Human mucosal IgA in health and disease
Yuvaraj, Saravanan

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Chapter 1

Literature Framework of work presented in the thesis.

Saravanan Yuvaraj¹, Maikel P. Peppelenbosch¹, Nicolaas A. Bos¹.

¹ Groningen University Institute for Drug Exploration (GUIDE), Department of Cell biology, Section Immunology, University Medical Center Groningen (UMCG), University of Groningen, Groningen, The Netherlands.

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Human Mucosal IgA in Health and Disease

The mucosal tissues such as gastrointestinal, urogenital and respiratory tracts are highly vulnerable to external environmental factors. These tracts are all covered with protective mucosal layer, which form together an area of approximately 400m$^2$ in an adult human (Brandtzaeg et al., 1999). Normally, the intestinal tract is in a state of peaceful co-existence with the complex milieu of microbes. Its epithelial barrier function is further enhanced by a pre-epithelial layer and a well-developed mucosal immune network. It responds to a range of microbial environmental conditions including colonization, commensalisms, symbiosis, persistent infections, and pathogen-induced diseases.

In general the mucosal immune system employs two different paths. One originates from innate cells and the other from adaptive immune cells. Innate immune cells rapidly sense pathogens, followed by the generation of effector functions like activation of cellular transcription and pro-inflammatory protein synthesis. For example, essential pathogen-associated motif sensing receptors on these cells are the Toll Like Receptors (TLR) recognize a wide range of antigens (Jiang et al., 2004) and prompt the cells to activate many signal transduction proteins. Though the action of these proteins, the innate immune cells can promptly respond to pathogen/commensal challenges and eliminate the microbes. After elimination the affected tissue returns back to its normal functional state with minimal pathology. The adaptive immune system mainly consists of two types of lymphocytes: B cells and T-cells. The former provides antigen specific humoral immune responses and the latter provides cell-mediated immune responses, respectively. In the present thesis we investigate and discuss the role of B cell responses in mucosal immunity.

The B cells in the mucosal immune systems produce high amounts of antibodies. Already as early as 1870, Alexandre Besredka, a Russian pathologist discovered the presence of antibodies in external secretion, specifically in gastrointestinal tract secretions. A lot of attention was given to this antibody whose presence was reported in most of the external secretions like intestinal fluid, milk and stool. Heremans and his co-workers demonstrated that the carbohydrate-rich, serologically peculiar $\beta$-globulin constituted a type of antibody, which was designated as Immunoglobulin A (IgA) in 1964 (Rockey et al., 1964). Since then it has been shown that IgA is an important component in the mucosal defence mechanism. An $\alpha$ constant region in the immunoglobulin defines the IgA antibody. In human two IgA subclasses are present they are IgA1 and IgA2, were in the later, 13 amino acid is deleted at the hinge region (Flanagan et al., 1984). The distribution and physiological function of these subclasses is not clear.
This thesis is focused on IgA and in the current chapter we shall discuss its biogenesis. IgA function and their production is described in the part-1 and the possible clinical application of IgA dependent therapy is depicted in part-2

1.1 PART-1 IgA Production and Physiological function

IgA production in the gut is a highly sophisticated process, the processes underlying this production are now reasonably well-defined in molecular terms. It starts with the recruitment of B cells to the various regions of the gut and following stimulation by antigen (e.g. normal gut flora, food constituents or pathogens) these cells can undergo a differentiation process yielding IgA as an end product. In this section, all the steps involved in the production of IgA are summarised.

1.1.1 Induction of IgA production

1.1.1.1 Recruitment of B cells.

Intestinal IgA+ B cells are produced from IgM+ B cells in two distinct gut microenvironments: organized follicular structures and lamina propria (LP). Upon gut environment experience, B cells express integrin α4β7 at high levels and this expression mediates migration of these B cells to the intestine. The origin, however of IgM+ B cells in the gut LP is as yet unidentified. Aly/aly mice are defective in all their organized follicular structures because of an NF-κB-inducing kinase (NIK) mutation. In addition, in vivo and in vitro experiments reported that aly/aly peritoneal cavity (PEC) cells have a defect that affects their homing capacity, especially to the GALT system (Fagarasan et al., 2000). The in vivo migration defect of aly/aly PEC cells correlates well with the in vitro impaired chemotactic response toward secondary lymphoid tissue chemokine (SLC) and B lymphocyte chemoattractant (BLC). It was found that SLC stimulation did not activate NF-κB in aly/aly PEC cells, whereas the same stimulation increases the nuclear NF-κB in aly+/PEC cells, thus demonstrating that NIK is located downstream of the signalling pathway through the receptors for SLC, and that aly-type NIK affects this pathway (Fagarasan et al., 2000). NIK is known to participate in the signalling cascade responsible for NF-κB activation through receptors of the TNF and IL-1R/toll-like receptor families (Figure-1) (Siebenlist et al., 2005; Malinin et al., 1997; Medzhitov et al., 1997). Transfer experiments to reconstitute IgM+ B cells and IgA plasma cells in LP of aly/aly mice revealed that naive B cells can directly migrate to the LP. This migration requires NIK-dependent activation of gut stromal cells. By contrast, the entry of gut-primed IgM+ B cells to the LP is independent of stromal cells with functional NIK. These data indicate that naive B cells directly migrate to the LP by a distinct pathway from gut-primed B cells (Suzuki et al.,
Apart from its role in migration the NF-κB dependent pathway is also an important factor in IgA class switching (Stavnezer, 1996).

