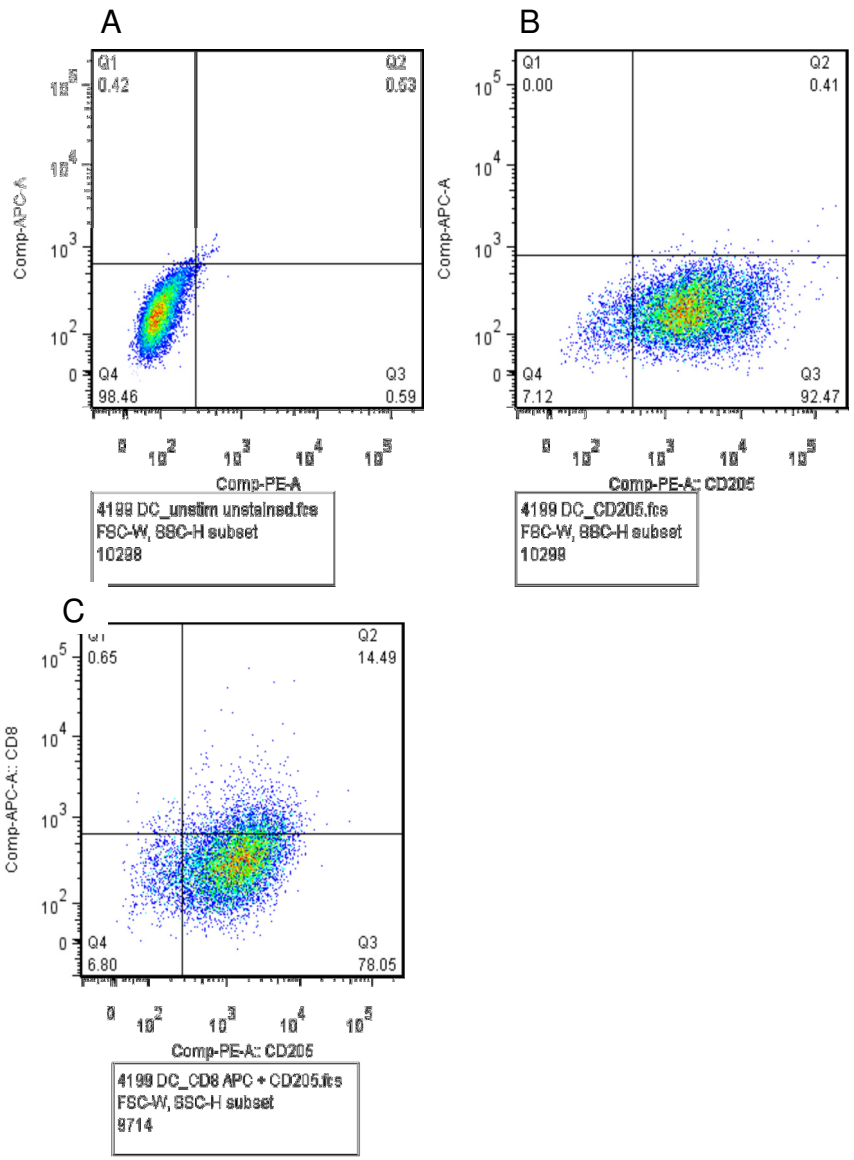

Figure 5.1. DEC205^{+}CD8α^{+} DC in bovine monocyte derived DC. Seven day old monocyte derived DC stained with isotype control **(A)** or DEC205 PE alone **(B)** or with CD8α for double positive cells **(C)**. 92.4 % are single positive for DEC205 and 14.4 % of the stained DC are double positive for DEC205 and CD8α APC. X-axis represents DEC205 PE and Y-axis denotes CD8α APC. Data representative of two independent experiments.

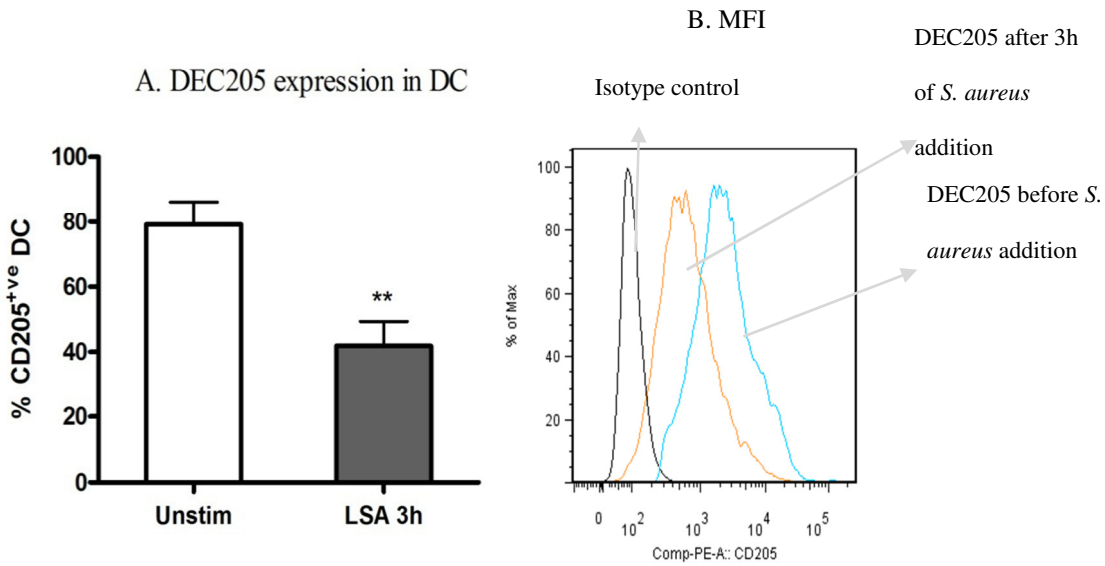

Figure 5.2. DEC205 expression in unstimulated and *S. aureus* loaded DC. After 3h of stimulation with *S. aureus* (MOI 50), DC were stained with anti-bovine DEC205 monoclonal antibody for DEC205 receptor expression, fixed and analyzed by flow cytometry. *S. aureus* infected DC showed a significant decrease in ($P = 0.005$) in (A) DEC205 expression compared to uninfected DC on a paired t test. Data represent mean $\pm$ SE. (B). Represents decrease in mean fluorescent intensity of DEC205 after *S. aureus* internalization. Data from four cows.

