Utrecht University

Scratching beneath the surface:

the Pathogenic Role of Staphylococcal Superantigens in Atopic Dermatitis

L.M.W. de Brouwer

Student number: 3383725

Examiner: Dr. DirkJan Hijnen

Master program: Biology of Disease

Department of Dermatology, UMCU

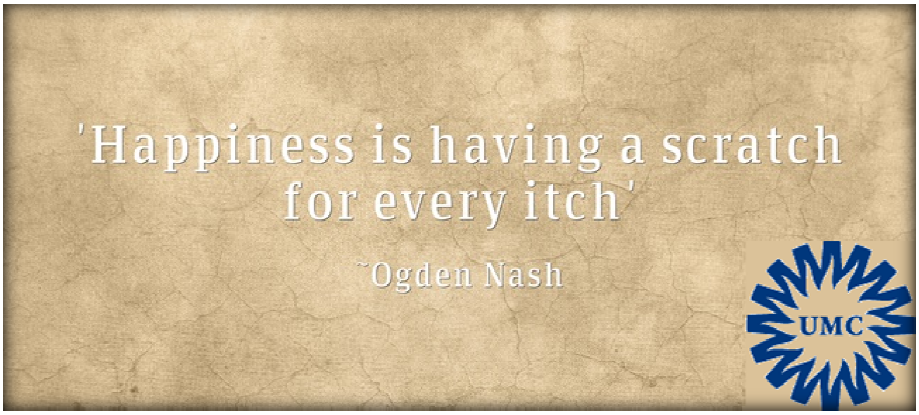


Table of content.

General introduction.

Atopic dermatitis (AD) is a chronic, highly pruritic, multifactorial and relapsing inflammatory skin disease, affecting 10-20% of all children worldwide.[1] It commonly presents during early infancy and childhood but can persist or even start in adulthood, making it one of the most common skin diseases in adults as well. Infants with a positive family history of AD or other allergic disorders are genetically predisposed and often go through the so-called 'atopic march' at later age stages in which their AD progresses into allergic rhinitis and asthma.[4] The geographic distribution of this disease is not random as it mainly concentrates in urban regions of industrial countries, in which it affects the higher social classes. Over the past decades, both prevalence and severity of atopic diseases like AD have significantly increased, which is a concerning development considering the major adverse effects AD can have on the patient's quality of life and the associated increasing health care costs. More effective therapeutic strategies are hence desired but require better knowledge about the underlying mechanisms of this complex disease. This review focuses on the pathogenic role of the bacterium *Staphylococcus aureus* in AD and covers more specifically all aspects of Staphylococcal superantigens in their contribution to AD.

Atopic Dermatitis.

Dermatitis (or eczema) is a collective noun for any type of inflammation of the skin. It is one of the most common skin diseases worldwide and includes frequent types like allergic and irritant contact dermatitis, nummular eczema and seborrheic dermatitis. AD is however the most common, severe and chronic type of dermatitis. It is a multifactorial disease in which immune dysregulation and abnormalities of the skin barrier together with increased exposure to environmental and infectious agents determine the pathophysiology of the disease.[1,3] AD patients express a wide spectrum of clinical manifestations such as pruritus, pityriasis alba, erythematous papules and chronic or relapsing eczematous lesions with excoriation and exudation. Symptoms are also dependent on the patient's age as infants generally develop more acute skin lesions on the scalp, face and extensor surfaces of the extremities whereas (young) adults suffer from a more chronic AD which mainly affects the flexural folds of the extremities and results in the development of lichenificated skin.[1] Due to the lack of specific diagnostic tests, AD diagnosis is based on the clinical manifestations combined with an assessment of the patients family history (to distinguish AD from other skin disorders like psoriasis).[3]

Atopic Dermatitis treatment.

There is currently no cure for AD, however multifaceted treatments in combination with lifestyle changes can calm the skin down, relieve pain and itchiness, decrease inflammation and prevent both infections and deterioration of the disease. Daily skin care is important in AD management during which patient specific irritants like fragranced soaps should be avoided. Topical corticosteroids (TC) are the mainstay of treatment in AD. TC are applied on the red and inflamed areas of the skin (most often in ointment, lotion or cream preparations) and wield anti-inflammatory, antiproliferative and immunosuppressive actions.[3] There are numerous FDA approved TC products which are, based on their potency, subdivided into either four

(Europe) or seven (US) classes. The least potent TC like hydrocortisones are available without prescription in the US but are only advisable for the treatment of very mild cases of AD. On the other hand, super potent TC like clobetasol are also effective in more severe cases of AD but long term use may result in side effects such as skin atrophy, which makes them less ideal for the treatment of sensitive skin areas like the face.[5] In very severe or therapy resistant cases of AD, systemic corticosteroids like prednisone can be prescribed temporarily, either orally or through intramuscular injections. However the amount, frequency and duration of systemic corticosteroid use should always (as with very potent TC) be kept as low and short as possible to reduce the risk of adverse side-effects.

To limit steroid exposure, a TC-based treatment can be interspersed with topical calcineurin inhibitors (TCI). Unlike TC, TCI do not contain steroids and thus are free from all the steroid-associated negative side-effects, however they are also far less effective. Currently, two types of TCI drugs are approved (pimecrolimus and tacrolimus) which both function as immune-suppressants.[5] In more severe cases of AD, systemic calcineurin inhibitors like cyclosporine can be prescribed as well although only for short intervals due to the risk of side-effects.[53]

Adult AD-patients can opt for a combinational treatment with ultraviolet (UV) phototherapy when topical and/or systemic treatment alone does not generate adequate relief. It is known that natural sunlight is beneficial in many skin diseases, however the associated heat and humidity can be irritating and more importantly, frequent exposure to sunlight damages the skin, accelerates aging and promotes the development of skin cancers. Instead of beaming full spectrum light, phototherapy lamps can be adjusted to only emit the most safe and beneficial fraction of light (narrow band UVB), which significantly reduces the patient's exposure to the damaging aspects of natural sunlight.[5]

AD-patients show an increased susceptibility to cutaneous bacterial, viral and fungal infections.[6] To manage heavy colonization and emerging skin infections, bleach baths are recommended and local or systemic antibiotics (like cephalosporins and penicillins), antivirals (like acyclovir) and antifungals (like zinc pyrithiones) can be prescribed.

Lastly, during flare ups, AD-patients may suffer from sleep deprivation as the pruritus usually intensifies at night. The prescription of antihistamines can offer some relief as they are found to have sedative properties and facilitate the process of falling asleep.[3] It might also be worthwhile to consider counseling when emotional triggers like stress and embarrassment worsen the symptoms.[5] If taken together, it is clear that for a successful management of AD, a multifaceted approach is necessary in which different aspects of the disease are treated.

Atopic Dermatitis pathogenesis.

When it comes to the pathogenesis of AD, two hypotheses have been proposed. For a long time, AD was thought to be primarily an immune disease in which observed skin barrier abnormalities were explained as downstream epiphenomenons of the immunologic dysfunction. This is referred to as the inside-outside view.[19] However recent findings, such as null-mutations in the *filaggrin* gene suggest that the reverse or outside-inside hypothesis, in which impaired barrier functioning resulting in increased allergens exposure, may be

the first step in the pathogenesis. Regardless of which one is the primary initiator, it is clear that both epidermal barrier defects and immune dysregulations are strongly intertwined and interdependent of each other in the pathogenesis of AD.

1. Skin barrier defects

Structural and functional abnormalities in the epidermal barrier are essential features of AD. Normally, the epidermal barrier permits the absorption of wanted substances (like water and essential nutrients) inwards whilst it simultaneously blocks allergens and infectious agents from penetrating the skin. Next to this, it also prevents the outward diffusion of important substances from the skin.[8] The major component of the epidermal barrier is the uppermost skin layer called stratum corneum (SC). Multiple abnormalities in the SC have been identified which all seem to contribute to the pathophysiology of AD.

1.1. Filaggrin deficiencies

Keratinocytes are the most abundant cell type in all layers of the epidermis and migrate from the stratum basale (where the epidermal stem cells reside) upwards to the surface whilst undergoing differentiation. In the final stage of migration as cells progress into the SC, an important precursor protein called profilaggrin is cleaved into multiple copies of filament-aggregating protein (filaggrin) by serine proteases. This filaggrin is able to bind the keratin cytoskeleton, forming the filament-matrix complex, which cross-links with the cornified envelope surrounding the keratinocytes at this stage. This organized intercellular network causes the keratinocytes to collapse, forming the characteristic SC corneocytes which are strongly anchored to each other to prevent percutaneous leaking. In the outermost layers of the SC, filaggrin is eventually proteolysed into free amino acids and subsequent derivatives, collectively known as natural moisturizing factors (NMFs) which help to maintain skin hydration, pH and buffering capacity.[8]

Filaggrin is encoded by the *FLG* gene and meta-analyses have shown that mutations in this gene genetically predispose towards AD.[11,12] Up to 60% of all European AD patients reveal single or double-allele *FLG* mutations, showing a very strong genetic link between mutation and disease.[13] Currently, over forty mutations have been identified in which the two most common variants (R501X and 2282de14) lead to a complete loss of filaggrin production.[8,10] Filaggrin deficiencies weaken the integrity of the intercellular matrix between corneocytes in the SC (leading to increased transepidermal water loss (TEWL) and percutaneous absorption), and significantly reduce the levels of NMFs.[9,13] Doing so, *FLG* mutations result in dehydration of the SC which contributes to the epidermal barrier dysfunction and subsequent xerotic phenotype often observed in AD patients. Decreased levels of NMFs also increase the alkalinity of the skin and impair its buffering capacity, making carriers of *FLG* mutations more susceptible to pH changes upon contact exposures.[8] Increased skin pH aggravates epidermal barrier dysfunction in AD,[14] as a more alkaline SC milieu heightens serine proteases (SP) activity which degrade specific processing enzymes and structural proteins like desmoglein-1, directly affecting the SC cohesion.[15] Next to this, bacterial colonization and subsequent infections profit from a more neutral surface skin pH as well.[8]

