Chapter 1

General introduction

In part adapted from:
The era of the Immunoglobulin A Fc receptor FcαRI; its function and potential as target in disease

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Human IgA is the dominant antibody subclass present in mucosal areas, where it plays a key role in mucosal defense. Mucosal surfaces (gastrointestinal and urogenital tracts and lungs) are the frontiers of the body. They are continuously exposed to environmental factors like inhaled antigens, food and ~10^{13}-10^{14} commensal microorganisms (mostly bacteria) that colonize healthy intestines. Mucosal immunity therefore faces the enormous challenge of tolerating harmless antigens (e.g. food) and commensals, while maintaining robust protection against pathogens. This balance is essential. On the one hand, inadequate immune responses lead to invasive mucosal infections while on the other hand, excessive immune responses to innocuous antigens result in a variety of unwanted inflammatory responses. IgA is involved in both anti- and proinflammatory responses, depending on the location and environmental stimuli.

In the blood circulation, 1-3 mg/ml IgA is present as a monomeric molecule (serum IgA). In mucosal areas, IgA is produced by local plasma cells as a dimeric molecule with an associating J chain (referred to as dimeric IgA or dIgA). It is then transported through the epithelial cells of the mucosal lining by the polymeric Ig receptor (pIgR), and released into the lumen. Part of the pIgR, secretory component (SC), remains attached, which provides secretory IgA (SIgA) with stability in the hostile milieu of the gut lumen.

Traditionally, IgA has been viewed primarily as a housekeeping antibody without activating properties that helps to maintain homeostasis in the mucosa. This is partly due to the fact that IgA deficiency is common, and it is estimated that up to one in 500-1000 Caucasian persons has selective IgA deficiency. Many of these people do not suffer from major illnesses, although increased susceptibility to recurrent (respiratory) infections, allergies, autoimmune diseases and inflammatory bowel disease has been reported. Additionally, most knowledge about the role of IgA has been based on experiments in rodents (mainly mice). However, mice lack a structural equivalent of the human IgA Fc receptor FcαRI. As such, interaction of IgA with its receptor and essential protective properties of triggering FcαRI on myeloid effector immune cells have therefore been overlooked in most rodent experimental studies. These led to the continuous misconception of IgA as a non-inflammatory and possibly even redundant antibody class.

Several compelling arguments support the importance of IgA and FcαRI. First, ~75% of the total daily antibody production is dedicated to IgA, which seems rather wasteful for a redundant molecule. Furthermore, a compensatory mechanism has been developed to overcome the lack of IgA, as increased production of IgM is generally observed in patients with selective IgA deficiency. In the absence of this compensatory mechanism, patients can develop severe disease. Perhaps the most convincing evidence backing the significance of IgA in protective immunity is the fact that bacterial evolution resulted in development of anti-IgA or anti-FcαRI bacterial proteins that obstruct IgA-FcαRI interactions. This likely represents an important evasion strategy for pathogens to escape IgA-mediated elimination by FcαRI-expressing phagocytes.

In the last decade it has become clear that IgA and FcαRI are multifaceted molecules that can be anti-, non- or proinflammatory depending on the environment. Moreover, evidence is increasing, which indicates that uncontrolled responses induced by IgA immune complexes can contribute to the severity of multiple diseases, including IgA...
nephropathy, rheumatoid arthritis, autoimmune skin blistering diseases and inflammatory bowel disease. As IgA has the ability to recruit neutrophils through binding to FcαRI, IgA autoantibodies or excessive IgA complexes will amplify neutrophil migration, which can result in tissue damage. However, it may be possible to exploit the destructive ability of FcαRI-expressing neutrophils in IgA monoclonal antibody (mAb) immunotherapy of cancer. Thus, harnessing the functions of IgA and FcαRI may represent attractive novel therapeutic strategies. In this introduction, I will address the role of IgA and FcαRI in mucosal immunity and disease as well as its therapeutic potential.

**Expression and structure of FcαRI**

FcαRI is a transmembrane receptor and a member of the Fc receptor immunoglobulin (Ig) superfamily, which also includes Fc receptors for IgG (FcγR) and IgE (FcεRI) \(^{6-9}\). However, the FcαRI gene is located on chromosome 19 (at 19q13.4) and lies within the so-called leukocyte receptor cluster (LRC) \(^{8,10}\), whereas genes encoding FcγR and FcεRI map on chromosome 1 (at 1q21-23) \(^9\). The LRC includes no other Fc receptor genes, but encodes killer inhibitory receptors (KIR) and leukocyte Ig-like receptors (LIR). The amino acid sequence of FcαRI shares more similarity with this type of receptors than with Fc receptors \(^6\). Orthologues of human FcαRI have been identified in several monkey species like chimpanzees and macaques, but not in mice due to a gene translocation \(^5,6\).