A B

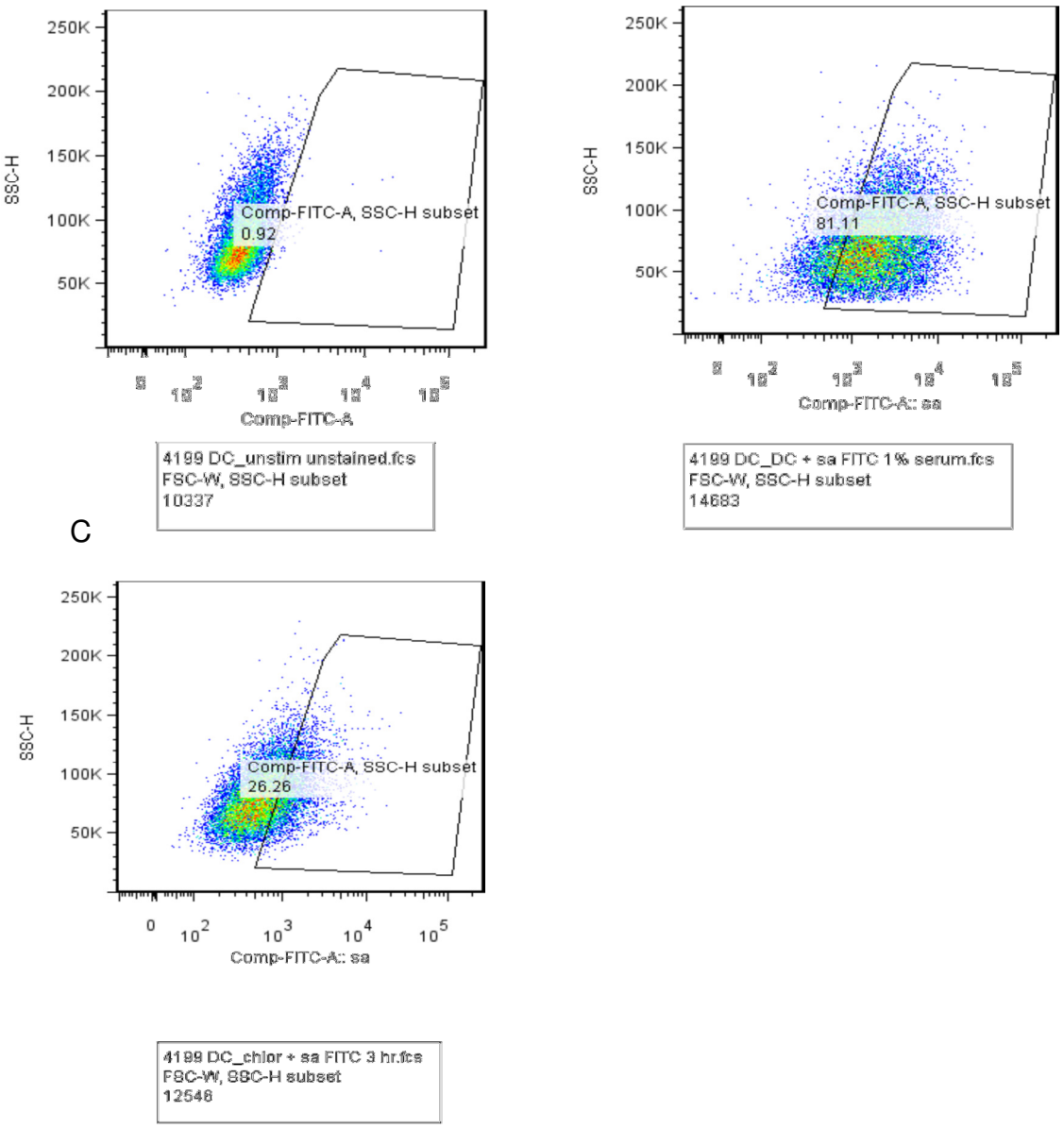

Figure 5.3. Blocking with chlorpromazine reduces *S. aureus* internalization by DC. DC were treated with or without chlorpromazine in 1% serum media for blocking receptor mediated endocytosis for 30 min. FITC labeled *S. aureus* was added to the DC cultures at MOI 50. After 3h, cells were washed, treated with lysostaphin, fixed and analyzed by flow cytometry. **(A)** unstained cells,**(B)** *S. aureus* uptake by control DC, and **(C)** Blocking with chlorpromazine significantly reduces *S. aureus* uptake by DC. Data representative of three individual experiments.

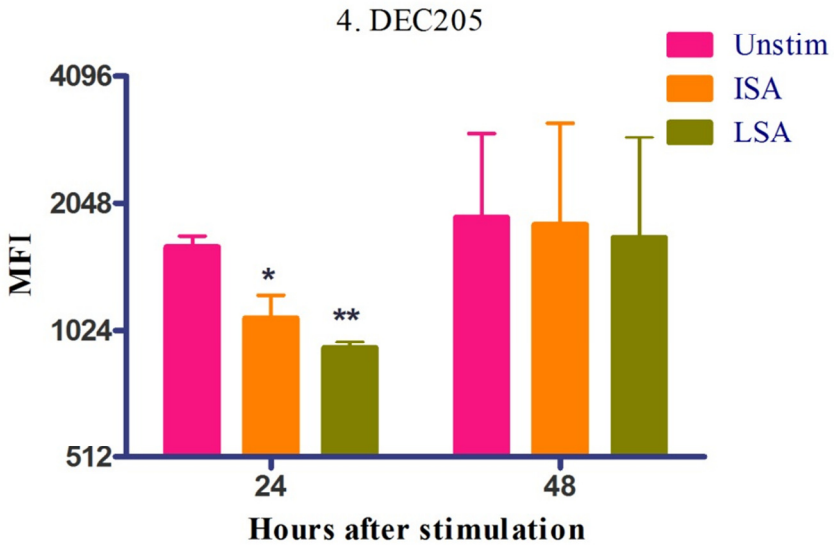

Figure 5.4. DEC205 internalization and recycling in DC at 24 and 48h of *S. aureus* loading. DEC205 DC were incubated with *S. aureus* (MOI50) for 3h. After 24 and 48h of initial stimulation, DC were stained for surface expression of DEC205 and analyzed by flow cytometry. Compared to unstimulated DC, ISA *(P* < 0.05) and LSA (*P* < .01) stimulation decreased DEC205 surface expression at 24h. However, both ISA and LSA stimulations didn't differ (*P* = 0.99) significantly from unstimulated DC. Data represents Mean with SEM of three independent experiments. The stars above the bars indicate significant differences (**$P < 0.01$, *$P < 0.05$) compared to unstimulated cells from three cows.

A. DEC205 B. DEC205 + FITC^{+ve} *S. aureus*

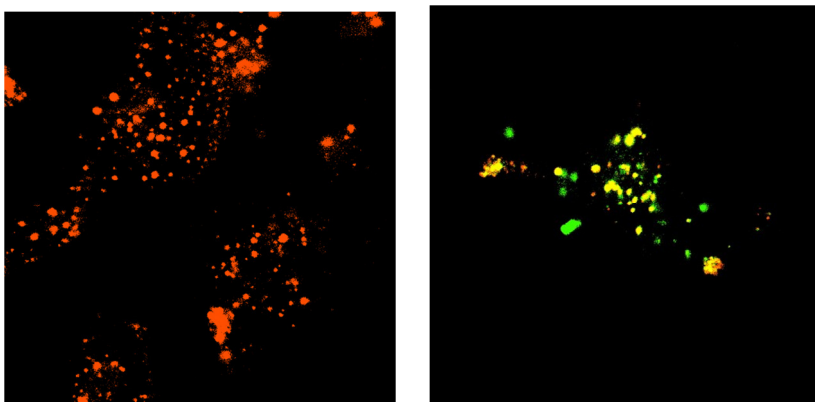

Figure 5.5A. Co-localization of DEC205 and *S. aureus* after 15min. DEC205 DC were stained with anti-bovine DEC 205 and visualized using Alexa 594 labeled IgG2b. Immediately after staining, FITC labeled *S. aureus* was added to the DC cultures. After 15 min DC were washed and pictures were taken. **(A)** Red staining areas indicate DEC205 receptor distribution in DC only and **(B)** yellow areas indicating DEC205 (red) and *S. aureus* co-localization (green). Data representative of two independent experiments.