1.2. Increased serine protease activity

Despite the strong genetic connection, *FLG* mutations cannot be held responsible for all abnormalities observed in AD. Moreover, at least half of all AD patients do not even carry a *FLG* mutation. It is already explained that AD patients have increased SP activity due to altered skin pH. However both gain-of-function mutations in the SP gene *KLK7* and loss-of-function mutations in the SP inhibitor gene *SPINK5* increase SP activity as well and have been associated with AD.[16] Overactive SPs decrease the total lipid content in the SC, directly affecting the intercellular lipid membrane which is there to preserve the SC integrity. Especially ceramide levels (which are the main components of the lipid membrane) are significantly reduced in AD patients and associated with increased TEWL and aggregation of disease.[17] SP hyperactivity also induces a local Th_2-based inflammation as it cleaves the precursors of cytokines IL-1α and IL-1β, subsequently generating active cytokines which stimulate the secretion of IL-4. IL-4 stimulates (amongst other things) the differentiation of naive T-helper cells into Th_2-cells and decreases the expression of crucial proteins like filaggrin, ceramide and dermoglein-3, affecting both integrity and functioning of the epidermal barrier.[16]

1.3. Sustainment of the Th_2-inflammation in atopic skin

The local Th_2-based inflammation can sustain and broaden when wound healing mechanisms are consistently activated due to the chronic or recurring SC defects in AD patients.[18] Also caspase 8, a protein involved in the process of tempering wound healing, is decreased in AD, contributing to the sustained inflammation as well. The Th_2-mediated inflammation is fueled by the higher degree of antigen exposure in the skin, which is a direct result of the increased percutaneous absorption and penetration of allergens and pathogens. In fact, over the past decades numerous mechanisms are discovered which all skew the AD associated inflammation towards a Th_2-based response which in turn plays a major role in the development, sustainment and aggravation of AD.

2. Immune dysregulation

Epidermal barrier defects are key features in the development of AD, however they cannot account for the entire pathophysiology of AD. Looking only at the recurring flare ups in between non-active intervals, many of the SC barrier abnormalities are present in both phases of the disease, indicating that other triggers are involved as well. Next to this, immunosuppressive drugs (like the calcineurin inhibitor cyclosporine) have a positive outcome on the SC integrity in AD, showing that barrier defects could be epiphenomenons of immune dysregulations.[53] As said before, for a long time AD was thought to be primarily an immune disease and even though this inside-outside view had to be adjusted, dysregulated immunity still plays a fundamental role in AD.

2.1. Impaired innate immunity

In AD, the adherence of pathogens to atopic skin is increased as extracellular matrix proteins like fibronectin (which serve as anchors for the pathogens) are upregulated by Th_2-inflammatory cytokines. This, together with the defective epidermal barrier often observed in AD facilitates the invasion of pathogens through the skin. Innate immunologic responses should however provide a first line of defense against the onset of these infections.

Specific pattern recognition receptors (PRR) like toll-like receptors (TLR), CD14-receptors, nucleotide-binding oligomerization domain-like receptors (NLR) and mannan-binding lectin (MBL) play a major role in this process as they recognize and bind invading pathogens by their pathogen-associated molecular patterns (PAMPs). After binding, PRRs mediate in the elimination of these pathogens by either inducing intracellular signaling cascades or attracting antimicrobial factors (depending if the PRR is membrane-bound or soluble). Unfortunately, AD is associated with a diminished capacity to generate adequate innate responses due to polymorphic mutations in the PRRs.[18] PRR mutations do not only impair fast microbial recognition but also disturb the Th_1/Th_2 balance even further as PRR signaling pathways otherwise promoted Th_1-immune responses and downregulated the (in AD already dominant) Th_2-response patterns.

Another major element of innate immunity are the antimicrobial peptides (AMPs), from which especially LL-37 (and its precursor cathelicidin) and α/β-defensins contribute to the antimicrobial and anti-inflammatory responses in the skin. AMP levels are however often diminished in AD as a direct result of the upregulated Th_2-inflammation and possibly genetic mutations as well.[18] A vicious cycle is put into motion as increased antigen exposure fuels the Th_2-inflammation which in turn decreases the expression of AMPs, subsequently only further increasing the exposure to potential pathogens.

2.2. Allergic sensitization

The abnormal high exposure levels in AD in combination with the ongoing Th_2-mediated inflammation can cause allergic sensitization to quite common environmental agents. In short, upon an exogenous challenge, Th_2-inflammatory responses are set in motion which (through IL-4 secretion) stimulate B-cells to differentiate into IgE producing plasma cells. These IgE antibodies are able to bind Fc-receptors on mast cells present at the site of allergen entry, and remain there for weeks whilst the mast cell is sensitized. During a second exposure, the relevant allergen is able to directly bind and cross-link this cell-bound IgE, inducing intracellular signaling pathways leading to the degranulation of the sensitized mast cell. The released mediators like histamines and cytokines develop a local type 1 hypersensitivity reaction in the skin which aggravates AD.

In summary, decreased levels of filaggrin and NMF's, combined with increased skin pH and SP activity all weaken the integrity and cohesion of the SC in AD patients. The affected barrier together with impaired innate immune responses facilitate the percutaneous penetration of allergens and pathogens, resulting in a higher degree of exposure which induces Th_2-based inflammation. Next to this, the increased exposure to potential allergens in combination with Th_2-based inflammation allow for detrimental allergic sensitization and increased IgE levels in AD patients. Thus, whilst AD has a complex pathophysiology in which numerous autonomous players are identified, the subsequent epidermal barrier defects and immune dysregulations are strongly intertwined and interdependent of each other in the manifestation of AD.

Human skin microbiome in health and Atopic Dermatitis.

Contrary to sterile internal tissues, epithelial surfaces like the skin and mucous membranes are colonized by micro-organisms because of

their direct environmental contact. The natural skin flora comprises mostly bacteria but also fungi, viruses and protists are part of the healthy skin microbiome.[20] Recent metagenomic studies have identified thousands of distinct species which in general convey a non-pathogenic character, living either in commensalism or mutualism. Age, skin site (most, dry or sebaceous) and personal hygiene are few of the many factors contributing to microbiome diversity and fluctuation, although Corynebacterium, Propionibacterium and Staphylococcus clearly are the three most abundant species colonizing the human skin.[20]

The skin microbiome holds protective values towards human health as a dense colonization of the skin surface crowds out pathogenic microbes. It also educates and interacts with the host immune system by triggering the upregulation of AMPs and activating immune cells. Next to this, skin microbes are able to produce bactericidal factors like AMPs, phenosoluble modulins (PSM) and free fatty acids (FFA) themselves, augmenting the host's innate immune defenses against infections even further.[20]

There is increasing evidence that disbalances in the skin microbiome contribute to the induction and progression of skin diseases. In AD, a markedly reduced cutaneous microbial diversity is observed at sites of disease manifestation in which both *Staphylococcus aureus (S. aureus)* and *S. epidermidis* are significantly more abundant. [23,21] The increased abundance of *S. aureus* is facilitated by the previously discussed epidermal barrier defects and immune dysregulations often observed in AD. *S. epidermidis* on the other hand is already part of the normal skin flora but thought to increase as a result of either a mutualistic relationship with the primary increasing *S. aureus* colonization or as an antagonistic response to it.[22] Clinical studies have shown that the increased Staphylococcal colonizations in AD are directly responsible for the drop in skin bacterial diversity, possibly through their production of antibacterial compounds affecting the normally abundant Corynebacterium and Propionibacterium. This decreased bacterial diversity of the skin associates with more severe forms of AD.[23,21] Antimicrobial and anti-inflammatory treatment prior or during exacerbations hamper *S.aureus* predominance, resulting in the sustainment of a higher degree of microbial diversity during the flare, which only further accentuates the importance of a balanced skin microbiome and the role of *S. aureus* in AD-affected skin.

1. The bacterium *Staphylococcus aureus*

Numerous bacterial, fungal and viral pathogens have been associated with AD, however none of them as strongly as *S. aureus*. *S. aureus* is a gram-positive spherical and cluster-forming coccus, first discovered in 1880 and within its genus, the most common species to cause disease. Staphylococcal infections are diverse, ranging from minor skin infections to more systemic and life-threatening conditions like sepsis. Over the past decade, *S. aureus* has gained global attention due to the emergence and rise of its drug resistant variant MRSA, which has been recognized as a problem of high priority in the field of medicine.