FcαRI expression is restricted to cells of the myeloid lineage, including neutrophils, eosinophils, monocytes and some subpopulations of dendritic cells and macrophages (e.g. alveolar, tonsilar and splenic macrophages as well as Kupffer cells, but not on small intestine macrophages) \(^6,11-14\). Additionally, it was demonstrated that platelets express functional FcαRI, although the physiological relevance has not yet been established \(^15\).

FcαRI expression is not observed on mast cells or basophils \(^16\). It is expressed as a 55-75 kDa molecule on monocytes and neutrophils, while the mass is 70-100 kDa on eosinophils due to of heavy glycosylation \(^17\).

The expression of FcαRI is constitutive and independent of its ligand, since IgA deficient patients express similar levels of FcαRI compared to healthy individuals \(^18\). However, the expression of FcαRI is modulated by exposure to inflammatory cytokines, chemoattractants or lipopolysaccharide (LPS) \(^6,8\). Additionally, adaptor protein binding to the intracellular domain of FcαRI modulates receptor expression \(^19\). Upregulation is induced by interleukin (IL)-1β, granulocyte-macrophage colony-stimulation factor (GM-CSF), tumor necrosis factor-α (TNF-α) or IL-8, whereas expression is downregulated by interferon-γ (INF-γ), transforming growth factor-β (TGF-β) or polymeric IgA \(^6,8\).

Upregulation of FcαRI on neutrophils is rapid and results from either de novo synthesis or transport from intracellular stores to the cell surface \(^20\).

**Binding of IgA to FcαRI**

FcαRI consists of two extracellular immunoglobulin-like domains (EC1 and EC2), a transmembrane region and a short cytoplasmic tail (Figure 1). The two extracellular domains are folded with an angle of approximately 90° to each other \(^21\). The binding site on FcαRI for IgA lies in the extracellular domain EC1, while FcαRI and FcγRs bind their
ligand in EC2 $^{6,8,21,22}$. FcαRI binds at the interface of the Ca2 and Ca3 domains of IgA in a 2:1 stoichiometry, as one IgA molecule is able to bind two FcαRI molecules $^{21,23}$. Because of the partial overlap of the IgA binding site for FcαRI and pIgR, binding of SIgA to FcαRI is (partly) hampered because of steric hindrance by SC (Figure 1) $^{14,21,22,24-26}$. Both monomeric and dimeric IgA bind to FcαRI with moderate affinity ($K_a=\approx 10^6 \text{ M}^{-1}$), while IgA-immune complexes bind avidly. For optimal binding immune complexes contain five to six IgA molecules (either monomeric or dimeric) per complex $^{27}$. Both FcαRI and IgA are heavily glycosylated proteins. FcαRI contains six $N$-glycosylation sites and several putative $O$-linked glycosylation sites $^3$. Deglycosylation of FcαRI N58 increases IgA binding $^{28}$, but IgA Fc glycosylation is not critical for binding to FcαRI $^6,28$.

Recently, it was described that the acute phase protein C-reactive protein (CRP) binds to FcαRI, leading to cell activation $^{29}$. CRP structurally belongs to the pentraxin family of proteins, which are characterized by a pentameric ring-like structure bearing five subunits or protomers. Although these proteins are structurally different from antibodies, it was shown that pentraxins recognize a similar site on FcαRI as IgA, resulting in competition for binding $^{30}$.

Ligand binding to FcαRI is regulated through inside-out signaling (Figure 2A). This entails that stimulation of cells with cytokines rapidly modulates binding capacity in response to intracellular signals, without affecting receptor expression levels $^{31-34}$. Experiments with transfectants, eosinophils and monocytes showed that FcαRI exhibits low capacity to interact with IgA-immune complexes in a resting state. However, ligand binding capacity increases profoundly after stimulation with cytokines like GM-CSF, IL-4 and IL-5 $^{32}$. In this way, FcαRI becomes primed but surface receptor expression is not affected. The intracellular domain of FcαRI and the presence of an intact cytoskeleton are necessary for this process, but FcR $\gamma$-chain is not required $^{32,33}$. Whether FcαRI priming is the result of increased conformational changes within the receptor (affinity) and/or lateral movement (avidity), as described for integrins $^{35}$, is still unknown. It is also unclear whether FcαRI priming on neutrophils is necessary, as activation of neutrophils during isolation precludes these experiments. However, it was demonstrated that neutrophils from patients with active and ongoing dermatitis herpetiformis had increased ability to bind IgA, without alternations in receptor expression, which is consistent with receptor priming $^{36}$.