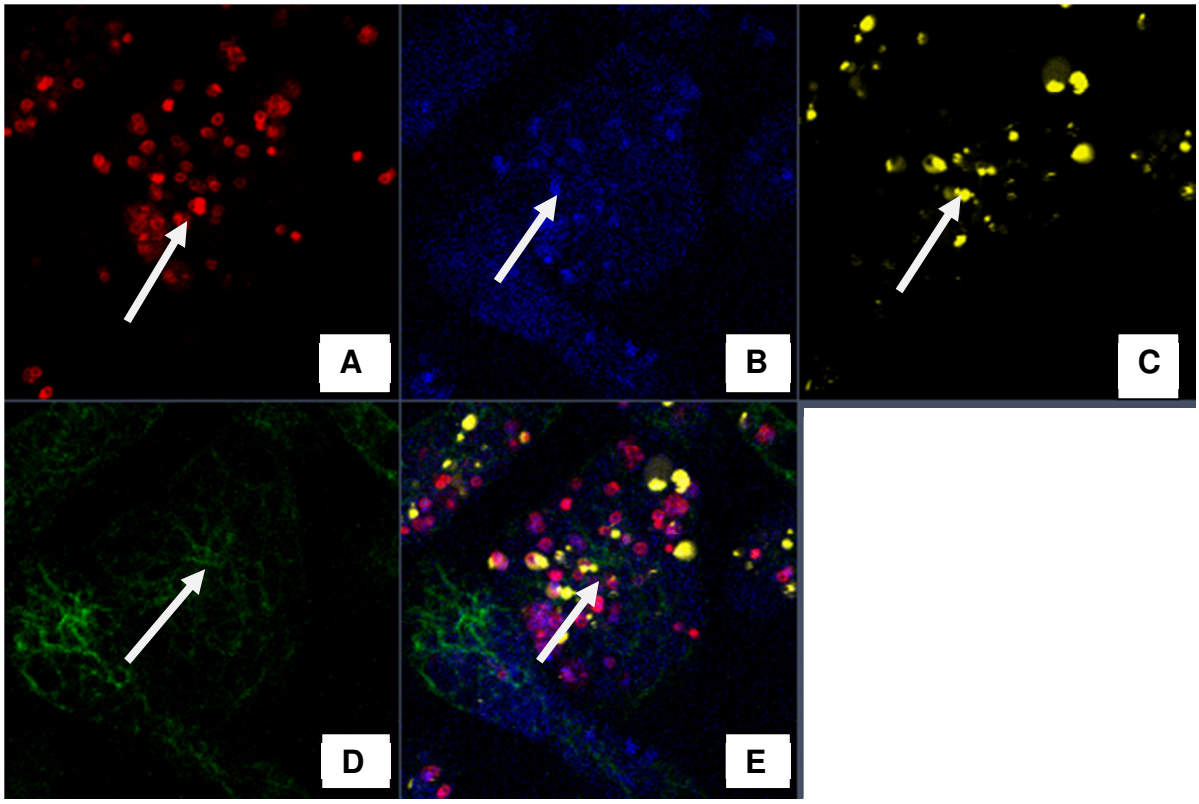

Figure 5.5B. Distribution of DEC 205 and *S. aureus* in DC. DEC205 DC were stained with anti-bovine DEC 205 and visualized using Alexa 647 labeled IgG2b. Immediately after staining, Alexa 555 labeled *S. aureus* was added to the DC cultures. After 3h, DC were washed and treated with lysostaphin. Cells were stained again with Oregon green tubulin and Lysotracker blue for lysosomes. **(A)** Red staining areas indicate internalized *S. aureus* and **(B)** blue stained areas indicates lysosomes/ endosomes **(C)** yellow areas indicating DEC205 receptor distribution after 3h **(D)** green staining areas indicate tubulin and **(E)** Composite image of A, B, C and D. Data representative of three independent experiments.