The first evidence of *S. aureus* involvement in skin diseases like AD dates back from the 1960s, when researchers discovered by accident abnormal high air-counts of *S. aureus* in clinical wards hospitalizing patients suffering from skin diseases.[24] Over time, the role of *S. aureus* in AD became increasingly prominent and is currently recognized as im-

portant contributor to the pathophysiology of AD. Over 90% of AD patients are colonized by *S. aureus* on both lesional and non-lesional skin which significantly differs from the only 5% of healthy individuals carrying this bacterium.[6] Aggravation of the disease is associated with an increased abundance of *S. aureus* colonization whilst treatment-induced removal of the bacterium improves the atopic skin.[6]

In order to colonize the skin, *S aureus* adheres with its cell wall receptors (adhesins) to extracellular matrix proteins like fibronectin and fibrinogen, present in the SC. Both of these protein-anchors are upregulated by Th_2-inflammatory cytokines like IL-4, and are more exposed due to the affected SC integrity, subsequently resulting in the enhanced binding and colonization of *S. aureus* to atopic skin.[25] Also scratching of the atopic skin enhances *S. aureus* binding as it damages the skin barrier and exposes extracellular matrix proteins. The subsequent disturbance of the skin microbiome, combined with the impaired epidermal barrier integrity and increased alkalinity of the atopic skin facilitate the penetration of *S. aureus*. As discussed before, innate immune responses are often inadequate in AD patients and subsequently give way for *S. aureus* to wield its harmful effects associated with AD.

S. aureus contributes to the pathophysiology of AD in numerous ways, which are broadly summarized in Table 1. Firstly, bacterial cell wall components like peptidoglycan and lipoteichoic acids interact with specific PRRs like the Toll-like receptors (TLR) through which a Th_2-based inflammation is promoted. *S. aureus* membranous lipoproteins dimerize for example TLR2 and TLR6 on keratinocytes which induces the release of Thymic stromal lymphopoietin (TSLP).[26] TSLP is known to link responses between host and bacteria to Th_2-based immune responses. It acts on a variety of cells including dendritic cells (DCs), mast cells, eosinophils and natural killer cells and induces the differentiation of naive T-cells into Th_2-cells, all resulting in the production and release of Th_2-related chemokines and cytokines.[26] Next to TSLP, also other keratinocytes-derived mediators like GM-CSF, RANTES and MCP-1 are induced by *S. aureus* cell wall components, showing that the presence of *S. aureus* alone already polarizes the inflammation in AD.[27]

Up to 60% of *S. aureus* strains isolated from AD patients are able to secrete α-toxin, a virulent exotoxin belonging to the family of pore-forming cytotoxins.[30] A-toxins (or α-hemolysins) bind and insert themselves into the cell membrane of various host cells (including keratinocytes, T-cells and macrophages) after which they aggregate into a ring-structured hexamer. This construction functions as a membrane perforating pore through which calcium, potassium and other molecules of low weight can leak in or out the cell. Doing so, α-toxins cause immune dysregulation, either by inducing apoptotic cell death or (in sublethal dosages) by affecting intracellular signaling pathways of processes like cytokine secretion.[28] As both innate and adaptive immune cells are targeted, α-toxins also attenuate the host's defensive responses to the percutaneous invasion of *S. aureus*. Compared to healthy individuals, AD patients are more vulnerable towards α-toxin induced cytotoxicity as their raised levels of Th_2-based cytokines (IL-4 and IL-13) significantly sensitize keratinocytes towards the lytic activity of α-toxins.[29] Carriage of α-toxin producing *S. aureus* strains is also associated with more severe forms of AD.[30]

Another major class of exotoxins produced by *S. aureus* and involved in AD are the Staphylococcal enterotoxins, commonly referred to as superantigens (SAg). SAg are considered to be the most important mediators by which *S. aureus* contributes to the inflammatory immune patterns in AD.[32] In the upcoming chapters, different SAg subtypes, their mechanisms of action and subsequent relation to AD will be discussed in greater detail.

TABLE 1.

***S. aureus* virulent components/toxins:**	**Mechanisms of promoting (Th_2-based) inflammation in AD:**
Cell wall components	activation of PRRs like TLR
α-toxin	Altering intracellular signaling pathways and induction of apoptosis through the formation of membrane perforating pores in host target cells
Enterotoxins	Induction of Vβ-specific polyclonal T-cell activation and cytokine storm

S. aureus superantigens: enterotoxins and TSST-1.

SAg are a family of bacterial, viral and fungal exotoxins, characterized by strong T-cell mitogenic activity and ability to stimulate cytokine release.[31] In inflammatory skin diseases, the secretion of SAg by invading pathogens is known to maintain and exacerbate the cutaneous immune responses. But also direct application of SAg on healthy skin and non-affected AD skin causes erythema and dermatitis. Up to 80% of *S. aureus* strains isolated from AD patients secrete SAg, and the presence of these SAg-producing strains is associated with more severe forms of AD.[30]

Staphylococcal SAg include toxic shock syndrome toxin-1 (TSST-1) and a variety of enterotoxins, which can be divided in two subgroups.[33] Staphylococcal enterotoxin (SE) type A (SEA), SEB, SEC, SED, SEE and SEI (as well as TSST-1) hold emetic activity, a key feature of enterotoxins as they affect the gastrointestinal tract (explaining the 'entero' in its designation). However the majority of the identified enterotoxins either lack or have not been tested for emetic activity, making it more properly to refer to them as Staphylococcal enterotoxin-like (SEl) types. This second group includes SEl-G, H, J, K, L, M, N, O, P and SEl-Q.[33] SAg grouping can however also be based on other criteria like sequence similarity, resulting in alternative subgroups.[37] The expression frequency varies among the enterotoxins, with SEA, B, C, D and TSST-1 being the most common and researched ones.[36] *S. aureus* is able to secrete multiple types of enterotoxins at once, and the production of both higher numbers and specific combinations of SAg are associated with more severe forms of AD.[33,34]

Conventional antigen presentation/recognition versus SAg mode of action.

As the name already implies, Staphylococcal SAg do not behave as conventional antigens inside the host. They hold a significantly stronger T-cell mitogenic activity which is achieved by alternative binding to both the major histocompatibility complex class II molecules (MHCII) and T-cell receptors (TCR) in the host. They are also able to directly interact with other immune cells like macrophages, langerhans cells, dendritic cells, keratinocytes and eosinophils and bypass the immune-suppressive activity of regulatory T-cells.

1. Conventional antigen processing and presentation on MHCII

The presentation of potential hazardous peptides by MHC molecules is a key feature of adaptive immunity. All nucleated cells express MHC class I molecules which present peptides of degraded endogenous self (e.g. tumor) and non-self (e.g. viral) proteins to $CD8^+$ cytotoxic T-lymphocytes (CTL). After activation, CTLs are able to induce apoptosis in all cells expressing that specific antigen, contributing to the cell-mediated immunity.[38] Professional antigen-presenting cells (APCs) like B-cells, macrophages and dendritic cells express MHC class II molecules on their membranes as well. These APCs are able to internalize exogenous proteins via phagocytosis and receptor-mediated endocytosis and process them via the endocytic pathway. Whilst traveling from early endosomes to late endosomes and lysosomes, the internalized proteins are degraded into antigenic peptides and bind MHCII molecules which are present in the endocytic compartment as well. Once loaded with an antigen in its antigen-binding groove, the MHCII complex is transported to the plasma membrane of the APC where it after insertion presents the antigen to naive CD4+ T-cells. TCR recognition of the presented antigen together with the presence of co-stimulatory signals results in the activation of the naive T-cells (Figure 1A).[63] After differentiation into T-helper cells, they generate specific immune responses through the secretion of cytokines and activation of other immune cells.[38]

2. Alternative MHCII α- and β-chain binding by SAg

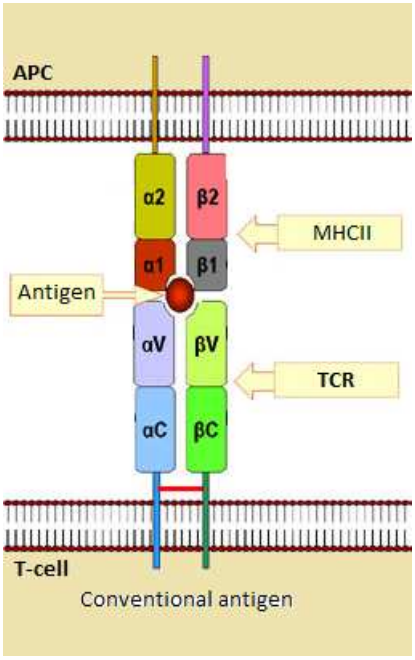
In contrast to conventional antigens, SAg do not undergo antigen processing inside the APC to be presented on MHCII for T-cell activation. Instead, SAg are able to bind directly to MHCII molecules already present on the membrane of APCs, and cross-link them with TCRs bearing specific Vβ-elements, resulting in the polyclonal activation of T-cells and massive cytokine release(Figure 1B).[63] Each SAg has its own distinct binding site on the MHCII molecule, however they all attach as intact proteins outside the antigen-binding groove without inducing large conformational changes. Moreover, SAg are constructed in a similar two-domain fashion and this structural homology among SAg together with the presence of conserved amino-acid residues (important in the interaction with MHCII) imply that some binding modes might be similar among SAg.[3]

All MHCII molecules comprise a conserved α-chain and a highly polymorphic β-chain. Both polypeptide chains consist of two domains (subsequently α1-α2 and β1-β2) in which the α2-β2 domains form the transmembrane domain of MCHII, and the α1-β1 domains the antigen-binding groove (see Figure 1). Major SAg like SEA, SEB, SEC, SED, SEE and TSST-1 all seem to bind to the MHCII α-chain α1-domain through conserved phenylalanine residues in their amino-terminal domain.[39,40,42,43] This binding also facilitates cross-linking with the TCR as it positions the SAg in a critical way for interactions with Vβ-elements.[39,40,41] Since the α-chain is a conserved feature of MHCII, α1-domain interacting SAg are able to bind numerous MHCII allotypes expressed by the host, only further strengthening its T-cell mitogenic activity.[41]