**Outside-in signaling via FcαRI**

For FcαRI to initiate optimal effector functions, association with FcR $\gamma$-chain is a prerequisite $^{37-39}$. FcR $\gamma$-chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular domains, which are necessary for induction of signaling. ITAMs are conserved sequences containing paired tyrosines and leucines (YxxLx$^6-8$YxxL). After receptor aggregation, tyrosines become phosphorylated and act as docking platforms for signaling molecules (see also below). In general, cells express FcαRI in complex with a FcR $\gamma$-chain homodimer on the cell surface (Figure 1) $^{37-39}$. The negatively charged aspartic acid 11 (D11) in the transmembrane region of the FcR $\gamma$-chain tethers to the positively charged arginine 209 (R209) located in the transmembrane region of FcαRI $^{37}$. Site-directed mutagenesis revealed that the orientation of the positively charged amino acid
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of FcαRI is critical for the association with the FcR γ-chain. Furthermore, several other amino acids contribute to stable complex formation, such as leucine 14 and 21, tyrosine 17 and 25 and cysteine 26 in the FcR γ-chain, and three leucines at position 217, 220 and 224 in the transmembrane region of FcαRI.

When FcαRI transgenic mice were crossed with FcR γ-chain-deficient mice, surface expression of FcαRI was lost, suggesting that association with FcR γ-chain is necessary for stable membrane expression in vivo. Nonetheless, “γ-less” FcαRI can be expressed in transfectants. Moreover, γ-less FcαRI was observed in human monocyte and neutrophil subpopulations, although functionality was limited to ligand binding and receptor recycling via early endosomes. A single nucleotide polymorphism (SNP) changing serine into glycine at position 248 in the cytoplasmic tail of FcαRI, enabled γ-less cells to induce IL-6 release after crosslinking the receptor with anti-FcαRI mAbs.

Complex formation with FcR γ-chain is also essential for other Fc-receptors such as the IgE receptor FcεRI and the IgG receptors FcγRI and FcγRIIIa. The receptor domains responsible for tethering to FcR γ-chain are, however, different, since the transmembrane regions are not homologous to the FcαRI sequence and the necessary residues are located on the C-terminal instead of the N-terminal. Additionally, complex formation does not involve any known electrostatic interactions, as is observed with the interaction between FcαRI and FcR γ-chain. In contrast, FcαRI shares the positively charged arginine at the N-terminus with other members of the LRC family, which also interact with FcR γ-chain. The KIRs are an exception, since they contain a positively charged lysine, but associate with DAP12 instead of the FcR γ-chain.

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**Figure 1. Schematic representation of the FcαRI-FcR γ-chain complex.** One monomeric IgA molecule can interact with two FcαRI molecules. Binding sites are located at Cα2 and Cα3 junctions of IgA (black dots) and in the extracellular (EC)1 of FcαRI, respectively. Dimeric IgA can bind with FcαRI as well, although the exact molecular interaction is not known. Secretory IgA binds poorly to FcαRI since secretory component (SC, red box) interferes with the binding site. FcR γ-chain homodimers interact via the negative aspartic acid 11 (D11) with the positive arginine 209 (R209) in the transmembrane tail of FcαRI. The FcR γ-chain contains ITAM motifs that are phosphorylated (P) after FcαRI triggering, which is the start of signal transduction. EC; extracellular, TM; transmembrane, IC; intracellular.
**ITAM signaling**