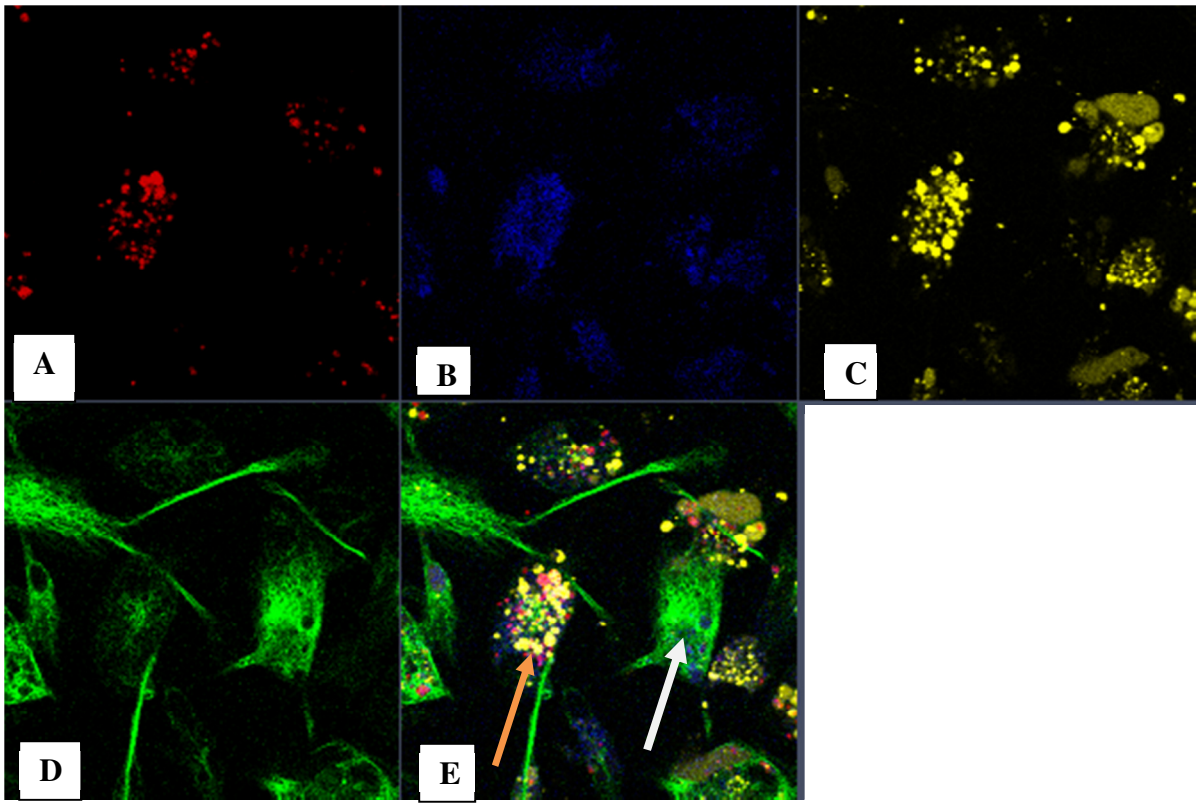

Figure 5.5C. Difference in *S. aureus* uptake by DEC 205 positive and negative DC at 3h. DEC205 DC were stained with anti-bovine DEC 205 and visualized using Alexa 647 labeled IgG2b. Immediately after staining, Alexa 555 labeled *S. aureus* was added to the DC cultures. After 3h, DC were washed and treated with lysostaphin. Cells were stained again with Oregon green tubulin and Lysotracker blue for lysosomes. **(A)** Red staining areas indicate internalized *S. aureus* and **(B)** blue stained areas indicates lysosomes/ endosomes **(C)** yellow areas indicating DEC205 receptor distribution after 3h **(D)** green staining areas indicate tubulin and **(E)** Composite image of A, B, C and D; white arrow indicates DEC205 low or negative DC with no *S. aureus* and yellow arrow indicates DEC205 positive DC with abundant *S. aureus* inside. Data representative of three independent experiments.

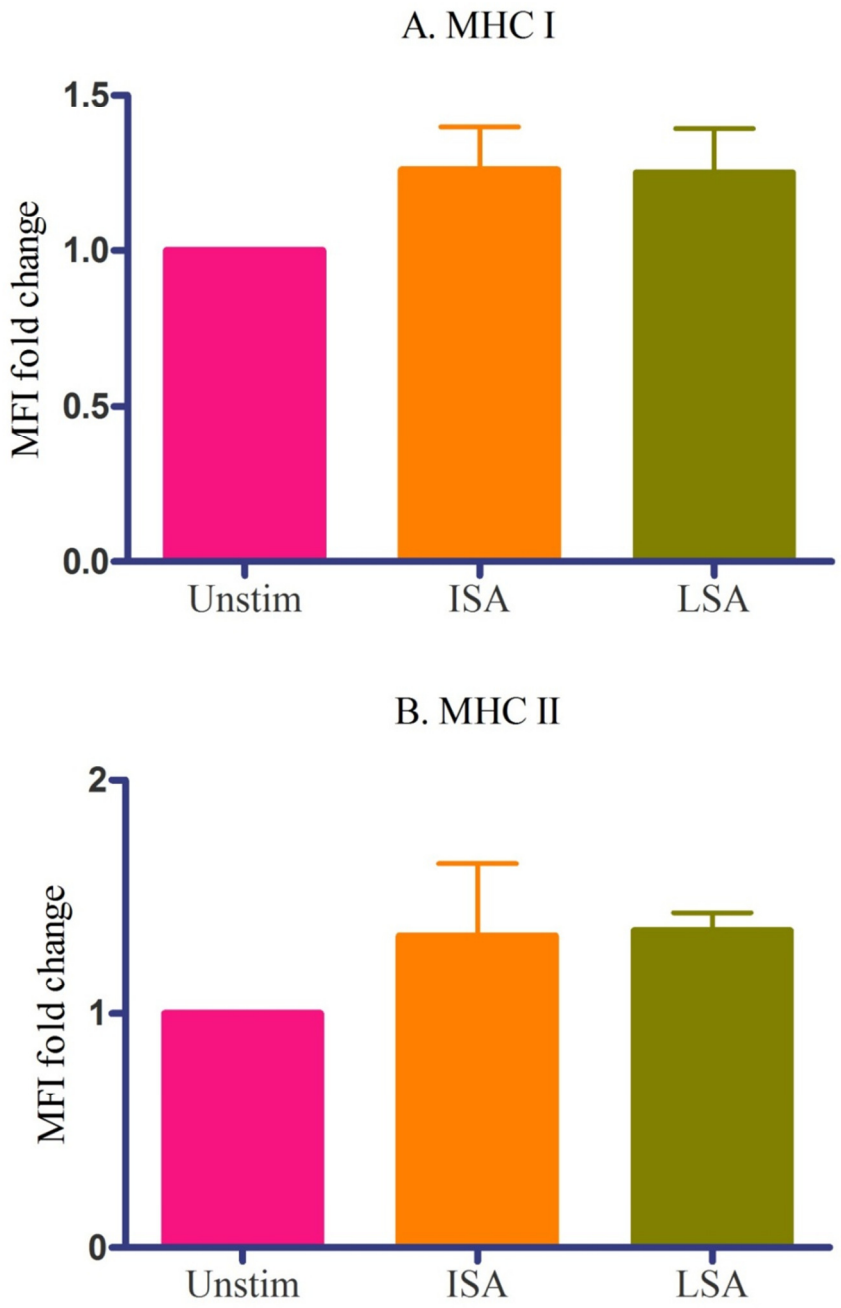

Figure 5.6. MHC expression in DC loaded with ISA and LSA after 24h. DC were loaded with ISA or LSA for 3h. MHC I and II expression was determined 24h post stimulation. Values expressed as fold change with respect to unstimulated DC. Data represents mean with SEM of mean fluorescence intensity of **(A)** MHC I and **(B)** MHC II from three cows.

mRNA induction of TLR2, cytokines and costimulatory molecules genes after 6h of ISA, LSA or LPS stimulation