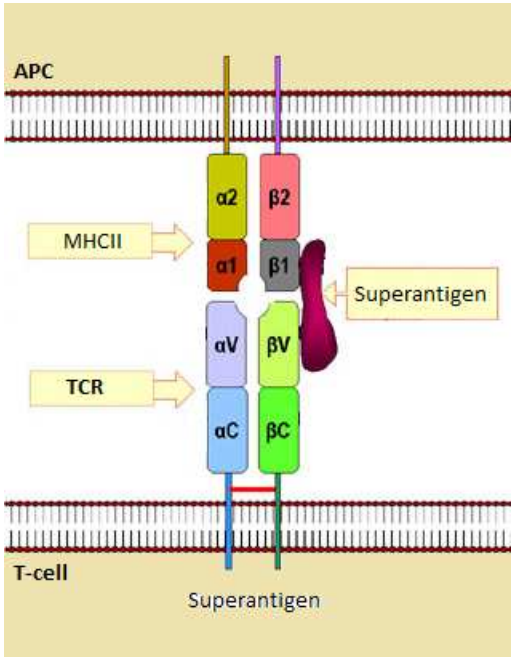
For high affinity binding to MHCII, some SAg like SEA, SED, SEE, SEI and SEl-H are dependent on the metal ion zinc

(Zn^{2+}).[43] Zn^{2+} is known to be involved in many protein and receptor interactions, and its role in MHCII binding by SAg has been recognized as well. Zn^{2+} ions function as a bridge between a conserved metal-binding site located in the carboxy-terminal domain of certain SAg and the specific Zn^{2+}-binding residue Histidine81 (His81) on the β-chain of MHCII. *In vitro* studies have shown that losing the ability to bind Zn^{2+} (as a result of induced mutations in the metal-binding site) diminishes both MHCII binding and activation of T-cells by SAg.[40] Unlike the α-chain, the β-chain is highly polymorphic among MHCII molecules, which could limit the extent of SAg interaction to only a small group of specific MHCII allotypes. However, the recruitment of Zn^{2+} most likely bypasses this limitation as it makes the SAg-MHCII interaction less dependent on the expressed residues, subsequently sustaining the interaction and promoting T-cell activation.[40] Next to this, Zn^{2+} is also able to homodimerize SAg like SED, during which two Zn^{2+} ions are sandwiched between two molecules of SED. This opportunity of SED to bind MHCII as either a monomer or a dimer allows for interaction with a broader array of Vβ-elements, subsequently activating a bigger population of T-cells.

SAg like SEA express distinct MHCII binding sites targeting both the α- and β-chain of MHCII. It allows the SAg to be active at lower concentrations and facilitates otherwise low affinity bindings (like α-chain binding in SEA) by first attaching strongly via the other binding site (in this case the Zn^{2+} mediated β-chain binding). Moreover, it renders opportunities to dimerize MHCII molecules in the membrane of the APCs, by binding two different MHCII molecules with either binding site. Doing so, the APC becomes activated and starts to secrete cytokines and express costimulatory signals (like B7) which all contribute to T-cell activation upon cross-linking with a TCR. This however will be discussed in greater detail in the upcoming chapter.



1A.



1B.

FIGURE 1:[63] Schematic overview of conventional antigen presentation and recognition and alternative binding of both MHCII and TCR by SAg.

A: Conventional antigen presentation by MHCII and subsequent recognition by the TCR. APCs express MHCII molecules on their membrane which are build up of a conserved α-chain and a highly polymorphic β-chain. Both chains consist of two domains (subsequently α1-α2 and β1-β2) in which the α1- and β1-domains together form the antigen-binding groove. Bound antigens are presented to the TCR, which is also build up of an α-chain (blue) and β-chain (green). Both chains of the TCR compose a variable domain (αV and βV), followed by a constant domain (αC and βC). The variable domains of both chains hold three complementarity-determining regions (CDR), which are responsible for the very specific antigen recognition.

B: Alternative binding of both MHCII and TCR by a SAg. In contrast to conventional antigens, SAg neither undergo antigen processing inside the APC nor are they presented on MHCII in the therefore destined antigen-binding groove. Instead, SAg are able to bind directly to either the β1 and/or α1 domain of the MHCII molecules. Next to this, Sag interact with specific Vβ-elements on the β-chain of the TCR as well. This SAg induced cross-linking between MHCII and TCR results in the polyclonal activation of T-cells and massive cytokine release.

3. Conventional TCR-mediated antigen recognition and T-cell activation

Both the recognition of MHCII presented antigens and subsequent T-cell activation is mediated by the TCR. Based on the composing polypeptide chains, two distinct types of TCRs are identified, the αβ- and the γδ-TCR. The αβ-TCR is by far the most prevalent and important receptor among T-cells, expressing high specificity for antigen recognition due to its hypervariable regions. In this thesis, all references to TCR denote the αβ-type. As shown in Figure 1,[63] both chains of the TCR contain a variable domain (Vα and Vβ) located at the amino-terminus, followed by a constant domain (Cα and Cβ). Together, these domains compose the extracellular antigen-binding unit of the receptor. The α- and β-chain are connected through a disulfide bridge, which is established directly in front of the chains membrane-anchoring transmembrane regions. The intracellular carboxy-terminus of each chain forms a cytoplasmic tail important for the TCR downstream signal transduction.

The Vα and Vβ domains of the TCR compose three hypervariable regions, referred to as the complementarity-determining regions (CDR) 1, 2 and 3. These regions are characterized by high, random sequence variations among T-cells which allow for very unique and hence specific antigen recognition by the TCR. The direct TCR interaction with the presented antigen takes place via the CDR1 and CDR3 regions whilst the CDR2 regions simultaneously interact with the conserved α-chain of the MHCII molecule. However, since these interactions are of low affinity, numerous T-cell accessory membrane molecules like CD3, CD4 and CD28 bind their ligands expressed on the APC membrane as well to strengthen the bound. These co-stimulators furthermore participate in the subsequent downstream signal transduction and T-cell activation. As mentioned before, SAg are able to induce the expression of these ligands through the dimerization of MHCII molecules on the APCs.

The interaction of a TCR with an antigen presented on MHCII, combined with all accessory co-receptors induces several signal transduction pathways which alter the gene expression profile of the naive T-cell. Amongst many other things, the newly

transcripted genes drive the T-cell into the G_1 phase of the cell cycle and cause secretion of IL-2 whilst simultaneously the IL-2 receptor is upregulated. All these stimulatory signals combined result in T-cell activation, proliferation and differentiation into various effector and memory T-cells. Depending on the cytokine environment, these effector cells will either have a Th_1 or Th_2 phenotype with distinct cytokine profile. The presence of INF-γ, IL-12 and IL-18 all drive towards Th_1, which is a key player in the cell-mediated immunity through its secretion of IL-2, TNF-β and IFN-γ. High IL-4 levels on the other hand (an important feature of AD) stimulates the differentiation into Th_2-cells, which contribute to humoral immunity through the secretion of IL-4, IL-5, IL-6 and IL-10.

4. Alternative TCR β-chain binding by SAg and polyclonal T-cell activation

The highly specific interaction between MHCII-presented antigens and TCRs activates only about 0.001% of all T-cells.[44] SAg on the other hand interact with TCRs in an alternative and far less specific manner, which enables them to associate with a significantly larger proportion of T-cells, activating up to 20% of the total T-cell population.[31] Contrary to conventional antigen-binding mechanisms, SAg bind specific residues mainly located in the CDR2 region of the TCR β-chain to interact with the receptor (see Figure 1B). These SAg-contact sites reside to a lesser extent in the CDR1, hypervariable region (HV) 4 and framework regions (FR) 2 and 3 of the Vβ-domain as well.[45,46,48] The CDR3 region is however not involved in the direct binding of SAg to the β-chain, although it could modulate SAg activity after TCR-binding has established. Each SAg requires different residues in the Vβ-domain to contact the TCR β-chain, explaining the unique binding specificity of SAg to only a subset of T-cells expressing the right Vβ-elements. Binding to other TCR regions than just described has only been observed for SEl-H, which is able to interact with the Vα-domain of the TCR α-chain.[43,49] However, this interaction is considered exceptional and is not part of the conventional (Vβ-based) binding mechanisms of SAg.

The TCR-binding site is located in a shallow cavity at the top of most staphylococcal SAg molecules. The majority of the Vβ-contacting residues present in this binding site differ among SAg, resulting in the distinct Vβ-binding specificities of each SAg.[45,46,47,48] However, it also comprises some conserved residues like asparagine (Asn23).[39,46] Mutational studies have shown that substitution of this Asn23 results in diminished levels of T-cell activation, which confirmed the involvement of this residue in T-cell mitogenic activity and delivered evidence for a common TCR-binding mechanism among SAg.[39,46] *Yafei et al.* showed however that Asn23 substitution in SED resulted in diminished activation of only a subset of T-cells (bearing TCR-Vβ5) whilst the affinity towards the remaining two subsets SED is able to interact with (TCR-Vβ8 and -Vβ12.1) was not affected.[39] It suggests that SAg like SED convey multiple and independent binding modes to different types of β-chains, next to the presence of conserved residues.