Fig. 1 Nuclear factor-κB (NF-κB) is activated by signalling through many receptors. These receptors can be grouped into two classes: first, receptors that only, or mainly, activate the classical pathway of NF-κB activation; and second, receptors that activate both the classical and the non-classical pathway of NF-κB activation. The first class includes tumour-necrosis-factor receptor (TNFR), interleukin-1 receptor (IL-1R) and members of the Toll-like receptor (TLR) family. These receptors signal through various kinases and adaptors, including members of the TNFR-associated factor (TRAF) family, which are recruited to these receptors and relay signals to various downstream targets. The B-cell receptor (BCR) and T-cell receptor (TCR) activate NF-κB through a phosphorylation cascade that includes (but is not limited to) BLK (B-lymphoid kinase), FYN, LYN, SYK (spleen tyrosine kinase), BTK (Bruton's tyrosine kinase), LCK and ZAP70 (ζ-chain-associated protein kinase of 70 kDa), which leads to activation of protein kinase C-β (PKC-β) following ligation of the BCR and PKC-θ following ligation of the TCR (PKC-β and PKC-θ are essential for signalling through NF-κB in mature lymphocytes but not in immature lymphocytes or thymocytes). After activation of PKC, the IκB kinase (IKK) complex is activated through CARMA1 (CARD (caspase-recruitment domain)–MAGUK (membrane-associated guanylate kinase) protein 1), BCL-10 (B-cell lymphoma 10) and MALT1 (mucosa-associated lymphoid-tissue lymphoma translocation gene 1). The second class of receptors, which activate both the classical and the non-classical pathway of NF-κB activation, includes the lymphotxin-β receptor (LT-βR), receptor activator of NF-κB (RANK), CD40 and the
B-cell-activating-factor receptor (BAFFR), although BAFFR only weakly activates the classical pathway. In the classical pathway, upstream signals induce phosphorylation of IkBa bound to cytosolic NF-κB. Phosphorylation is carried out by the IKK complex, which is composed of IKK-γ and two catalytic subunits, IKK-α and IKK-β. Phosphorylation tags IkBa for ubiquitylation and, ultimately, for proteasomal degradation, liberating NF-κB for translocation to the nucleus and activation of target genes. (Modifications of NF-κB proteins occur in the cytoplasm or nucleus to regulate activation of transcription and other functions.) The non-classical pathway is controlled through TRAFs, NF-κB-inducing kinase (NIK) and IKK-α; this regulation occurs independently of the classical IKK complex and leads to processing of the p100 form of NF-κB2, generating p52–REL-B heterodimers (and other NF-κB dimers), which migrate to the nucleus.

1.1.1.2 The role of B cells in positive and negative immune regulation

Naïve, long lived memory and/or gut experienced B cells internalize specific antigen efficiently in the M-cell1 pockets. They provide efficient stimulation of adjacent CD4+ CD40L+ T cell by antigen presentation and B7-CD28 ligation. This interaction likely leads to a diversified T-cell response that can further diversify the B cell response (Ha et al., 2001).

In comparison to ordinary antigen-presenting cells (APC), naïve B cells have low or absent levels of co-stimulatory molecules, thus internalization of antigen, either specifically or non-specifically and its presentation to T cells produces tolerance. Moreover, interaction of B and T-cells via B7-CTLA-4 might also result in tolerance.

1.1.1.3 Germinal Center (GC) formation.

Follicular dendritic cells (FDC) also present native antigens to B and T-cells to induce an immune response. Primary lymphoid follicles contain recirculating naïve B lymphocytes (sIgD+ IgM+). They pass into the network formed by antigen-capturing FDC which are turned into secondary follicles by the GC reaction. The GC is of crucial importance for T cell-dependent generation of memory B cells, affinity maturation of the B cell receptor, and Ig class switching. It has been shown that naïve B cells are first stimulated at the edge of the primary follicle by cognate interaction with activated CD4+ T cells. These T-cells have previously been exposed to processed antigen in MHC class II expressing interdigitating DCs (Garside et al., 1998; Manser, 2004). The naïve B cells now designated as founder B cells re-enter the follicle to become a proliferating GC. The GC founder cells

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1 The M cell is a extraordinary cell type found in the epithelium that covers mucosa-associated lymphoid tissue in the digestive tract and the airways. M cells internalize macromolecules and micro-organisms efficiently and deliver them to the underlying lymphoid tissue. In the gut, M cells, unlike the neighbouring absorptive enterocytes, lack a highly organized apical brush border and glycocalyx, and are poorly equipped with digestive enzymes.
activated in the extrafollicular compartment then migrate to the dark zone, where they proliferate and differentiate.

The stimulated B cells in the GC produce low affinity IgM that can bind to circulating antigen. Later the soluble immune complexes subsequently become deposited on the FDCs where antigen is maintained for prolonged periods to maintain B cell responses (Ahmed and Gray, 1996; MacLennan et al., 1997; Lindhout et al., 1997). Such a role for IgM in the induction of secondary immune responses with antibody affinity maturation has been strongly supported by observations in knockout mice lacking natural background IgM antibodies (Ehrenstein et al., 1998). In the GC, B cells of different subsets undergo somatic hypermutation (SHM) and class switching recombination (CSR) to increase the diversity of the antibody responses. Let us now review these two important processes:

1.1.1.4 **Somatic Hypermutation (SHM).**

SHM is a process which introduces non-templated point mutations in the variable region of rearranged immunoglobulin heavy and light chain genes. SHM leads to affinity maturation, which results in the selected outgrowth of B cells expressing an immunoglobulin that has high affinity for its cognate antigen. Activation-induced cytidine deaminase (AID) is essentially required for SHM and its expression is restricted to GC B cells. Initially AID was thought to be a RNA-editing enzyme that targets mRNA (Muramatsu et al., 2000), but no experimental evidence was acquired to prove this. In contrast, accumulating evidence supported that AID acts directly on DNA. AID begins SHM by the deamination of cytidine (C) nucleotides on single-stranded DNA of the immunoglobulin gene. As first proposed by Neuberger (Neuberger et al., 2003) and colleagues, the mismatch of C results in uridine (U) and guanosine (G) that can be repaired by one of following the three pathways. (1) For a mutation to be fixed at the site of deamination, error-free DNA repair must be perturbed to become error-prone. If the mismatch is carried unpaired to replication, DNA polymerase will insert an adenosine (A) opposite the U nucleotide, ultimately creating C to T and G to A transition mutations. (2) In base-excision repair (BER), replication over an abasic site created by uracil-DNA glycosylase (UNG) will give rise to both transition and transversion mutations. (3) Mismatch repair (MMR) machinery might create mutations on A to T base pairs.
1.1.1.5 **Class Switching Recombination.**

Immunoglobulin is made of two heavy chains and two light chains. Each heavy (H) and light (L) chain contains a variable (V) region which binds to the antigen and a constant (C) region which is responsible for the effector function. Upon activation by antigen and accessory signals, naive IgM^+IgD^+ B cells undergo Ig CSR, resulting in expression of a different C_H gene, while maintaining expression of the same V region gene. CSR allows optimization of the antibodies for elimination of different pathogens. It is mediated by DNA recombination between switch (S) region sequences located 5' to each C_H gene, except C_{\mu}. This results in a new Ig isotype producing IgA^+ B cells as well as the switch circles (Figure-2). The isotype to which the B cells will switch is regulated by Th cells, the particular APC involved, and the anatomical site of antigen presentation.