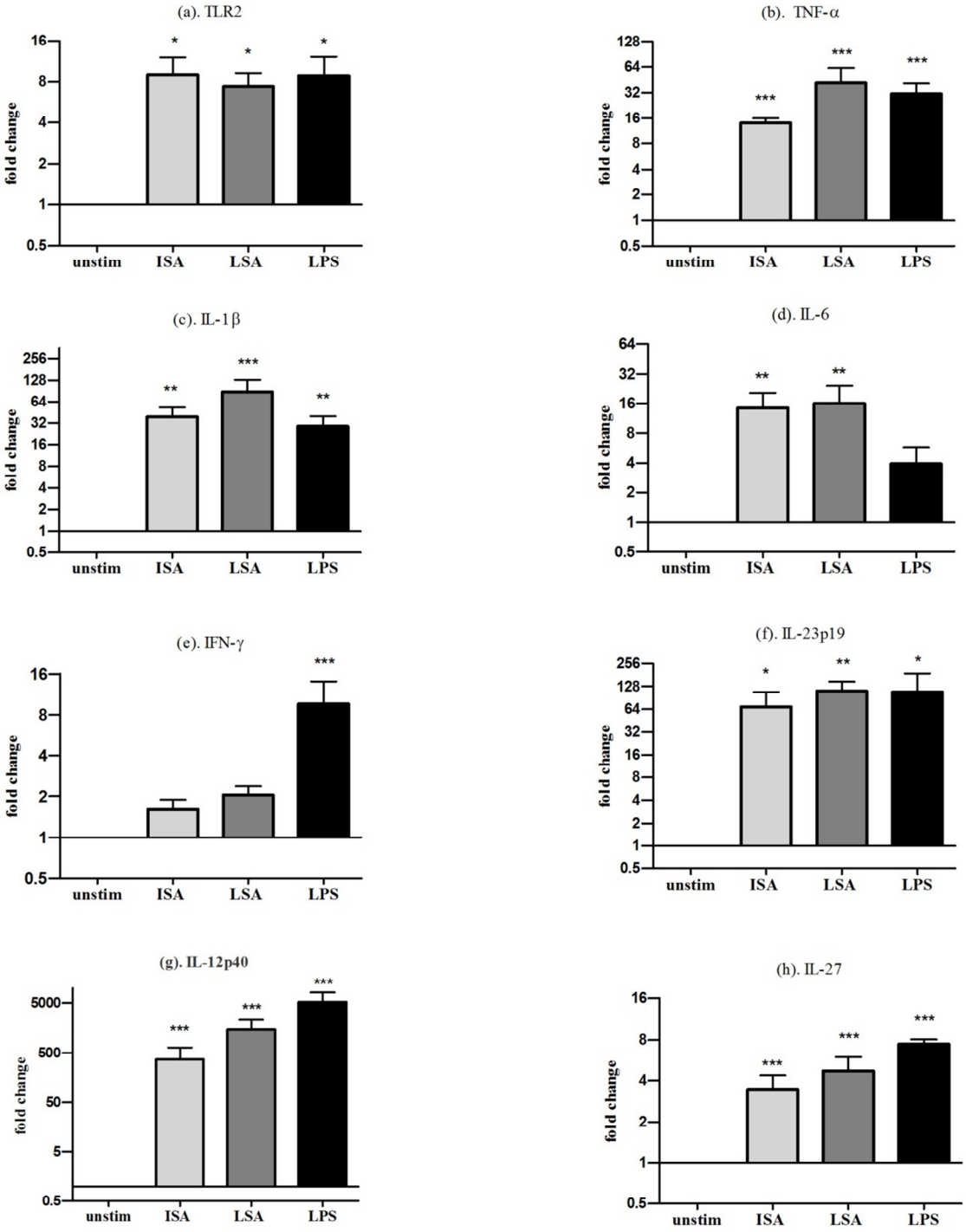

Figure 5.7. mRNA induction of (a)TLR2, (b) TNF-a, (c) IL-1b, (d) IL-6, (e) IFN-g, (f) IL-23p19, (g) IL-12p40, (h) IL-27, (i) CCR7 and (j) CD80 in DC stimulated with ISA, LSA, LPS or unstimulated. DEC205+ve DC were stimulated with ISA and LSA (MOI50) and LPS (0.5mg/mL) for 3hs. After 3hs, cells were washed extensively, added fresh media with gentamicin and incubated for another 3h. Total RNA was collected at 6h of initial stimulation and mRNA expression was determined by real time PCR. All the results were normalized with the GAPDH. All results are expressed as fold change from unstimulated DC calculated using the ddCT method. The expression levels are mean values of four independent experiments. Bars on bar graphs represent standard error. Statistical analysis was conducted using ANOVA with Tukeys post-test. The stars above the bars indicate significant differences (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$) compared to unstimulated cells from three cows.

A. IL-12p40 B. TNF-α

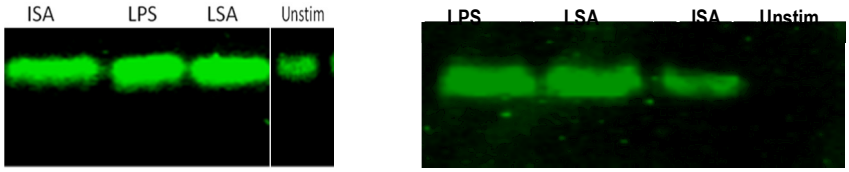

Figure 5.8. Secreted cytokines in supernatants of ISA, LSA, LPA and unstimulated DC. DC were stimulated with ISA, LSA for 3h and incubated for 24h. The 24h cell culture supernatants were analyzed by western blot for IL-12p40 and TNF-α. Increased secretion of (A) IL-12p40 and (B) TNF-α in the supernatants of ISA, LSA and LPS stimulated DC compared to unstimulated DC. Data representative of three independent experiments.

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CHAPTER 6 CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS

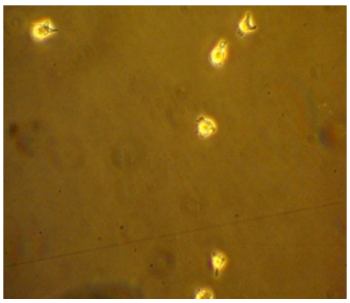
My dissertation focused on DC cell based immunological strategies to enhance the efficiency of anti-staphylococcal therapy in a bovine model. The novel finding in our first study was that $CD14^{+}$ monocytes when stimulated with irradiated or live *S. aureus* up-regulated gene expression of granulocyte-macrophage colony stimulating factor (GMCSF) and tumor necrosis factor-α and stimulated monocytes in an autocrine fashion to differentiate into $CD11c^{high}CD11b^{high}$ DC phenotype. Subsequent characterization of monocyte-derived DC response revealed that intact *S. aureus* stimulated DC more efficiently than its purified structural components (peptidoglycan or lipotechoic acid), up- regulated MHC molecules and induced T cell proliferation. In our third study, we identified CD8 memory T cells against *S. aureus*, which being the first ever report of this cell type in *S. aureus* infection in any species. Our study also depicted the role of DC DEC205 in *S. aureus* internalization. In addition the role of DEC205as an endocytic receptor, DC that possess this C-type lectin, undergo enhanced maturation and antigen presentation by up regulating MHC I and II expression in response to *S. aureus.* In conclusion, monocytes have the capacity to differentiate into DC upon *S. aureus* stimulation and DC elicits an effective innate and adaptive immune response.

FUTURE DIRECTIONS

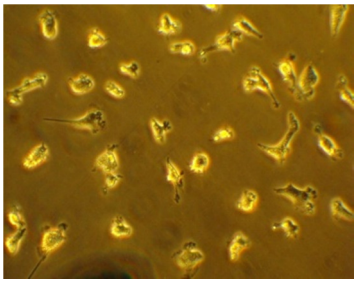
Future plans should be targeted to develop an effective anti-staphylococcal immunotherapy to combat *S. aureus* infections. The versatility of *S. aureus* poses serious challenges to the design of a working vaccine against this important animal and human pathogen. A vertically integrated approach should be used to characterize *S. aureus* antigens that are

specifically recognized by CD4 and CD8 T cells and elucidate the molecular and cellular mechanisms involved in the protection. For that purpose, conduct a detailed study for the identification of appropriate target antigens of *S. aureus* that could be used for vaccine formulation. The functional outcomes of DC-T cell interactions in response to target antigens are critical in the differentiation of an effective T cell memory and protective immunity. Characterization of the signals and molecules involved in DC-T cell interactions in response to *S. aureus* infection might be useful for the design of a successful vaccine. The possibilities of using DC endocytic receptor DEC205 (C-type lectin) as a potential target for immunotherapy to *S. aureus* should be further explored. Targeting this endocytic receptor on DC for antigen delivery would generate long-lived memory T cells that confer lasting immunity with appropriate specificity. To achieve this, future studies will investigate three key components: (a) identification of target antigens, (b) antigen specific T cell clones (Th1/Th17), and (c) combination technologies using targeted delivery and novel adjuvants.