Even though SAg are neither processed nor presented in the therefore destined groove, the primary binding to MHCII remains necessary as it properly positions the SAg, exposing the TCR-binding site. Binding assays have shown that SAg complexed with MHCII express increased TCR affinity and T-

cell mitogenic activity compared to soluble unbound SAg.[46] This ternary MHCII-SAg-TCR complex is stabilized by the α-chain of the TCR, which binds with its CDR2 region the β1-domain of the MHCII β-chain. Doing so, the activation of T-cells bearing TCR with similar Vβ-domains but different Vα-domains is still modulated as some α-chains will strengthen the complexing with MHCII whilst others destabilize it as a consequence of unfavorable residues.[46,47]

If taken together, the MHCII-TCR cross-linking mediated by SAg involves three separate sets of interactions: (1) the SAg to the MHCII α- and/or β-chain, (2) the SAg to the TCR β-chain and (3) the TCR α-chain to the MHCII β-chain. Through these alternative interactions, SAg circumvent the conventional binding mechanisms preceding T-cell activation (based on antigen specificity of the TCR) and instead activate a repertoire of T-cells bearing the correct TCR Vβ-elements. Since similar Vβ-domains are expressed by large subsets of T-cells, SAg stimulation results in polyclonal T-cell activation and expansion in a Vβ-selective manner. This overstimulation of the immune system results in a massive release of cytokines, commonly referred to as the cytokine storm, which induces and exacerbates immune-mediated diseases. In AD, naive T-cells will mostly differentiate into Th_2-cells upon SAg stimuli, due to the already skewed cytokine environment in these patients. The subsequent vigorous production of key cytokines like IL-4, IL-5, IL-6 and IL-10 maintain and exacerbate the AD related skin inflammation.

Other immune modulatory effects of SAg through various cell-types.

It is important to realize that the negative effects of SAg in AD extend beyond their ability to activate big groups of T-cells. Macrophages, dendritic cells, langerhans cells, keratinocytes, eosinophils, mast cells, basophils, regulatory and CLA^+ T-cells are all cell-types affected by SAg in unique ways and all contribute to the pathogenesis of AD. SAg are even found to intervene with the working mechanism of typically prescribed drugs like corticosteroids, which only further complicates disease management.

1. Professional APCs

A range of immune cells are indirectly activated by SAg through the massive cytokine release following SAg induced polyclonal T-cell activation. Next to this, professional APCs like macrophages, dendritic cells and langerhans cells can be activated by SAg in a more direct manner as well. The adherence of SAg to MHCII already triggers a variety of downstream signaling pathways in the APCs, regardless of additional interactions of this SAg with a TCR. Moreover, certain SAg contain multiple MHCII binding sites which allows them to activate the APC by dimerization of the MHCII molecules in the membrane. This SAg induced downstream signaling of MHCII results in cytokine secretion which affects both the APCs themselves and other immune cells, subsequently contributing to the skin inflammation in AD in multiple ways. For example, SAg adherence to MHCII on macrophages leads to the activation of TNF-α converting enzyme (TACE), which cleaves proTNF-α as well as other cytokines into their active form, resulting in a primary wave of cytokine secretion.[74] The cytokine TGF-α in particular is a ligand for the EGF-receptor, which is present on the macrophage's cell

membrane. Secreted TGF-α (from both SAg-activated macrophages and T-cells) induces even more (self-)activation as it binds the EGF-receptor, triggering a P38MAPk-dependent signaling pathway resulting in a second wave of cytokine release by the macrophage. Next to TACE, SAg induced MHCII signaling also involves the transcription factor NFκβ which after translocation to the nucleus induces the expression of cytokine IL-12.[74]

2. CLA^+ T-cells

In the presence of the cytokine IL-12, SAg do not only activate subsets of Vβ-bearing T-cells, but upregulate their expression of the cutaneous lymphocyte-associated antigen (CLA) as well. The CLA receptor is responsible for skin-selective migration and subsequent homing of the T-cell. Over 80% of all skin-infiltrating T-cells are CLA positive.[32] Interaction of CLA with its vascular ligand E-selectin (present on endothelial cells) is very important for T-cell extravasation into inflamed skin areas. Thus by increasing CLA expression, SAg stimulate T-cell infiltration into the skin, subsequently aggravating the cutaneous inflammation associated with AD.

3. Keratinocytes

The cytokine IFN-γ is known to induce MHCII expression on non-professional APCs like keratinocytes. This induced MHCII expression broadens the array of cell-types SAg are able to interact with to induce polyclonal T-cell activation. Keratinocytes themselves are also activated in the same way as observed with professional APCs by the adherence of SAg to their MHCII molecules, resulting in the secretion of cytokines and chemo-attractants like eotaxin-3. Next to this, research has shown that IFN-γ also upregulates the expression of the FAS death receptor on exposed keratinocytes, whilst activated and skin infiltrating T-cells both secrete Fas-ligand and express it on their membrane. The interaction between Fas and Fas-ligand subsequently generates a death signal inside the keratinocytes. Apoptotic keratinocytes play a role in many eczematous disorders as they contribute to the spongiotic process inside the epidermis and augment skin infiltration by T-cells through the secretion of specific T-cell recruiting chemokines.[54,57] This could also be the case in AD, although the direct contribution of induced keratinocytes apoptosis in the aggravation of AD remains to be determined.

4. Eosinophils

Th_2-cytokines IL-5 and IL-3 play important roles in eosinophilopoiesis, as they stimulate the generation, maturation and survival of eosinophils, as well as their release from the bone marrow into the bloodstream.[32] In AD, activated keratinocytes secrete increased levels of chemotactic factors like eotaxin and regulated on activation, normal T expressed and secreted (RANTES), which results in the subsequent heightened recruitment of eosinophils into the inflamed areas of the skin.[57,32] Numerous SAg like SEB have been found to inhibit eosinophil apoptosis and increase the expression of membrane receptors like CD11b and CD69, both known activation markers for the eosinophils. This makes it tempting to speculate that SAg are able to activate eosinophils in AD as well.[55] Once activated, eosinophils secrete their granule proteins and lipid mediators which promote inflammation and tissue damage by increasing microvascular permeability, attracting and activating other immune cells and triggering flare reactions.[31] Both blood and skin tissue

eosinophilia are common features of AD and correlate with disease severity.[56,32]

5. IgE antibodies, basophils and mast cells

The continuous release of SAg into the skin in combination with the Th_2-inflammatory environment can induce a local type 1 hypersensitivity reaction. This allergic sensitization in which SAg-specific IgE antibodies are generated is a common feature of AD, affecting up to 80% of all AD patients.[58] The presence of these IgE antibodies correlates with both the total serum IgE level of the patient and the severity of disease.[58] In response to SAg exposure, IgE antibodies are generated which bind Fc-receptors present on a variety of immune cells and remain there for weeks during which the cells are sensitized. Upon re-exposure, the SAg are now able to directly activate all Fc-receptor bearing cells which are sensitized with the correct IgE antibody and subsequently exacerbate AD by inducing IgE-mediated inflammation. Especially basophils and mast cells play an important role in allergic sensitization as SAg induced cross-linking of their membrane-bound IgE induces degranulation, resulting in the local release of histamine and Th_2-based cytokines.[32,61] Interestingly, research has shown that AD patients express increased numbers of Fc-receptors on their peripheral blood monocytes and Langerhans cells compared to healthy controls, which might enhance their activation by SAg as well as their SAg presentation on MHCII, both fueling the inflammation in AD.[59,60]

6. Regulatory T-cells

AD patients are found to carry non-functional regulatory T-cells (Tregs),[50,51] which could explain why all the above mentioned augmented immune responses in the inflamed areas of the skin are neither controlled nor suppressed. Tregs are a subset of T-cells characterized by their immunosuppressive activity.[52] They need to be activated before they can exert these immune-modulating effects, however this requires much lower antigen concentrations compared to conventional activation of naive T-cells.[52] Also, the activation of Tregs is not restricted by the need for costimulatory signals, allowing immature DCs to activate Tregs solely by antigen presentation.[52] Once activated, Tregs suppress other lymphocytes via both cell-contact dependent and independent mechanisms, expressing key molecules like CTLA-4 on the cell surface whilst simultaneously secreting anti-inflammatory cytokines like IL-10 and TGF-β. Doing so, Tregs modify and kill APCs and prevent the activation of antigen-stimulated naive T-cells.[50]

The percentage of CD4+CD25+ Treg cells in the peripheral blood of AD patients is significantly increased compared to nonatopic healthy controls,[50] although this rise seems to be insufficient, considering the inflammatory nature of the disease. In papers by *Ou et al.* and Lin et al. *in vitro* analysis showed that Tregs isolated from both AD patients and healthy controls were equally anergic to stimulation and capable of suppressing T-cell proliferative responses. However once exposed to the SAg SEB, the Tregs became activated and lost their immunosuppressive effects.[50,51] These findings suggest that in a subgroup of AD patients, Treg functioning is hampered as a result of the presence of SAg producing *S. aureus* strains, subsequently leading to increased levels of T-cell proliferation. Treg dysfunction was already associated with severe forms of atopic dermatitis, and this could be an additional mechanism of SAg to augment

T-cell activation and aggravate the skin inflammation associated with AD.[50]

7. SAg-induced corticosteroid resistance of T-cells

SAg affect a vast array of different cell types, and does so in quite unique ways, making treatment options with a more general approach (like the use of immunosuppressive drugs) more suitable for AD patients. As said before, topical corticosteroids (TC) are successfully deployed worldwide in the treatment of inflammatory skin diseases like AD. Unfortunately, corticosteroid resistance (CR) occasionally develops during which the immunosuppressive and anti-inflammatory properties of the TC are diminished, subsequently complicating the patient's disease management. *Li et al.*[62] discovered that SAg are able to induce T-cell resistance to the corticosteroid dexamethasone, and does so most likely through enhanced CD28 signaling.