CSR occurs only in the presence of cytokines (for example TGF-β, IL-4, IL-5, IL-6, etc) and AID. AID is an essential enzyme to regulate CSR also other proteins are important. The histone methyltransferase Suv39h1 increases CSR specifically to IgA (Bradley et al., 2006). A crucial role for Smad2 is to mediate signals for the TGF-β-directed class switch to IgA. It also influences induction of IgA responses in vivo (Klein et al., 2006). It has been proposed by Qiao et al (Qiao et al., 2006) that negative factor (Nef) protein, is an immunosuppressive HIV1 protein expressed and released by infected cells, penetrates B cells both in vivo and in vitro. Nef suppresses immunoglobulin CSR by inducing IkBα and SOCS proteins. These induced proteins block CD154 and cytokine signaling via NF-κB and STAT transcription factors.

RUNX proteins like RUNX3 are important mediators of TGF-β which induce B cell CSR to IgA. RUNX3 is induced by TGF-β1 in the mouse IgM B cell lines 1.29μ, CH12.IX (which can switch to IgA antibody expression), and activated spleen B cells. This is not observed in mouse IgG2a B cell line A20.3 (which is unable to switch to IgA antibody expression), suggesting a role for RUNX3 in regulating antibody class switching to IgA. Over expression of RUNX3 or Smad3 and Smad4 was found to increase the amount of IgA secreted from normal spleen cells and IgM C12.LX.4927 mouse B cells treated with TGF-β. Consistent with the synergistic activation of Ig Ca promoter constructs by RUNX3, and Smad3 and Smad4, their co-transfection further increased the surface expression and secretion of IgA in LPS-activated spleen B cells following TGF-β treatment (Figure-3) (Blyth et al., 2005; Whiteman and Farrell, 2006). Together, these observations highlight the importance of TGF-β signalling for IgA responses.

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2 Contains 7 families of variables in heavy chain
3 Contains γ, δ, ε, α, μ constant regions in heavy chain
Fig. 2 Top line shows Ig heavy chain genes in a B cell that expresses IgM and IgD (by alternative RNA processing). CSR occurs after AID deaminates dC residues within S regions that are transcriptionally active. Transcripts from the unrearranged heavy chain genes are called germline transcripts. Middle line illustrates the intrachromosomal deletional recombination between two switch (S) regions during CSR from IgM to IgE. The DNA in between Sm and Se is excised from the chromosome as a circle. Bottom line illustrates the chromosome after CSR showing that the identical V_H region originally expressed with the C_m gene is now expressed with C_e gene in cells expressing IgE.

1.1.1.6 Repertoire development.

Recent studies show that the peripheral circulating B cell repertoire, which reflects a complex group of cells expressing IgV genes, might have been influenced by a variety of immunological stimuli. The IgV genes of B cells infiltrating the different tissues may provide a more skewed population owing to the local selection and/or (antigen-dependent) proliferation. Clonal B cell expansions in the target tissues of many different autoimmune diseases are well established (Steiman-Shimony et al., 2006). Molecular analyses of Ig heavy chain rearrangements from peripheral blood and tonsils have shown a polyclonal pattern of IgV gene usage. IgA heavy chain repertoire has a restricted pattern in intestine and salivary gland (Dunn-Walters et al., 2000). In other mucosal tissues the IgA repertoire is unknown.
1.1.1.7 **Inductive site for the switch to IgA producing B cells.**

The evidence for the inductive site comes from the presence of AID expression and switch circles (Figure-2). Lamina Propia (LP) stromal cells can recruit naïve B cell to the gut through an independent pathway that involves the activation of NF-κB by NIK (Suzuki et al., 2005). Later the recirculating IgM⁺ B cells undergoes CSR to generate IgA⁺ antibody secreting Cells (ASC) or IgA⁺ memory cells in the GC of Peyer’s patches (PP) and mesenteric lymph nodes. Butcher et al demonstrated this phenomenon for the first time in mice (Butcher et al., 1982). In mice approximately half of these IgA ASCs in the gut wall are derived from a particular (B-1) lineage of B cell precursors (Kroese et al., 1989). Fagarasan et al. showed that both the organized Gut-Associated Lymphoid Tissue (GALT) and the intestinal lamina propria (i-LP) were sites of generation and diversification of plasma cell precursors, which were largely B-1 cells in mice (Fagarasan et al., 2001). In humans no data support that i-LP is the inductive site for IgA. If this occurs, it is a rare and possibly transient phenomenon.

Bronchus Associated Lymphoid Tissues (BALT) is not constitutive feature of the normal human lungs. The human airway epithelium constitutively produces IL-2, TGF-β, IL-5, and IL-10, factors essential for IgA isotype switch and differentiation into IgA-producing plasma cells. The close proximity of B cells to the airway epithelium probably guarantees a constant supply of growth and differentiation factors needed for mucosal IgA production (Salvi and Holgate, 1999).

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Fig. 3 This diagram illustrates the central role of RUNX–CBF complexes in the orchestration of cell fate in response to exogenous factors (yellow circles) and environmental signals (green circles). A subset of the known RUNX target genes is depicted (grey boxes), and these targets have been selected for their potential relevance to cancer. This model might be helpful in understanding the effects of loss of function or
dominant-negative inhibitors that require collaborating events to drive tumour-cell proliferation (indicated by the red box). By contrast, ectopic expression of RUNX factors leads to activation of both growth-promoting and suppressive signals (indicated by both the red and purple boxes, respectively). In the absence of collaborating mutations that prevent growth-suppressive effects (purple box), cell growth is restricted. The role of individual target genes in these phenomena remain to be established and it is conceivable that the consequences of RUNX-gene deregulation involve multiple downstream effectors. BMP, bone morphogenetic protein; C/EBPδ, CCAAT/enhancer binding protein-δ; FGF, fibroblast growth factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IgA1, immunoglobulin A1; IL-3, interleukin-3; M-CSFR, macrophage colony-stimulating factor receptor; MPO, myeloperoxidase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TCRα, T-cell receptor-α; TGF-β, transforming growth factor-β; TGFβR1, TGFβ type I receptor; VEGF, vascular endothelial growth factor.