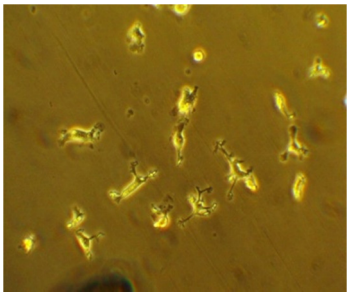
APPENDIX A. SUPPORTING DATA



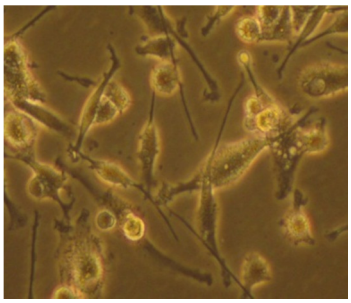
A. Unstimulated 7 d monocyte



B. 7 d LSA stimulated monocyte



C. 7d ISA stimulated e



D. 7d GMCSF and IL-4 DC

Figure A. 1. Images of 7d unstimulated, ISA and LSA stimulated monocytes showing a DC phenotype compared to unstimulated DC and the positive control, GMCF and IL-4 derived DC. Fresh monocytes were stimulated with ISA or LSA (MOI 10) for 2h. After 2h, cells were washed three times, added monocyte media with gentamicin and cultured for 7d. Microscopic appearance of **(A)** unstimulated, **(B)** LSA and **(C)** ISA stimulated monocytes are shown. Our **(D)** positive control was GMCSF and IL-4 stimulated 7d DC

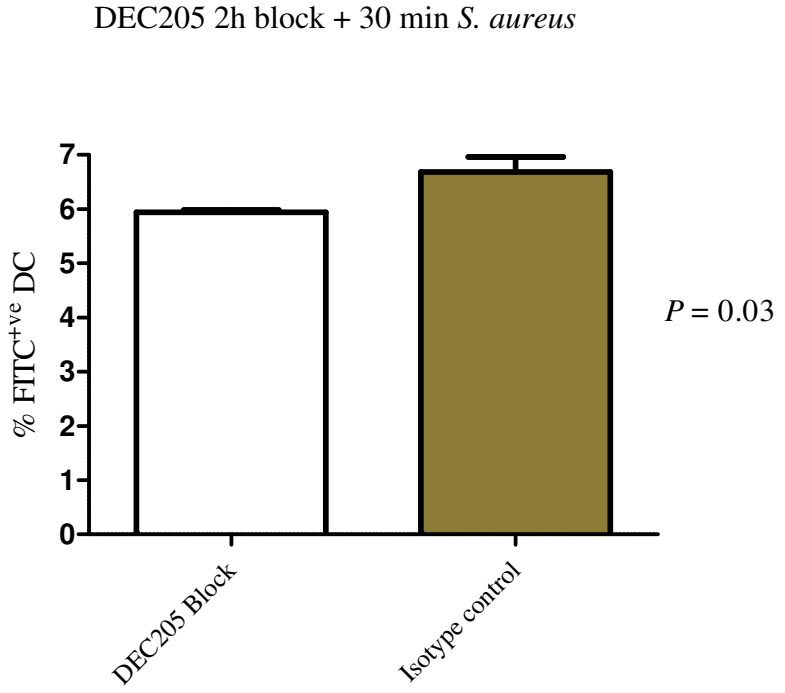


Figure A. 2. DEC205 blocking using monoclonal antibody reduces *S. aureus* uptake. DEC205 DC were treated with antibody or isotype control for 2h. FITC labeled *S. aureus* was added to DC cultures at MOI 50 for 30 min. After incubation, cells were washed, treated with lysostaphin, fixed and analyzed by flow cytometry. Results indicate that blocking of DEC205 with its antibody shows a tendency ($P = 0.03$) to reduce *S. aureus* uptake.

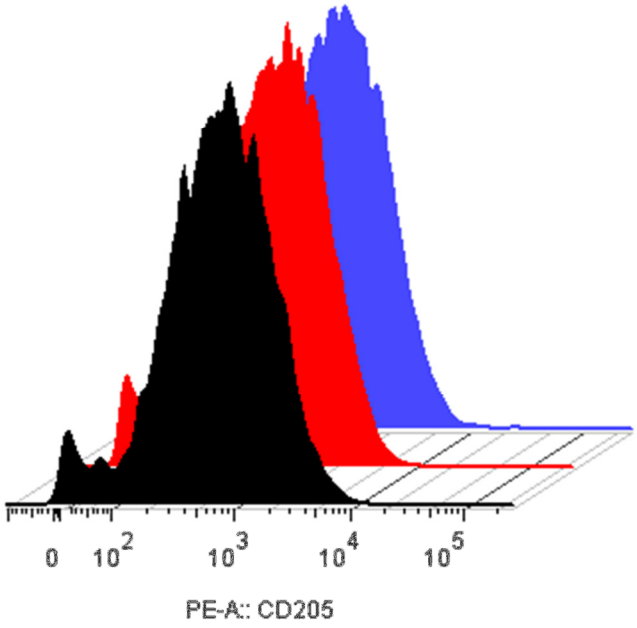


Figure A. 3. DEC205 expression on DC after SiRNA treatment. 4d old DC were treated with DEC205 or control SiRNA (Invitrogen, NY, USA) and analyzed for the surface expression of DEC205 after 48h. Histograms showing DEC205 receptor expression after 48h of DEC205 SiRNA at 50nM (Blue), 100nM (Red) or control SiRNA treatment (Black). SiRNA treatment did not reduce the expression of surface DEC205 on DC.

APPENDIX B. DETAILED PROTOCOLS

ALEXA 555 & FITC LABELING OF RN6390B STRAIN *S. AUREUS*

Reagents:

TSB

Carbonate bicarbonate buffer (pH 9)

Succinidimyl ester of Alexa 555

Fluorescein isothiocyanate

HBSS

Protocol:

1. Culture *S. aureus* in TSB for 4 h
2. Wash 3x with HBSS at 1500xg for 10 min at 4°C
3. Resuspend the bacteria in carbonate bicarbonate buffer (pH 9) @ 10^9 CFU/mL
4. Add 250uL of succinidimyl ester of Alexa 555 (1mg/mL) in HBSS or 100 μg of fluorescein isothiocyanate (FITC) isomer I /ml
5. Incubate for 2 h in dark at 37ºC
6. Wash 3x with HBSS and aliquot 500uL/tubes
7. Stored at -20C until use.

GAMMA IRRADIATION OF *STAPHYLOCOCCUS AUREUS*

1. Culture single colonies of RN6390B strain of *S. aureus* in tryptic soy broth for 4 h with rigorous shaking (12400 Incubator Shaker, New Brunswick Scientific C0, Inc.).

2. Wash the cultures three times with HBSS, and pellet at 1500xg for 10 min at 4°C.

3. Serially dilute the cultures and drop plate to get the actual colony counts.

   (1 ml of *S.aureus* suspension in a 1.5 mL tube (10^9 to 10^{11} CFU/mL) and put 2 -3 tubes in a 50ml Falcon tube)

4. Irradiate bacteria in a Model 109 research cobalt irradiator (JL, Shepherd and Asscocites, San Fernando, CA) for 3 h.

5. Streak the colonies in EBA plate and incubate overnight at 37ºC to ensure irradiation.

6. Before use, the irradiated *S. aureus* (ISA) should be washed twice with HBSS and diluted to 10^9 CFU/mL with RPMI 1640 medium.