Corticosteroids wield their immunosuppressive actions by diffusing into the host target cells and binding to the glucocorticoid receptor α (GCRα) which resides in the cytosol. In response, bound GCRα travels to the cell nucleus and induces the expression of target genes involved in the suppression of inflammatory and proliferative responses whilst it simultaneously suppresses the expression of pro-inflammatory genes. In accordance with this, *Li et al.* showed that T-cell proliferation was indeed successfully inhibited by dexamethasone *in vitro*. However, they also found that this inhibitory effect of dexamethasone was significantly decreased in the presence of additional CD28 co-stimulation, which seemed to make the T-cells more resistant. It is already known that CD28 co-stimulation is very important for T-cell activation as it strengthens TCR-signaling to the mitogen-activated protein kinase (MAPK) cascade and augments ERK activation, making it faster, stronger and longer persisting. Interestingly, ERK proteins function as a kinase which directly phosphorylate many target proteins, amongst which the glucocorticoid receptor α (GCRα). Once phosphorylated by ERK, the corticosteroid-induced translocation of the GCRα to the nucleus is inhibited, resulting in diminished damping of the inflammatory processes, creating a state of CR. Interestingly, *Li et al.* discovered that SAg stimulation also reduced the dexamethasone induced GCRα translocation into the nuclei, subsequently enabling the T-cell to proliferate whilst still being exposed to dexamethasone. As said before, SAg are capable of upregulating costimulatory signals like CD28 on the APC, which might be the underlying working mechanism of this process. By inducing a state of CR, SAg only further contribute to the severity of AD as it complicates the treatment and disease management of the patient.

Future perspectives: potential of SAg-specific VHHs as novel therapy in AD.

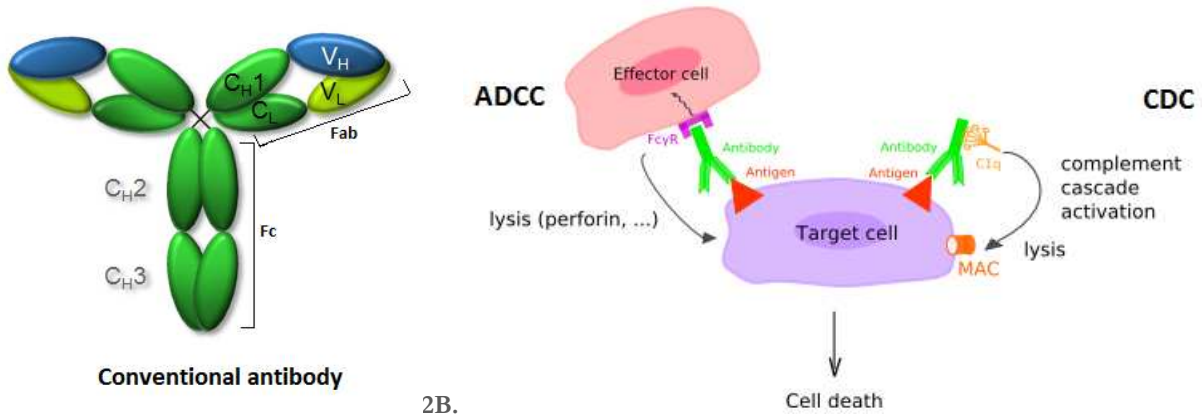
Over 90% of all AD patients are colonized by *S. aureus* and up to 80% of *S. aureus* strains isolated from AD patients secrete SAg.[6,30] Both the colonization and secretion of SAg are important trigger factors in AD and associate with disease aggravation. There is still no cure for AD, however in most cases the disease can be adequately treated with TCs. Sometimes a more multifaceted approach is necessary in which anti-inflammatory steroids are combined with other drugs like antibacterials. Unfortunately, both the ability of SAg to induce CR and the increasing

prevalence of MRSA strains impedes the treatment, subsequently complicating disease management. It is clear that for this subgroup of patients new options for therapy are desirable and the application of SAg-specific antibodies could hold a lot of potential.

1. Conventional antibody structure, functioning and therapeutic use

As shown in Figure 2A[72], antibodies (or immunoglobulins) are glycoproteins build up of two identical heavy-chains and two identical light-chains, which are linked together by disulfide bonds. Each chain composes a variable domain (VH and VL) followed by a conserved domain (CH and CL). Whereas the light-chains only have one conserved domain, the heavy-chains compose (depending on the specific class of antibody) either three or four tandem conserved domains, respectively CH1, -2, -3 and -4. The variable domain of each chain has three complementarity-determining regions (CDR1, -2 and -3), which allow for very unique and specific antigen recognition by the antibody. A target antigen is bound by the fragment antigen-binding (Fab) region of the antibody, which encloses the first variable and conserved domains of each chain, or in layman's terms the arms of the Y-shaped molecule. Doing so, antibodies are able to opsonize and neutralize several bacterial toxins and tag microbes and infected cells for further processing by the immune system.[38] The base of the Y-shaped molecule (consisting of the CH-domains) is referred to as the fragment crystallizable (Fc) region and interacts with Fc-receptors present on cytotoxic effector cells like natural killer cells, macrophages and eosinophils. The interaction of an antibody with the Fc-receptor will activate the effector cell and induce the secretion of lytic substances like perforins and granzymes which will destroy the cell expressing the target antigen. This process, visualized in Figure 2B, is known as antibody-dependent cellular cytotoxicity (ADCC).[38] The Fc region of an antibody is also able to interact with the C1q complement protein which triggers the classical complement cascade. This complement-dependent cytotoxicity (CDC) results in the formation of a lethal membrane attack complex (MAC) on the target cell, as shown in Figure 2B[73]. Next to this, the binding of antibodies and complement proteins marks the target cell for opsonin-dependent phagocytosis as well.[38]

In the medical world, both mono- and polyclonal antibodies have proven to hold diagnostic and therapeutic value. They have been successfully applied in the diagnosis and treatment of cancerous, autoimmune and inflammatory diseases like rheumatoid arthritis, Crohn's disease, plaque psoriasis and several types of cancer.[67] Unfortunately, even though antibodies have benefited numerous patients, there are still some major disadvantages clinging to their therapeutic use.[66] To start, the production process of conventional antibodies is time, money and effort consuming and requires the use of mammalian species. Moreover, the binding repertoire of conventional antibodies is restricted by a size limit as small target antigens might not be recognized. Next to this, cross-reactions have been observed during which the antibodies unexpectedly showed affinity to unrelated antigens.[66] These are just a few examples which drive research in the field of antibody technology to design new and improved generations of therapeutic antibodies and develop alternative scaffolds.[66,67]



2A. 2B.

FIGURE 2:[72,73] Schematic overview of (IgG) antibody and its effector functions.
A. Antibodies are build up of two identical heavy-chains and two identical light-chains. Each chain comprises a variable domain (VL and VH) with three CDR regions. Next to this, the light-chains have one conserved domain (CL) whereas the IgG heavy-chains have three (CH1, CH2, CH3). Target antigens are bound with the Fab-region of the antibody whilst the Fc-region interacts with complement proteins and Fc-receptors on cytotoxic cells.
B. Antibodies bind target antigens expressed on the surface of an infected cell or microbe, which subsequently tags the cell for destruction either via antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC).

2. Camelidae-derived VHHs

In 1993, *Hamers-Casterman et al.*[64] discovered a unique feature of a class of antibodies descending from the family of Camelidae, which encloses species like camels, dromedaries, alpacas and llamas. This class of camelid antibodies is devoid of both light-chains and comprise only a heavy-chain dimer (Figure 3A)[72]. These antibodies lack the CH1-domain in both heavy-chains as well, which is known to be important in light-chain binding.[65] As with conventional antibodies, the camelid heavy-chain antibodies do undergo selective refinement in antigen specificity and affinity, which enables them to still express an extensive antigen binding repertoire despite their lack of light-chains.[64] It is from these heavy-chain antibodies that the first single-domain antibodies (VHH or nanobodies) were engineered (Figure 3B[72]).

The production of a VHH fragment starts with the immunization of a camelidae with the target antigen. In response to the antigen exposure, mRNA molecules encoding for heavy-chain antibodies are produced, which are isolated and stored in a gene library. Through subsequent screening tests the most potent antigen-binding antibody is identified, from which the DNA sequence is then optimized to improve certain biological properties like the stability of the VHH. As a last step, the optimized DNA sequence of the VHH is build into a suitable microorganism like *E. coli* for translation, resulting in the microbial production of the desired VHH.[65]

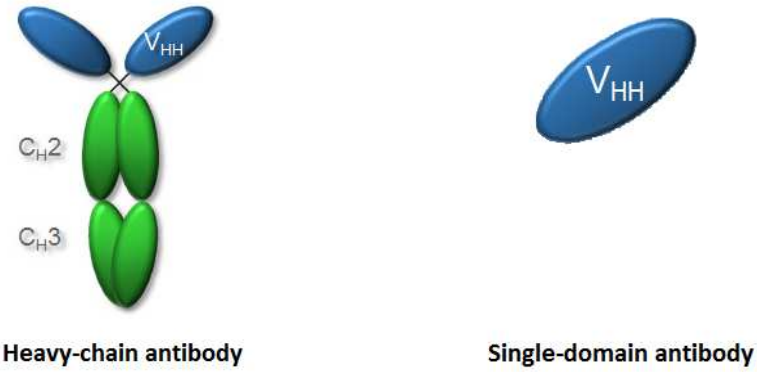


FIGURE 3:[72] Schematic overview of camelid heavy-chain antibody and derived VHH.
A. In contrast to conventional antibodies, heavy-chain antibodies are build up of only two identical heavy-chains as the two light-chains are absent. Each heavy-chain comprises a single variable domain (VHH) with three CDR regions, and two conserved domains (CH2 and CH3). They lack the CH1-domain, present in conventional antibodies.
B. The new generation of single-domain antibodies comprising only the VHH domain of the original heavy-chain antibody. They are referred to as VHHs or nanobodies and express full antigen-binding capacity.