1.1.1.8 High and low affinity IgA antibody.

SHM can provide certain advantages like generation of high-avidity antibodies. Autoantibodies encoded by germline genes could lose self-reactivity following mutation. In order to maintain B cell memory, higher-avidity BCRs might provide a survival advantage, particularly when nominal antigen availability is a limiting factor. SHM may generate B cells with high-avidity Ig receptors, that could also become efficient activators of T cells who are specific for peptide fragments (Longo and Lipsky, 2006).

Diversity might be more important than avidity in response to SHM-inducing pathogens. It is important to note that in the context of efficient B cell responses apart from the obvious advantages, SHM has disadvantages as well: (1) The BCR might no longer recognize the stimulating antigen, which can result in the lack of selection and death or, if selected, the B cell responds to a different antigen. (2) It may alter the tertiary structure, resulting in B cell death. (3) The mutated BCR recognizes self antigens instead of foreign antigens. This is usually not a problem because autoreactive peripheral B cells fail to receive cognate T-cell help, as autoreactive T cells are usually deleted or tolerized in the thymus, preventing their functional presence in the periphery.

In vitro experiments have suggested that stochastic or non-selective mechanisms are of primary importance in the regulation of plasma cell differentiation (Hasbold et al., 2004). On the other hand, indirect evidence suggests that plasma cell differentiation of GC B cells may be more selective, with only those cells that exceed a threshold antigen affinity contribute to the antibody response (Smith et al., 1997;Smith et al., 2000). Affinity maturation is therefore driven by a tightly
controlled mechanism that ensures only antibodies with the highest potency of neutralizing foreign antigens (Phan et al., 2006).

Low affinity B cells that are fully able to generate antibody-forming cells have a physiologic significance. Transgenic models demonstrate that low-avidity antigen–antibody interactions are sufficient for deletion, receptor editing, and induction of an immune response against antigens like gut flora (Macpherson et al., 2000).

1.1.2 Migration of IgA producing B cells to the effector site.

1.1.2.1 Chemotaxis of antibody producing cells or memory IgA cells.

Several molecules like chemokines and adhesion molecules are involved in lymphocytes trafficking into mucosal tissues. Selective expression of MadCAM-1 on the mucosal high endothelial venules (HEV) indicates a role for this molecule in homing of lymphoid cells to mucosal tissues (Hamann et al., 1994). MadCAM-1 expression on HEV is increased by cytokines, such as TNF-α and IL-1, suggesting that recruitment of lymphocytes to the mucosa can be modulated. There are two major adhesion molecules, L-selectin and α4β7 integrin on lymphocytes that bind to MadCAM-1. Another integrin, αEβ7 is suggested to be involved in the migration of lymphocytes into the intestinal epithelium.

Intestinally induced IgA ASCs can populate the bone marrow and produce antibodies, which are present in the circulation (Forster et al., 1994;Hargreaves et al., 2001;Youngman et al., 2002). CXC4 is probably the chemokine mediator in trafficking of IgA-secreting plasma cells to the bone marrow(Kunkel et al., 2003;Lazarus et al., 2003), where its ligand CXCL12 is highly expressed by stromal cells. It also plays a crucial role for the development and retention of B cell progenitors (Ma et al., 1998;Egawa et al., 2001). The chemokines CCR9 and CCR10 are mainly involved in the trafficking of IgA ASCs in the mucosal tissues.

1.1.3 The Function of IgA in the immune response

1.1.3.1 In serum.

In addition to barrier protection, IgA performs other vital functions as well, which are discussed below. Using cellular Fc receptors, IgA is efficient way of delivering antigens to the immune system. IgA is in this manner important for recruitment of effector leukocytes and is a trigger for the initiation of potent protective pathways or pathological and inflammatory reactions. IgA can interact with various cell types like neutrophils, eosinophils, monocytes and macrophages which express FcαRI(CD89). Serum IgAs in immune complexes are very effective at initiating a wide range of inflammatory responses such as: phagocytosis, antibody dependent
cellular cytotoxicity, oxidative burst, and cytokine release (Monteiro and Van De Winkel, 2003). In humans, serum IgA, is the second most abundant serum antibody class. It plays pro- and anti-inflammatory roles and interfaces with the mucosal and systemic immunity. Serum IgA is predominantly of the IgA1 subclass, and this antibody subclass also predominates in mucosal effector sites such as the mammary glands, salivary glands, nasal mucosa, bronchial mucosa and the upper digestive tract. Parental immunisation with protein antigens tends to elicit serum IgA1. The IgA2 subclass is more abundant in the colon and is induced by polysaccharide antigens. These antibody subclasses are highly homologous except for a short mucin-like hinge region which is unique to primate IgA1. This region is responsible for some of the unique receptor and lectin-binding properties (Wines and Hogarth, 2006).

1.1.3.2 In mucosal tissues.

Mucosal antibodies effectively reduce absorption of soluble or particulate antigens on mucosal surfaces. However, the antibody isotype essentially influences the quantity of absorbed antigen. In the mucosal tissue IgA exists as secretory IgA (SIgA). Two monomeric IgA molecules are held together by a J chain that is produced by the plasma cells. This entire IgA complex binds to the poly immunoglobulin receptor (pIgR) present on epithelial cells of the mucosa and gets subsequently transported to the lumen of the gut. Then extracellular part of the pIgR is also released along with the dimeric IgA into the lumen as SIgA (Johansen and Brandtzaeg, 2004). Its anti-inflammatory properties and multiple antigen-binding sites make SIgA an effective inhibitor of immune responses. At mucosal surfaces antigen-specific SIgA bound to commensal as well as pathogenic microorganisms effectively inhibit their adherence to epithelial cells. These cells display corresponding receptors to bacterial antigens on their surfaces (van der Waaij et al., 2004). SIgA confers microbes with a negatively charged and hydrophilic 'coat' that repels their attachment to mucosal surfaces. Interactions of SIgA with antigens such as enzymes, toxins, and viruses may neutralize the biological activities by binding to their critical epitopes. SIgA antibodies during pIgR-mediated transport might be able to neutralize intracellular viruses as demonstrated in vitro with Sendai, influenza, and human immunodeficiency viruses (Fujioka et al., 1998;Bomsel et al., 1998).