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM BOVINE BLOOD

Reagents:

EDTA

HBSS or PBS (1x and 10x)

Ficoll-paque

2X MEM

Hanks Balanced Salt Solutions (HBSS, Sigma H4891)

Procedure:

1. Collect 250 ml blood using 10% (vol/vol) 40 mM EDTA containing bottle. Transfer blood to 50 ml centrifuge tubes.
2. Centrifuge in 50 ml tubes at 670xg, 30 min, 15°C. BRAKE OFF.
3. Remove 10 ml buffy coat and using 10 ml pipette in the smallest possible volume with the least amount of RBC's transfer into a 50 ml centrifuge tube containing 20 ml HBSS.
4. Layer over 12.5 ml Ficoll-Paque in a 50 ml centrifuge tube.

    a. Pick up 25 ml of cell in 25 ml pipette

    b. Tip tube so that ficoll-paque almost reaches front edge of the tube, and carefully place one drop of cells just in front of ficoll. Tip the tube so that cells run onto the ficoll, then slowly add the remaining cells into the tube. As you add more cells, bring the tube back to a near vertical position.
5. Centrifuge 330xg, 45 min 15°C. Brake OFF.
6. Remove mononuclear layer at interface using 10 ml pipette and transfer to another 50 ml centrifuge tube with HBSS.

a. Bring up to 50 ml with HBSS

7. Centrifuge 170xg, 10 min 15°C
8. Pool the cells and Resuspend in 5 mL of HBSS and add 10 mL of sterile water to lyse RBC. Pipette up and down for 18 sec and add 10 mL of 2x MEM. Mix well and fill the tube with HBSS

12. Centrifuge 170xg 10 min, 15°C
13. Wash 2x with HBSS
14. Resuspend in 25 ml and count
15. Use cells in functional assay or culture

MAGNETIC CELL SORTING

Purpose: To purify pure population of cells based using Milteny Biotech MACS sorting system.

Reagents:

HBSS

Column Wash: PBS + 2mM EDTA + 1% BSA (or 2%FBS)

LS/LD column (Miltenyi Biotech)

Magnets and Stand

Ice

Magnetic bead labeled antibody

Protocol:

1. Isolate PBMC

2. Centrifuge cells at 240xg 10 min, 4°C

3. Resuspend pellet in column wash solution (10 µl beads per 10^7 cells).

4. Incubate 20 minutes on ice.

5. Add 15ml HBSS

6. Centrifuge cells at 240xg 10 min, 4°C

7. repeat step 5 and 6

8. Resuspend pellet in Column Wash (2.5 ml)

    a. Filter cell prep through nylon mesh (70µ) into 50 ml conical tube

9. Equilibrate column with 5 ml column wash

10. Load cells onto column (~1×10^{8} magnetically labeled cells from up to 2×10^{9} total cells/column)

11. Wash 5 times with 3 ml Column Wash

12. Use plunger to elute cells off of column

    a. 2 times with 5 ml Column Wash

13. Centrifuge cells at 240xg 10 min, 4°C

14. Resuspend in Cell Culture Media at $1x10^{6}$ cell/ml after counting.

CFSE STAINING PROTOCOL

Reagents:

PBS

CFSE stock + DMSO

BSA

Cold monocyte media

Protocol:

1. Dilute 1 vial of CFSE in 18µL of DMSO (Molecular probes, Invitrogen, USA)

2. Pellet isolates lymphocytes

3. Dilute @ $10^{6\ cells}$/ml of PBS + 0.1% BSA

4. Add 1µL of CFSE/ mL of cells (final concentration of 2.5µM). Mix well and incubate at 37°C

for 10 min

5. Add 5 x volumes of cold monocyte culture media, mix and incubate for 5min on ice.

6. Wash 4x in media

7. Dilute the labeled lymphocytes at desired concentration. (Make sure you have some unlabeled lymphocytes for staining compensation controls for flow cytometry)

DENDRITIC CELL ANTIGEN LOADING AND LYMPHOCYTE CO-CULTURE

Reagents:

Bacterial 4 h culture in TSB

Dendritic Cells (DC)

DC media with and without antibiotics

CFSE or CellTiter 96-AQueous (Colorimetric) cell proliferation reagents (Promega)

Procedure: Use DC media without 2-betamercaptoethanol

Antigen Loading:

1. Culture DC for in 6-well plates at a density of 10^6/well in 2 mL media without gentamicin and cytokines
2. Add bacteria at a multiplicity of infection (MOI) of 50 for 3 h in antibiotic-free medium
4. After 3 h, wash the wells three times with HBSS and add 3mL of fresh media with gentamicin.
5. Culture DC overnight prior to addition of lymphocytes.
6. Isolate lymphocytes
7. Label lymphocytes with CFSE (2.5μM final concentration)

   Take out 200 μL of the supernatant and add lymphocytes cells at a ratio of 10 lymphocytes per DC in 1mL.

   Concanavalin A (2.5 μg/ mL) should be added to lymphocytes as positive control.

   Unstimulated lymphocytes are used as negative control.
8. Incubate for 48 – 72 hr (4 d).
9. Harvest supernatants for cytokine assay and cells for flow cytometry (CFSE labeled) or

add Cell Titer Solution (promega).

a. Add cell proliferation reagent (10µl /well) and read on plate reader 15-45 min. later (mix on nutator).

b. CellTiter 96-AQ - read on colorimetric plate reader at 490 nm.

ASSAY FOR ENDOCYTOSIS AND ENDOCYTOSIS INHIBITION

Reagents:

FITC labeled *S. aureus*

DC/ Monocytes

Lysostaphin, HBSS

Chlorpromazine, Sucrose, Filipin

Media with 1% serum and without 2-betamercaptoethanol

Procedure:

1. For endocytosis assay, add FITC labeled *S. aureus* (MOI 50) to 7d old DC cultures in antibiotic free media and incubate for 3h at 37ºC.
2. For endocytosis inhibition assay, pretreat immature DC with 1% serum media containing 10µg/mL of chlorpromazine or 5µg/mL of Filipin or 450mM (Sigma-Aldrich, MO, USA) or media alone for 30 min at 37ºC.
3. Incubate with FITC labeled *S. aureus* (MOI 50) for an additional 3h.
4. Wash the cells twice with HBSS and treat with lysostaphin 100µg (Sigma-Aldrich) for 7 minutes.
5. Collect the cells using cell stripper solution and immediately fixed with 1% formaldehyde and analyze in flow cytometer for $FITC^{+ve}$ DC.