Even though VHHs are composed of only one VH-domain of a heavy-chain camelid antibody, the antigen-binding affinities are preserved and remain equal to that of conventional antibodies. Moreover, the single domain nature of these VHHs makes them advantageous over conventional antibodies in multiple ways.[65] Firstly, VHHs are more resistant to chemically and thermally induced denaturation and efficiently refold if denaturation does occur. This thermodynamic stability enables VHHs to remain functional up to 90°C. VHHs are also much smaller than conventional antibodies which facilitates both tissue penetration and blood clearance, whilst it also enables the VHH to recognize antigenic sites which are hidden for larger antibody fragments. Compared to conventional antibodies, VHHs are also more soluble, easier to genetically manipulate and can be efficiently manufactured by microbial systems without the use of mammalian cells.[65]

However, despite the superior properties of VHHs over conventional antibodies, their therapeutic applicability is still challenging.[65] A downside of VHHs is their inability to induce antibody associated effector functions like ADCC and CDC by themselves. Next to this, the rapid renal clearance causes the VHHs to have only a short serum half-life which limits their efficacy as well. Both problems can however be resolved through additional manipulative steps during the engineering phase of the VHH fragment. Moreover, even without the induction of processes like ADCC and CDC, VHH-antigen binding can still block specific molecular interactions and neutralize microbial toxins. Doing so, numerous oncogenic and inflammatory disorders like solid tumors and rheumatoid arthritis have already been successfully treated with VHHs (see table 2).[65]

TABLE 2. Therapeutic applicability of camelid VHHs.[65]

Disease	Pathogen	Target antigen	Reference
Sleeping sickness	Trypanosomes	VSG Oligomannose	*Baral et al. 2006*
Sepsis	N. meningitidis	LPS	*El Khattabi et al. 2006*
Cancer	-	CEA	*Cortez-Retamozo et al. 2004*
Cancer	-	EGF-receptor	*Roovers et al. 2007*
Rheumatoid arthritis	-	TNF-α	*Coppieters et al. 2006*
Brain disorders	-	A (2,3)-Sialoglycoprotein	*Muruganandam et al. 2002*
Neurodegenerative diseases	-	BAX	*Gueorguieva et al. 2006*
Infant diarrhea	Rotavirus	unknown	*Van der Vaart et al. 2006 & Pant et al. 2006*

3. Applicability of VHHs in Atopic Dermatitis

When it comes to the applicability of VHHs in AD, not a lot of research has been done. However considering its toxin neutralizing abilities, VHHs hold a lot of potential for the subgroup of AD-patients colonized by SAg-producing *S. aureus* strains. In 2006, *Goldman et al.* constructed an extensive library of semi-synthetic llama single-domain antibodies and successfully selected VHHs specific for a variety of toxins, including SEB.[68] *Graef et al.* also isolated VHHs with high affinity and specificity for SEB and furthermore ruled out any cross-reactivity to related molecules like SEA and SED.[69] Both reports demonstrated the applicability of their VHHs as capture and reporter molecules in antibody-based detection assays. In 2009, *Adems et al.* demonstrated that their llama-derived VHHs directed against TSST-1 could efficiently remove TSST-1 from spiked human and pig plasma *in vitro*, even in very low (pathologically relevant) concentrations. In an attempt to further explore the applicability of SAg-specific VHHs, *Horrevoets et al.*[71] set up a proliferation assay in which isolated human peripheral blood mononuclear cells were stimulated with various doses of TSST-1, SEA or SEB. Subsequent addition of their llama-derived anti-TSST-1 VHHs significantly reduced the radioactive thymidin incorporation in the TSST-1-stimulated subgroup. This inhibition of proliferation was specific as the thymidin incorporation was not reduced in either SEA- or SEB-stimulated subgroups after addition of the anti-TSST-1 VHH. These results showed that through the specific binding of their target SAg, VHHs are able to neutralize SAg-associated effector functions like the induction of cell proliferation *in vitro* as well.

All these data serve as stepping stones towards the development of novel therapies based on SAg-specific VHHs. Even though further *in vivo* research is necessary, one could already speculate about the possibilities of VHHs in the treatment of AD-patients colonized by SAg-producing *S. aureus* strains. For instance, after identification of the SAg secreted by the colonizing *S. aureus* strains, specific llama-derived VHHs could be generated and processed in the form of an ointment. In an ideal situation, topical application of this crème neutralizes the locally secreted SAg and inhibits their inflammatory effector functions, resulting in an improved disease management of AD which ultimately contributes to the patients quality of life.

Reference list.

1. Leung, D.Y.M. et al., (2003), Atopic dermatitis, The lancet, Jan;361:151-60
2. Shultz-Larsen, F. et al., (2001), Epidemiology of atopic dermatitis, Immunology and allergy clinics of North America, 22:1-24
3. Watson, W. et al., (2011), Atopic dermatitis, Allergy, Asthma & Clinical Immunology, 7 (Suppl 1):S4
4. Zheng, T. et al., (2011), The Atopic March: Progression from Atopic Dermatitis to Allergic Rhinitis and Asthma, Allergy, asthma and immunology research, Apr;3(2):67-73
5. National Eczema Association, (2014), Topical corticosteroids: risks of topical corticosteroids, https://nationaleczema.org/eczema/treatment/topical-corticosteroids/risks-of-topical-corticosteriods/, 21;Jan;2014
6. Baker, B.S. et al., (2006), The role of microorganisms in atopic dermatitis, Clinical and experimental immunology, Apr;144(1):1-9
7. Barnes, K.C. et al., (2010), An update on the genetics of atopic dermatitis: Scratching the surface in 2009, The journal of allergy and clinical immunology, Jan;125(1):16-29
8. Levin, J. et al., (2013), Atopic Dermatitis and the Stratum Corneum. Part 1: The role of filaggrin in the stratum corneum barrier and atopic skin, The Journal of Clinical and Aesthetic Dermatology, Oct;6(10):16-22
9. Kezic, S. et al., (2008), Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum, The journal of Investigative Dermatology, Aug;128(8):2117-2119
10. Palmer, C.N. et al., (2006), Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis, Nature Genetics, Apr;38(4):441-6.
11. Rodriques, E. et al., (2009), Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease, The journal of allergy and clinical immunology, Jun;123(6):1361-70
12. Baurecht, H. et al., (2007), Toward a major risk factor for atopic eczema: meta-analysis of filaggrin polymorphism data, The journal of allergy and clinical immunology, Dec;120(6):1406-12
13. Elias, P.M. et al., (2009), Abnormal skin barrier in the etiopathogenesis of atopic dermatitis, Current Opinion in Allergy and Clinical Immunology, Oct;9(5):437-46
14. Eberlein-König, B. et al., (2000), Skin surface pH, stratum corneum hydration, trans-epidermal water loss and skin roughness related to atopic eczema and skin dryness in a population of primary school children, Acta-Dermato Venereologica, May;80(3):188-91
15. Integrit Hachem, J.P. et al., (2005), Sustained serine proteases activity by prolonged increase in pH leads to degradation of lipid processing e nzymesand profound alterations of barrier function and stratum corneum, The journal of investigative dermatology, Sep;125(3):510-20
16. Levin, J. et al., (2013), Atopic Dermatitis and the Stratum Corneum. Part 2: other structural and functional characteristics of the stratum corneum barrier in atopic skin, The Journal of Clinical and Aesthetic Dermatology, Nov:6(11):49-51
17. Di Nardo, A. et al., (1998), Ceramide and cholesterol composition of the skin of patients with atopic dermatitis, Acta-Dermato Venereologica, Jan;78(1):27-30