The indirect function of IgA is to give a protective effect by interacting with mucin and several other humoral factors of innate immunity present in external secretions (Russell et al., 2005). SIgA is incapable to fix complement efficiently or to act as
an opsonin. This is an advantage in secretions, where initiation of an inflammatory reaction would affect the integrity of the mucosal surface.

In contrast, antigen-specific IgG antibodies may cause complement activation and subsequently inflammation, influx of polymorphs, and altered mucosal integrity with increased absorption of bystander antigens (Russell et al., 1997; Brandtzaeg and Tolo, 1977).

1.1.4 Receptors for IgA

The receptors for Ig are specific for their Fc fragment. Fc receptors are defined by their specificity for the Fc fragment of immunoglobulin isotypes. Five different IgA receptors are recognized so far. They are polymeric Ig receptor (pIgR), FcαRI (or CD89), FcαRI, asialoglycoprotein (ASGP-R) and transferrin (TfR).

pIgR is expressed on the basolateral surface of mucosal epithelial cells, and is specific for polymeric IgA and IgM. It is responsible for the transcytosis of polymeric IgA and IgM across the mucosal epithelium to form Secretory IgA or IgM. pIgR has no inflammatory role and functions primarily in transcytosis.

FcαRI (CD89) is the major activating IgA receptor on myeloid cells and is capable of eliciting respiratory burst, phagocytosis, degranulation and cytokine production. Neutrophils, macrophages, Kupffer cells and eosinophils are the cell types that express FcαRI. On myeloid DCs, it is involved in the capture of IgA complexes for subsequent antigen presentation. This receptor is absent on lymphocytes, mast cells, basophils, and platelets (Wines and Hogarth, 2006).

FcαRI binds both IgM and IgA (Shibuya et al., 2000). This receptor is widely expressed in non-hematopoietic tissue with mRNA found mainly in kidney and intestine and at low levels in the lung, liver and heart. The in vivo function of this receptor is not clear, although, FcαRI mediates similar responses to IgA and IgM immune complexes.

Eosinophils are key mucosal mediators in protective and pathological reactions in the gut and lung. Human eosinophils express all the above-mentioned IgA receptors. A unique receptor which is not yet characterized for secretory component (SC) is expressed on eosinophils (Lamkhioued et al., 1995). This receptor binds SC and IgA, but not serum IgA. Further, it triggers degranulation and release of eosinophil cationic protein and peroxidase.

A novel mouse IgA receptor was described on Peyer’s patch M cells. An epithelial cell located exclusively within the follicle-associated epithelium overlying MALT (Mantis et al., 2002). Its molecular and other functions are not well known yet.
The liver plays an important role in maintaining homeostasis through regulation of IgA catabolism. The ASGP-R expressed on hepatocytes (Stockert et al., 1982; Grossetete et al., 1998) recognizes terminal Gal residues on serum glycoproteins, including IgA, and conveys bound ligand for intracellular degradation. The rapid clearance of IgA2, compared to IgA1 in the liver may contribute to the higher serum levels of IgA1 (Sakamoto et al., 2001). Transferrin receptor 1, TfR (or CD71), selectively binds IgA1 (Moura et al., 2001). In contrast to FcαRI, this IgA receptor is not fully expressed on mature blood leukocytes, but it is well-expressed on cultured renal mesangial cells as well as on glomerular mesangial cells in patients with IgA nephropathy.

1.1.5 IgA in (auto)immunity

IgA associated diseases are characterized by increased serum IgA levels, often paralleled by IgA tissue deposition. These disorders include IgA nephropathy (IgAN), ankylosing spondylitis, Sjogren’s syndrome, alcoholic liver cirrhosis (ALC), HIV infection, and dermatitis herpetiformis.

1.1.5.1 IgA in nephropathy

IgA nephropathy (IgAN) is the most common form of glomerulonephritis, characterized by deposition of IgA immune complexes, followed by the infiltration of inflammatory immune cells, mesangial cell proliferation and mesangial matrix expansion (Monteiro et al., 2002). IgAN patients display several major abnormalities in the IgA system like increased levels of serum IgA and IgA-immune complexes (Monteiro et al., 2002), increased ratio of polymeric to monomeric (Valentijn et al., 1984; Novak et al., 2001) and the generation of abnormally glycolated IgA1 (Tomana et al., 1999). Studies in IgAN patients and patients with other IgA-associated disease including HIV infection, ALC and spondyloarthropathies reveal reduced FcαRI expression levels on circulating monocytes and neutrophils. In some IgAN patients soluble FcαRI is present in serum. This results in the IgA/FcαRI complexes into the circulation which later deposit in kidney. TfR is over expressed on the kidney cells in IgAN patients (Barratt et al., 2000). TfR specifically binds IgA1 but not IgA2 with clear preference for polymeric IgA1. TfR may be responsible for trapping IgA1 in the mesangium, leading to the well-known mesangial IgA1 deposits that are hallmarks of IgAN.
1.1.5.2 IgA in multiple sclerosis (MS)

IgA1 and IgA2 are found in the cerebrospinal fluid of patients with MS (Budka et al., 1985; Henriksson et al., 1985). Zhang et al. (Zhang et al., 2005) have demonstrated the clonal expansion of IgA-bearing plasma cells in MS lesions with somatic mutations and ongoing intra-clonal mutations occurred in their VH genes. Via immunohistochemical studies, infiltration of dimer and polymer IgA1- and IgA2 positive plasma cells in perivascular spaces, parenchyma of MS lesions, and adjacent white matter was demonstrated. Double immunofluorescence staining showed binding of IgA antibody on axons and walls of micro vessels in the areas of chronic active and inactive demyelination.

1.1.5.3 IgA in Sjögren’s syndrome (pSS)

High levels of serum monomeric IgA, SIgA, IgA-rheumatoid factor and IgA containing circulating immune complexes have been reported in pSS (Atkinson et al., 1989). In pSS patients enhanced sialyltransferase activity in B lymphocytes was observed (Basset et al., 2000b). Also, increased N-linked glycosylation leading to oversialylation of monomeric IgA1 was found (Basset et al., 2000a). Evidence for IgA producing plasma cells was first observed in the parotid glands after rituximab treatment (Pijpe et al., 2005) but the repertoire of these cells is largely unknown.