LIVE CONFOCAL LASER SCANNING MICROSCOPY

Reagents:

LysoTracker blue DND-22 (L-7525; Molecular Probes, Invitrogen, USA)

Oregon green tubulin (T34075; Molecular Probes, Invitrogen, USA)

Mouse antibovine CD205 (MCA1651G; Abd serotec, Raleigh, NC, USA)

Alexa 647-conjugated goat anti-mouse IgG2b Ab (Invitrogen/Caltag lab, New York, USA)

HBSS, DC media without phenol red and cytokines

36mm glass bottom dishes (Mat Tek Corporation, Ashland, MA, USA)

Alexa 555 labeled *S. aureus*

Procedure:

1. Culture monocyte-derived DCs (10^4) in 36mm glass bottom dishes in phenol red free media.
2. Incubate DC with antibovine CD205 primary antibody for 30 min at 37°C.
3. Wash with HBSS three times.
4. Use Alexa 647-conjugated IgG2b Ab (1 in 200) to visualize the staining of primary Ab for another 30 min.
5. Wash with HBSS three times.
6. Incubate DC with Alexa 555 labeled *S. aureus* (MOI 50) for 3h.
7. Washed DC three times and treated with lysostaphin 100µg (Sigma -Aldrich)

8. Wash and stain for another 30 min each with 250nM Oregon green tubulin (T34075; Molecular Probes, Invitrogen, USA) and 1µM LysoTracker blue DND-22 (L-7525; Molecular Probes, Invitrogen, USA).

9. Wash DC three times and add RPMI media without phenol red to the DC culture.

10. Visualize DC at a magnification of 40x or 100x with a Zeiss confocal microscope (LSM510 META microscope) and analyze using ZEN 2009 software (Carl Zeiss Microimaging, Germany).

WESTERN BLOT FOR CYTOKINES IN CELL CULTURE SUPERNATANTS

Reagents:

Comassie Blue

Novex Midi Gel

Starting Block™ blocking buffer (Thermo Scientific, IL, USA)

Immobilon-FL PVDF membrane

1. Bradford assay was performed to assess the total protein in samples to ensure equal protein loading.
2. Separate the proteins by Novex Midi Gel (Invitrogen Co, CA, USA) electrophoresis at 100V for 90 min and transfer on to a Immobilon-FL PVDF membrane at 60mA for 90 minutes.
3. Block the resulting membrane with Starting Block™ blocking buffer (Thermo Scientific, IL, USA) for an hour.
4. Incubated the membrane with the primary antibody (TNF-α; polyclonal Q06599, AHP852Z, AbD Seortec Raleigh, NC, USA; and GMCSF; GM-CSF 17.2, VMRD, Pullman, WA, USA) at 4ºC overnight in blocking buffer.
5. Wash the membrane five times with PBS tween (0.05%)
6. Visualize immunoreactive proteins were by incubating with goat anti-mouse (1 in 10000) or anti-rabbit IgG (1 in 10000) secondary antibody (Bio-Rad).
7. Wash the membrane five times with PBS tween (0.05%)

8. Detect the signals with an ODYSSEY Infrared Imaging System (LI-COR, Lincoln, NE, USA) and the gray value of protein bands quantified with Photoshop (Adobe, San Jose, CA).

BOVINE CCL2 VETSET™ ELISA DEVELOPMENT PROTOCOL

Included Components:

Description	Quantity	Component Number
Bovine CCL2 Coated Plate	2 each	VS0083B-CP
Bovine CCL2 Standard	2 each	VS0083B-ST
Bovine CCL2 Detection Antibody	2 each	VS0083B-DA
Streptavidin-HRP	1 each	AR0100-001
Plate Sealer	6 each	N/A

Additional Reagents Required:

Reagent	**Formulation**
DPBS	0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01M potassium chloride, pH 7.4
Standard and Sample Diluent	Complete cell culture medium used to generate cell culture supernatant samples. It is critical that this medium contain at least 1% carrier protein. If the medium does not contain carrier protein, use Reagent Diluent to dilute the Standard and samples.
Reagent Diluent	4% BSA in DPBS, 0.2 µm filtered
Wash Buffer	0.05% Tween®-20 in DPBS
Substrate	3,3',5,5'-tetramethylbenzidine (TMB) Substrate
Stop Solution	0.18 M Sulfuric Acid

Procedure:

1. Prepare standard and cell culture supernatant sample dilutions in standard and sample diluent. (Reconstitute Standard in 1 mL standard and sample diluent. Dilute 500 µl of

the reconstituted standard in 500 µl of standard and sample diluent. The standard now has a concentration of 10,000 pg/mL. Prepare 1:1 serial dilutions of the standard by mixing 500 µL Standard with 500 µL standard and sample diluent. Repeat 1:1 serial dilutions until reach a final concentration of 156.25 pg/mL. Use standard and sample diluent as a zero standard.)

2. Add 100 µL of Standard or sample to appropriate wells.

   (Note: Run each Standard or sample in duplicate.)

3. Cover plate with Plate Sealer and incubate at room temperature (20-25°C) for 1 hour.
4. Wash plate FOUR times with wash buffer.

   (Note: Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.) Reconstitute detection antibody in 500 µL Reagent diluent. Dilute the 500 µL of reconstituted detection antibody in 11.5 mL Reagent diluent.

5. Add 100 µL of Detection antibody working solution to each well.
6. Cover plate with plate sealer and incubate at room temperature for 1 hour.
7. Wash plate FOUR times with wash buffer as described in step 4.
8. Dilute 7. 500 µL Strepavidin-HRP in 11.5 mL Reagent diluent.
9. Add 100 µL of Streptavidin-HPR working solution to each well.
10. Cover plate with plate sealer and incubate at room temperature for 30 minutes.

11. Wash plate FOUR times with wash buffer as described in step 4.

12. Add 100 µL of TMB substrate solution to each well.

13. Develop the plate in the dark at room temperature for 30 minutes.

(Note: Do **NOT** cover plate with Plate Sealer)

14. Stop reaction by adding 100 µL of Stop Solution to each well.

15. Measure absorbance on a plate reader at 450 nm.

STAPHYLOKINASE ASSAY

Purpose: To measure levels of Staphylokinase production by Staphylococcus strains

Reagents:

Todd Hewitt Broth or TSB

Tris/HCl buffer (50 mM, pH 7·4)

D-Val–Leu–Lys paranitroanilide (S-2251; Chromogenix)

PBS

Protocol:

1. Isolate single colonies of bacteria by culturing for isolation on Blood agar plate.

2. Inoculate 2 ml Todd Hewitt Broth or TSB with one colony from blood agar plate.

    a. Incubate at 37°C overnight

3. Centrifuge at 4000g for 10 min

4. Collect supernatant for determination of secreted SAK activity and place on ice.

5. Wash pellet in 10 ml PBS

6. Take OD readings.

7. Resuspend at 10^9 CFU/ml

8. Add calculated amount of 20 μg/ml human Glu-plasminogen and incubate for 4 hrs.

9.Wash pellet twice in PBS and resuspend in Tris/HCL buffer

    a. Add plasmin substrate (with 0·2 M human Glu-plasminogen)

    b. Incubate 30 min.

10. Add 0·4 _M H-D-Val–Leu–Lys paranitroanilide (S-2251; Chromogenix)

11.Create standard curve of recombinant SAK

12. Take absorbance reading at 405 nm.

    a. 30 min for surface-bound SAK

b. 2 hr for secreted SAK

* Run in duplicates*