18. Levin, J. et al., (2013), Atopic Dermatitis and the Stratum Corneum. Part 3: The immune system in atopic dermatitis, The Journal of Clinical and Aesthetic Dermatology, Dec:10
19. Elias, P.M. et al., (2008), Basis for the barrier abnormality in atopic dermatitis: Outside-inside-outside pathogenic mechanisms, The Journal of Allergy and Clinical Immunology, Jun;121(6):1337-43
20. Chen, Y.E. et al., (2013), The skin microbiome: Current perspectives and future challenges, The Journal of American Academic Dermatology, Jul;69:143-55
21. Bourrain, M. et al., (2013), Balance between beneficial microflora and Staphylococcus aureus colonisation: in vivo evaluation in patients with atopic dermatitis during hydrotherapy, European Journal of Dermatology, Dec;23(6):786-94
22. Iwase, T. et al., (2010), Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization, May;20;465(7296):346-9
23. Kong, H.H. et al., (2012), Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis, Genome Research, May;22(5):850-9
24. Selwyn, S. et al., (1965), Dispersal of bacteria from skin lesions: a hospital hazard, The British Journal of Dermatology, Jul;77:349-56
25. Cho, S.H. et al., (2001), Fibronectin and fibrinogen contribute to the enhanced binding of Staphylococcus aureus to atopic skin, The Journal of Allergy and Clinical Immunology, Aug;108(2):269-74
26. Vu, A.T. et al., (2010), Staphylococcus aureus membrane and diacylated lipopeptide induce thymic stromal lymphopoietin inkeratinocytes through the Toll-like receptor 2-Toll-like receptor 6 pathway, The Journal of Allergy and Clinical Immunology, Nov;126(5):985-93, 993
27. Matsubara, M. et al., (2004), Staphylococcus aureus peptidoglycan stimulates granulocyte macrophage colony-stimulating factor productionfrom human epidermal keratinocytes via mitogen-activated protein kinases, FEBS Letters, May 21;566(1-3):195-200.
28. Berube, B.J. et al., (2013), Staphylococcus aureus α-Toxin: Nearly a Century of Intrigue, Toxins, Jun;5(6):1140-66.
29. Brauweiler, A.M. et al., (2014), Th2 Cytokines Increase Staphylococcus Aureus Alpha Toxin Induced Keratinocyte Death Through the Signal Transducer and Activator of Transcription 6 (STAT6), The Journal of Investigative Dermatology, doi: 10.1038/jid.2014.43
30. Ong, P.Y. et al., (2010), The infectious aspects of Atopic Dermatitis, Immunology and Allergy Clinics of North America, Aug;30(3):309-21
31. Guedes, A. et al., (2008), Role of superantigens in Atopic Dermatitis, US Dermatology, Vol;3(1)
32. Ou, L.S. et al., (2007), Cellular aspects of Atopic Dermatitis, Clinical Review in Allergy and Immunology, Dec;33(3):191-8
33. Schlievert, P.M. et al., (2008), Superantigen profile of Staphylococcus aureus isolates from patients with steroid-resistant atopic dermatitis, Clinical Infectious Diseases, May 15;46(10):1562-7
34. Leung, D.Y. et al., (2009), Effects of pimecrolimus cream 1% in the treatment of patients with atopic dermatitis who demonstrate a clinical insensitivity to topical corticosteroids: a randomized,multicentre vehicle-controlled trial, The British Journal of Dermatology, Aug;161(2):435-43
35. Pragman, A.A. et al., (2004), Characterization of virulence factor regulation by SrrAB, a two-component system in Staphylococcus aureus, Journal of Bacteriology, April; 186(8): 2430–2438
36. Cardona, I.D. et al., (2006), Role of Bacterial Superantigens in Atopic Dermatitis: Implications for Future Therapeutic Strategies, American Journal of Clinical Dermatology, 7(5):273-9

37. Sundstrom, M. et al., (1996), The crystal structure of Staphylococcal enterotoxins type D reveals Zn^{2+}- mediated homodimerization, The EMBO Journal, Dec 16;15(24):6832-40
38. Kuby et al. Immunology, sixth edition.
39. Li, Y. et al., (2006), Mutational analysis of the binding of staphylococcal enterotoxin D to the Tcell receptor V chain and major histocompatibility complex class II, Immunology letters, May 15;105(1):55-60
40. Hudson, K.R. et al., (1995), Staphylococcal enterotoxins A has two cooperative binding sites on major histocompatibility complex class II, The Journal of Experimental Medicine, Sep 1;182(3):711-20
41. Jardetzky, T.S. et al., (1994), Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen, Nature, Apr 21;368(6473):711-8
42. Hurley, J.M. et al., (1995), Identification of class II major histocompatibility complex and T cell receptor binding sites in the superantigen toxic shock syndrome toxin 1, The Journal of Experimental Medicine, Jun1;181(6):2229-35
43. Pumphrey, N. et al., (2007), Cutting Edge: evidence of direct TCR α-chain interaction with superantigen. The journal of immunology, Sep 1;179(5):2700-4
44. Mak, T.W. et al., (2006), The immune response: Basic and clinical principles, Elsevier; Academic press
45. Li, H. et al., (1998), Structure-function studies of T-cell receptor-superantigen interactions, Immunological Reviews, Jun;163:177-86
46. Li, H. et al., (1999), The structural basis of T cell activation by superantigens, Annual Review of Immunology, 17:435-66
47. Leder, L. et al., (1998), A Mutational Analysis of the Binding of Staphylococcal Enterotoxins B and C3 to the T Cell Receptor β Chain and Major Histocompatibility Complex Class II, The Journal of Experimental Medicine, Mar16;187(6):823-33
48. Dinges, M.M. et al., (2000), Exotoxins of Staphylococcus aureus, Clinical Microbiology Reviews, Jan;13(1):16-34
49. Saline, M. et al., (2010), The structure of superantigen complexed with TCR and MHC reveals novel insights into superantigenic T cell activation, Nature communications, Nov 16;1:119
50. Ou, L.S. et al., (2004), T regulatory cells in atopic dermatitis and subversion of their activity by superantigens, The Journal of Allergy and Clinical Immunology, Apr;113(4):756-63
51. Lin, Y.T. et al., (2011), Skin-homing CD41 Foxp31 T cells exert Th2-like function after staphylococcal superantigen stimulation in atopic dermatitis patients, Clinical and Experimental Allergy, Apr;41(4):516-25
52. Sakaguchi, S. et al., (2009), Regulatory T cells: how do they suppress immune responses?, International Immunology, Oct;21(10):1105-11
53. Sowden, J.M. et al., (1991), Double-blind, controlled, crossover study of cyclosporin in adults with severe refractory atopic dermatitis, The lancet, Jul 20;338(8760):137-40
54. Trautmann, A. et al., (2000), Role of dysregulated apoptosis in atopic dermatitis, Apoptosis, Nov;5(5):425-9.
55. Wedi, B. et al., (2002), Staphylococcal exotoxins exert proinflammatory effects through inhibition of eosinophils apoptosis, increased surface antigen expression (CD11b, CD45 and CD69), and enhanced cytokine-activated oxidative burst, thereby triggering allergic inflammatory reactions, The Journal of Clinical and Allergic Immunology,Mar;109(3):477-84

56. Kiehl, P. et al., (2001), Tissue eosinophilia in acute and chronic atopic dermatitis: a morphometric approach using quantitative image analysis of immunostaining, British Journal of Dermatology, 145:720–729
57. Esche, C. et al., (2004), Keratinocytes in atopic dermatitis: inflammatory signals, Current Allergy and Asthma Reports, Jul;4(4):276-84
58. Leung, D.Y. et al., (1993), Presence of IgE antibodies to Staphylococcal exotoxins on the skin of patients with atopic dermatitis. Evidence for a new group of allergens, Journal of Clinical Investigations,Sep;92(3):1374-80
59. Maurer, D. et al., (1994), Expression of functional high affinity immunoglobulin E receptors (Fc epsilon RI) on monocytes of atopic individuals, The Journal of Experimental Medicine,Feb 1;179(2):745-50.
60. Wollenberg, A. et al., (1996), Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema, The Journal of Investigative Dermatology, Mar;106(3):446-53.
61. Metzger, H. et al., (1986), The receptor with high affinity for immunoglobulin EgE, The annual Review of Immunology, 4:419-470
62. Li, L.B. et al., (2004), Superantigen-induced corticosteroid resistance of human T cells occurs through activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK-ERK) pathway, The Journal of Allergy and Clinical Immunology, Nov:114(5): 1059-69
63. Microbiology and Immunology online, University of South Carolina School of Medicine, (12;July;2010), http://pathmicro.med.sc.edu/bowers/ant-pres.htm, 23;April;2014
64. Hamers-Casterman, C. et al., (1993), Naturally occurring antibodies devoid of light chains, Nature, Jun 3;363(6428):446-8.
65. Harmsen, M.M. et al., (2007), Properties, production, and applications of camelid single-domain antibody fragments, Applied Microbiology and Biotechnology, 77:13-22
66. Denovebiotech, (2014), What are the advantages and disadvantages of using Monoclonal antibodies Vs Polyclonal antibodies?, http://www.denovobiotech.com/what-are-the-advantages-and-disadvantages-of-using-monoclonal-antibodies-vs-polyclonal-antibodies/itemid-1705, 02;May;2014
67. Leavy, O. et al., (2010), Therapeutic antibodies: past, present and future, Nature reviews. Immunology, May;10(5):297
68. Goldman, E.R. et al., (2006), Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library, Analytical Chemistry, Dec 15;78(24):8245-55.
69. Graef, R.R. et al., (2011), Isolation of a highly thermal stable lama single domain antibody specific for Staphylococcus aureus enterotoxin B, BMC Biotechnology, Sep 21;11:86
70. Adems, H. et al., (2009), Specific immune capturing of the staphylococcal superantigen toxic-shock syndrome toxin-1 in plasma, Biotechnology and Bioengineering, Sep 1;104(1):143-51
71. Horrevoets, W. et al., Neutralization of the Staphylococcal Superantigen Toxic-Shock Syndrome Toxin-1 by a Llama single-domain antibody, confidential draft version
72. Ablynx: Nanobody Technology: Understanding Nanobodies, (2014), http://www.ablynx.com/en/research-development/nanobody-technology/understanding-nanobodies/, 05;may;2014
73. Lefranc, M.P. et al., IMGT®, the international ImMunoGeneTics information system®, http://www.imgt.org/IMGTeducation/IMGTlexique/A/ADCC_and_CDC.html, 05;may;2014
74. Khan, A.A. et al., (2008), SEB-induced signaling in macrophages leads to biphasic TNF-alpha, Journal of Leukocyte Biology,Jun;83(6):1363-9