1.1.5.4 Asthma and allergy

Various studies have shown that mucosal and systemic production of allergen-specific IgA occurs in patients with asthma and/or allergic rhinitis (Peebles, Jr. et al., 2001, Nahm et al., 1998, Terada et al., 1996). Reports have shown that IgA induces an inflammatory type of immune response (Seminario and Gleich, 1994), while few reports show that IgA can suppress inflammatory responses in asthma and allergy.

1.1.5.5 Ankylosing spondylitis (AS)

Elevated level of monomeric IgA1 in serum was reported in Ankylosing spondylitis, which is a chronic inflammatory form of arthritis that affects the spinal joints. At the same time, IgA deficient patients were shown to exhibit severe AS (Pilette et al., 2004).

1.1.5.6 Inflammatory bowel Disease (IBD).

IBD includes Crohn’s disease (CD) and ulcerative colitis (UC). In IBD an imperfection occurs in bacterial antigen sampling by the epithelium, maybe mediated by TLRs and controlled by genetic factors. A variety of autoantibodies,
including ANCA, antierythrocyte antibodies, pancreatic antibodies, lymyototoxic antibodies and antibodies to epithelial cells component, have been described (Seibold et al., 1996). These autoantibodies are not primarily responsible for disease pathogenesis but they mark for disease-related autoantigens. These autoantigens prone to cross-react with bacterial antigens from the normal intestinal flora. The importance of most of these antibodies in the pathogenesis of IBD is unclear. IgA autoreactivity was most severe in celiac disease patients. Furthermore, it was shown that IgA deficiency is associated with a tenfold increase risk of celiac disease.

1.1.6 Syndromes related to IgA deficiency

1.1.6.1 Selective IgA syndrome.

IgA deficiency is the most common primary immune deficiency disease in humans, affecting as many as 1:400 individuals. Most IgA-deficient individuals are healthy, but have an increased level of secretory IgM in mucosal secretions that compensate for SIgA (Cunningham-Rundles, 2001). Low IgA secreting plasma cells were observed in IgA deficient patients. The reason for this may be due to blockade in post-IgA switch differentiation of B cells (Wang et al., 1999). IgA production was attenuated in IgA-deficient patients which might be due to T-cell defect (Cunningham-Rundles, 2001). TGF-β is a key cytokine in CSR which is less in IgA-deficient sera when compared to normal donors (Muller et al., 1995).

1.1.7 Treatment of diseases that are correlated with an IgA imbalance

1.1.7.1 IgA producing B cell depletion.

B cell-targeted therapy has been used to treat B cell lymphoma and autoimmune diseases⁴. Rituximab is a potent B cell cytolytic chimeric IgG1 CD20-specific monoclonal antibody. The success of treating rheumatoid arthritis (Dass et al., 2006) with rituximab opened a new avenue in addressing autoimmune diseases.

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⁴ thrombocytopenia, SLE, vasculitis, auto-antibody associated neuropathies, multiple sclerosis, myasthenia gravis, myositis, blistering skin disorders, mixed cryoglobulineamia, thrombotic thrombocytopenic purpura, Sjogren’s syndrome and anti-factor VIII syndrome
Epratuzumab, that targets the B cell epitope CD22, is also used for the treatment of autoimmune diseases. Recent studies have confirmed the efficacy and safety of epratuzumab in several autoimmune diseases, including systemic lupus erythematosus and primary Sjogren's syndrome (Steinfeld and Youinou, 2006). Another way to deplete B cells is the neutralization of survival factors for B cells interfering with signalling mechanisms in B cells that are required for cell maintenance or activation. Bruton’s tyrosine kinase or spleen tyrosine kinase are interesting potential targets (Edwards and Cambridge, 2006). In this way plasma cells which are not targeted by rituximab can be specifically targeted.

1.1.7.2 Intravenous immunoglobulin (IGIV) therapy

There has been a considerable increase in the use of IGIV therapy for the treatment of autoimmune disease, systemic inflammatory diseases and for supportive therapy of immunodeficient patients. IGIV is beneficial in several diseases, including acute and chronic/relapsing diseases, autoimmune diseases and inflammatory disorders. Therapeutic efficacy of IGIV has also been established in a number of dermatologic diseases. The mode of action of IGIV is complex, involving modulation of expression and function of Fc receptors. It also interferes with the activation of complement and the cytokine network, modulation of idiotype network, regulation of cell growth, alteration of cellular adhesion process, and effects on the activation differentiation and effector functions of T and B cells and antigen-presenting cells. The therapeutic effects of IGIV most likely reflect the functions of natural antibodies in maintaining immune homeostasis in healthy people (Misra et al., 2005).

1.1.8 IgA mediated therapy

Antibody-based immunotherapy has become an effective treatment for a number of different cancers. Therapeutic monoclonal antibodies (mAb) can induce tumor cell death by a variety of mechanisms. Some act by simply cross-linking antigens on tumor cells, leading to apoptosis, cell cycle arrest or the inhibition of cell proliferation (Glennie and Johnson, 2000).

A number of studies showed that therapeutic antibodies able to trigger immune responses via FcαRI show exciting potential. These antibodies can either be intact IgA or bispecific antibodies (BsAb) that recognize both the ectodomain for FcαRI and the tumor antigen of interest (Valerius et al., 1997). In a comparison of hapten-directed antibodies of different human isotypes, IgA2 was found to be more effective that IgG isotypes at recruiting neutrophils and inducing tumor cell death (Valerius et al., 1997). Similarly, an IgA1 antibody against EpCAM proved more effective than IgG1 at recruiting neutrophils to kill EpCAM-positive, solid tumor cells (Huls et al., 1999).
FcαRI has the unique ability to mediate either activation or inhibitory inflammatory responses depending on the nature of its interactions with IgA (Pasquier et al., 2005). Monoclonal Fabs targeting the FcαRI ectodomain or engineered IgA constructs could therefore play a valuable therapeutic role in inhibiting inflammatory processes in autoimmune disorders or allergy and asthma. Leukocyte infiltration, a key characteristic of asthma, was markedly inhibited by treatment with anti-FcαRI mAb in a murine model of IgE-triggered asthma.

1.1.8.1 IgA therapy to treat cancer and autoimmune disease

Targeted therapies that are developed to selectively induce apoptosis in cancer cells are presently among the most promising anti-cancer strategies. Bremer et al and others have provided proof of principle for target-cell-restricted apoptosis induction using recombinant fusion proteins in which a tumour-selective antibody fragment is fused to either sTRAIL or sFASL (Bremer et al., 2004; Bremer et al., 2005). Many scFv like anti-TNF-α (Scallon et al., 1995), anti-BAFF, anti-CD25 (Onizuka et al., 1999) and many anti-interleukins etc is in clinical trial to treat autoimmune diseases.

1.2 Part II

1.2.1 2.1. Delivery systems

1.2.1.1 GMO

Functional human proteins are constitutively produced in genetically modified bacteria that survive happily on human mucosal surfaces to benefit the host. The successful phase 1 clinical trial with IL-10 producing Lactococcus lactis for Crohn’s disease has opened new avenues to use transgenic bacteria as delivery vehicle (Braat et al., 2006). The major advantage of this novel strategy is to avoid systemic side effects associated with conventional therapies. This methodology opens up an alternative method for local delivery of therapeutic proteins to various mucosa tissues.

1.2.1.2 Lactococcus lactis as a delivery vehicle of therapeutic molecules.

Due to technological advances the insight into the molecular basis of disease is rapidly expanding and as a consequence, a multitude of possible therapeutic
proteins, including cytokines and signalling molecules have been identified. The therapeutic use of these possible clinically relevant proteins is hampered, however by functional and economical considerations. The most important of these considerations are delivery and production of these proteins. Very recently, it has emerged that by using genetically engineered bacteria important barriers in this respect can be circumvented. Conventionally, therapeutic proteins are purified as to become endotoxin free (which is very expensive) and subsequently systemically administered with a suitable carrier into the bloodstream. The obvious drawbacks of this conventional strategy are the unwanted side effects as a consequence of the systemic delivery as well as often prohibitively high production costs. Genetically Modified (GM) bacteria that are qualified with high experience to co-exist with humans to produce human therapeutic proteins are opening new ways for those who are suffering from disease conditions: the delivery of proteins in the tractus circumvents the need for endotoxin free preparations, by their very nature bacteria are cheap producers of recombinant proteins and for digestive tract related diseases delivery can be topical rather as systemic.

Microbes have been consumed from ages in a variety of fermented foods and drinks that reside in the intestinal milieu to benefit the host, and these probiotic microbes are classified as GRAS (Generally Regarded As Safe) organisms. Furthermore, these food grade organisms have been genetically engineered to produce various foreign proteins for immune modulation. For example, antigens from pathogens as a prophylactic immunization, and proteins like cytokines, interleukins, and interferons have been expressed. Local delivery of recombinant therapeutical proteins to the disturbed intestine in many disease conditions has many advantages: Targeting to the mucosa for delivering therapeutic proteins can be easily achieved by administering GM bacteria that produce therapeutic proteins to prevent and combat infections. In the oral cavity GM *Streptococcus mutans* were used to prevent dental carries by a novel approach postulated as replacement therapy (Hillman, 2002). A different approach was reported by Paton *et al* that uses engineered *E. coli* that express a range of toxin binding receptor expression (Focareta *et al*., 2006; Paton *et al*., 2006; Paton *et al*., 2005) to capture bacterial toxins to prevent enteric infections. Similarly, GM *Streptococcus gordonii* were used to producing single chain antibodies (ScFv) specific for *Candida albicans* (Oggioni *et al*., 2001) and recombinant IL-1Ra (Ricci *et al*., 2003). The ScFv *S. gordonii* could eliminate experimental *Candida albicans* in rats after successful colonization in the vagina along with the anti-inflammatory effects of the IL-1Ra expressing *S. gordonii*. In addition, oral immunization of *Listeria monocytogenes* expressing Gag protein of HIV results in Gag specific CD8⁺ cells increase in mice (Peters *et al*., 2003). Non-pathogenic strains of *Salmonella typhimurium* are used to express virulence factors of *L. monocytogenes*. A second use of GM bacteria is to restore metabolic enzyme deficiencies or other human proteins. For instance, *L. lactis* expressing eukaryotic lipase was effective in treating pancreatic insufficiency in pig (Drouault *et al*., 2002). Thirdly, GM bacteria can be used to modulate the
immune system. *Lactobacillus casei* is used to deliver bovine beta-lactoglobulin during neonatal colonization to prevent milk allergic reactions in mice (Hazebrouck et al., 2006). Likewise *Lactobacillus* is extensively use in active vaccination and other immune interventions that will be discussed below. Thus both by niche occupation as well as by the production of recombinant proteins, GM bacteria may be clinically interesting.

Especially interesting in this context is that next to pancreatic insufficiency *L. lactis* has been used to deliver different therapeutic proteins orally. Trefolin factors (TTFs) involved in the protection and repair of the intestinal epithelium were expressed in *L. lactis* and delivered orally they had a striking protection against DSS-colitis in mice (Vandenbroucke et al., 2004). *Streptococcus gordonii* that was genetically modified to express IL-1Ra (pro-inflammatory) improved the condition of IL-2/− mice that spontaneously develop ulcerative-colitis-like pathology. The delivery of IL-10 locally by *L. lactis* has proven many advantages over systemic administration in mice. This improved status in mice IBD was followed by the first clinical trial of human IL-10 producing *L. lactis* to IBD patients (see below) (Braat et al., 2006; Steidler et al., 2003).

Likewise, *Lactobacillus jensenii* that were modified to secrete cyanovirin-N successfully inhibited HIV when delivered vaginally. *Lactobacillus zeae*, engineered to produce a ScFv against *S. mutans* antigen I/II adhesion molecule markedly reduced *S. mutans* counts and caries scores in caries rat model (Kruger et al., 2002). Intranasal application of live bacteria that express antigens has beneficial effect to increase the antigen specific antibodies during infection (Steidler et al., 1998), at the same time it also lower the allergic epitope antibodies (Daniel et al., 2006).

*Braat et al* conducted the first successful human trial with a GM *L. lactis* in which the thymidylate synthase gene was replaced with a synthetic sequence encoding mature human IL-10 (Braat et al., 2006). This novel treatment was safe with minor adverse events present and a decrease in disease activity was observed. Fecally recovered *L. lactis*-thy12 were dependent on thymidine for growth and IL-10 production indicated that the containment strategy was efficient. Bacterial-based topical delivery of biological active proteins is a novel and highly promising avenue for combating mucosal disease.

One possible disadvantage of the usage of GM probiotica is that the delivery of powerful recombinant proteins is not further targeted within the intestinal tract. Potentially this could lead to unwanted side effects. Therefore a new generation of targeted GM *Lactococci* are now being designed.
The execution of a first clinical trial is obvious importance for further development of this field, but future studies are likely to include more specifically targeted recombinant proteins, for instance is by membrane expression instead of continuous secretion of the recombinant protein by the GM organisms. In support of this view, membrane expression of ScFv instead of secretion turned out to be more efficient in removal of *S. mutans* from the oral cavity (Kruger et al., 2002). Nevertheless, already the current generation of clinically interesting GM organisms has already substantial better local delivery of heterologously expressed proteins as compared to conventional systemic strategies. In this context it is important to note that where GMO-delivered IL-10 seemed effective in the first placebo-uncontrolled trial, systemic IL-10 delivery did not seem clinically interesting, dramatically highlighting the power of local delivery. Further refinement of targeting strategies by this novel class of therapeutics will only further add to the promise of GMOs in clinical medicine. As an alternative to conventional therapy, they are particularly attractive, given the increasing problem of side effect and the high production cost. The most convincing evidence for the clinical effectiveness of recombinant probiotica certainly comes from first clinical trail using recombinant *L. lactis* secretion IL-10 and other animal studies. One important point to remember is that the various transgenic probiotica are different and each may have a different clinical effect in a specific disease. Apart from modulating the immune response, transgenic probiotica are used to deliver growth factors, silencer RNA, peptides that inhibit appetite. Significant safety issues will need to be addressed. There is a seemingly limitless area for future research on the use of transgenic probiotica and this novel technology clearly has great potential. Broad usage of such golden bullets both as pharmaceutica as well as functional food might revolutionize the usage of probiotica in the near future.

### 1.3 Scope of this thesis.

This thesis is focused on two main themes: 1) IgA and its repertoire and 2) *in vitro* production of the scFv SIgA molecule on the surface of *L. lactis*.

IgA is the most abundant mucosal immunoglobulin present in different mucosal tissues. It is produced by the experienced B cells in different tissues upon stimulation under different situations. Functions of IgA are prevention of attachment and translocation of gut bacteria (immune exclusion) (Kroese and Bos, 1999), neutralisation of pathogens both in the gut lumen and the inside of gut epithelial cells (Fernandez et al., 2003), export of immune complexes from the lamina propria into the lumen (Lamm, 1997) and anti-inflammatory responses against food antigens (Frossard et al., 2004). In IgA deficiency patients, IgA is not produced while in autoimmune disease like IgA nephropathy an altered IgA production is seen. In the former conditions, these patients can be compensated by giving IgA intravenously and in later situation, the abnormal B cell can be deleted.
by antibodies against them, for example Rituximab. It is obvious with the use of advanced molecular techniques these types of proteins delivery of IgA using bacteria like L. lactis would constitute a superior strategy. On the other hand B cell depletion can control the disease conditions when the disease is caused by autoantibodies.

This First chapter provided an overview of various aspects of IgA and its repertoire in various disease conditions and also the current state of the art with regard to delivery of IgA and different proteins was reviewed.

The intestine is exposed to gut microflora and food antigens. This influences the development of the mucosal immune system and tolerance respectively. When the B cell is exposed to this wide range of antigens then it is obvious to expect antibodies against these antigens. Therefore in the second chapter, to investigate the IgA repertoire, IgA genes from normal human ileum were cloned and sequenced. In this chapter we shall report that IgA producing cells in the intestinal tract are derived from a limited number of precursors that undergo local expansion. While almost sequences showed evidence for SHM, only a relatively minor group of these sequences have an R/S ratio that is consistent with an antigen-driven selection.

Human and humanised antibodies are now poised to become a new leading class of protein-based therapeutic agents. Following the development, a model L. lactis expressing scFv SIgA as a membrane protein was constructed in chapter three. In this chapter it will be shown that the Fab regions specifically bound to the Epithelial glycoprotein-2 (EGP-2) which is highly expressed on colon cancer cells. We shall discuss the release from L. lactis of membrane bound scFv SIgA occurs as a free molecule.

In Crohn’s disease, few regions of the intestine are inflamed while the rest remains healthy. The Fourth chapter specifically addresses the differences of the IgA repertoire and the gut flora populations in the inflamed and healthy regions of human or mouse intestines. In particular we shall report a difference in the VH gene usage between the inflamed and uninflamed intestinal regions in the gut of such patients.

The Fifth chapter we shall describe the involvement of the gut microflora in the development of Inflammatory Bowel Disease (IBD) in a mouse model for experimental colitis. Moreover, in this chapter it will be shown that an anti-inflammatory response differentially induces iNOS responses in epithelial cells by gut bacteria (different types of bacteria).
Excessive IgA may lead to autoimmune diseases like IgA nephropathy or Sjögren’s syndrome. Treatment of these diseases by depleting the B cells is reported to be beneficial. However, the relapsing nature of these diseases remains an important clinical challenge. In chapter six the IgA repertoire after anti-CD20 treatment in Sjögren’s syndrome patients is studied. A high influx of the IgA1 subtype in the diseased salivary glands is found. Moreover it will be shown that the rapid appearance of IgA producing cells in the salivary glands after treatment is not caused by regeneration of the B cell compartment, but rather by local expansion of pre-existing B cells.

Cancer in general can be elegantly targeted by introducing the mutated or missing proteins, whose absence underlies development of pathology. In case of colon cancer in particular, epithelial BMP-2 signalling is lost. In the Seventh chapter we shall show the production of recombinant human BMP-2 and its apoptotic effect on the colon cancers cells employing in vitro co-culture of recombinant bacteria producing BMP-2 with colon cancer cell lines.

In the Eighth chapter of this thesis, the main results of the experiments will be summarized, and discussed, followed by a short exposure on future perspectives.

In conclusion, this thesis shall show that IgA production is heterogeneously regulated in number of different conditions and understanding IgA repertoire in various disease state can provide insight about the pathogenesis of the diseases. Application of the advanced technologies provides the opportunity to use GMO as a production factory to produce different proteins like scFv IgA, which potentially should have important clinical applications.

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Literature Framework


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