The cellular response following triggering of the FcαRI-FcR γ-chain complex depends on the nature of the ligand. Multimeric IgA immune complexes trigger potent proinflammatory responses after binding to FcαRI. After crosslinking, the FcαRI-FcR γ-chain complex caps transiently and redistributes to plasma membrane rafts, which involves the actin cytoskeleton. By isolating lipid raft fractions of transfected cells and by immunoprecipitating FcαRI before and after crosslinking, several signaling molecules were identified that play a role in the FcαRI signaling cascade. The following model has been proposed. After crosslinking, the FcαRI-FcR γ-chain complex redistributes to membrane rafts. The src family kinase Lyn is recruited to membrane rafts and aggregates with the receptor complex to phosphorylate the tyrosines in the ITAMs of the FcγRIIγ chain. Phosphorylated ITAMs subsequently act as docking platforms for a diverse repertoire of signaling molecules. B-lymphocyte kinase (Blk) and SH2-domain containing proteins such as Syk, phospholipase Cγ (PLCγ), Shc and growth factor receptor-bound protein 2 (Grb2) are recruited to phosphorylated ITAMs. Syk plays a pivotal role in the signaling process by activating multiple interconnecting pathways. First, Syk can directly activate phosphoinositide 3-kinase (PI3-K), which results in the transformation of phosphatidylinositol(4,5)-biphosphate (PI(4,5)P2) into phosphatidylinositol(3,4,5) triphosphate (PI(3,4,5)P3) and triggers the downstream effectors phosphoinositide-dependent kinase-1 (PDK1) and protein kinase Ca (PKCa) (Figure 2B; 1). PI(3,4,5)P3 directly stimulates protein kinase Bα (PKBα, also known as Akt), which was found to reside in lipid rafts after FcαRI crosslinking. Additionally, PI(3,4,5)P3 can trigger Bruton’s tyrosine kinase (Btk), thereby activating the family of Rho GTPases and inducing actin remodelling. Btk also influences PLCγ and protein kinase Ca (PKCa) activity in lipid rafts. Second, Syk can directly stimulate PLCγ, which leads to the transformation of PI(4,5)P2 into inositol triphosphate (I(1,4,5)P3) and diacylglycerol (DAG) (Figure 2B; 2). The second messenger DAG can mediate protein kinase Ca (PKCa) directly or via release of intracellular calcium, induced by PLCγ. DAG can recruit PKCa, exemplifying additional interconnections between the PI3-K and PLCγ pathways. After FcαRI triggering, calcium is released from intracellular stores, independently of store-operated Ca2+ entry pathways. Finally, Syk was shown to phosphorylate Shc after FcαRI activation. A similar role for Syk after FcαRI triggering is hypothesized, since FcαRI ligation was shown to modulate a multimolecular adaptor protein complex which was disturbed by Src kinase inhibitors. After Shc phosphorylation, the stable complex of p120-Cbl with SLP-76 binds Shc. Additionally, SH2 domain-containing inositol polyphosphate 5-phosphatase (SHIP) associates with phosphorylated Shc, suggesting that signaling molecules capable of down regulating immune responses can also be recruited to the receptor complex. Furthermore, the adaptor molecule Grb2 can recruit phosphorylated Shc. Grb2 binds to Crkl and guanine nucleotide exchange factor Sos. Given that Sos is able to exchange GDP-Ras to GTP-Ras, this multimolecular adaptor complex controls the Ras/Raf-1/MAP kinase cascade. Of note, different signaling molecules displayed a spatial and temporal divergence in kinetics. Whereas Syk and Blk showed sustained association with lipid rafts, Shc and Grb2 exhibited a transient interaction before dissociating.
rafts, PLCγ and PKBα had an intermediate interaction profile while FcαRI, FcR γ-chain, PKCε, PI3-K and downstream effectors of PI3-K displayed a transient association. The activation of these interconnecting signaling pathways leads to the activation of several transcription factors, including nuclear factor-κB (NF-κB), AP-1 and Sp1. Multimeric stimulation of the downstream signaling pathways of FcαRI leads to proinflammatory functions.

**ITAMi signaling**
Non-targeted monomeric serum IgA or Fab fragments of anti-FcαRI antibodies bind FcαRI but are not able to crosslink FcαRI, and induce anti-inflammatory responses (Figure 2C). Monovalent targeting of FcαRI results in partial phosphorylation of the FcR γ-chain, and involves ERK-dependent recruitment of tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 (SHP-1) to sphingolipid-cholesterol-rich membrane domains. Subsequently, cytoplasmic clusters referred to as “inhibisomes” are formed, containing signaling molecules and inhibitory and activating receptors. SHP-1-initiated actin depolymerization is essential for the formation of these intracellular clusters. Inhibisomes hamper Syk, LAT and ERK phosphorylation and thereby impair the function of other activating Fc receptors (like FcεRI or FcγRI) 56. The process by which these anti-inflammatory responses are mediated via ITAMs is referred to as inhibitory ITAM receptor signaling (ITAMi). Collectively these results suggest that serum IgA plays an important role in maintaining homeostasis and that ITAMi signaling dampens proinflammatory responses that are induced by other activating Fc receptors 55. Of note, the same may hold true for monomeric IgG, as signaling via ITAMi has also been described for FcγRIIa and FcγRIIIa 57,58. In conclusion, activating the FcαRI-FcR γ-chain complex can lead to either anti- or proinflammatory responses, depending on the ligand and the subsequent recruitment of tyrosine kinases or phosphatases.

**FcαRI-mediated functions**
Fc receptors contribute in host defense through engagement with antigens that are complexed with antibodies. Crosslinking of Fc receptors induces different processes, including phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), superoxide generation and the release of cytokines and inflammatory mediators. FcαRI mediates phagocytosis of IgA-coated beads, bacteria or yeast particles by resting neutrophils, which is enhanced after priming of neutrophils with cytokines like GM-CSF or IL-8. This was also demonstrated for monocytes and eosinophils after stimulation with either IL-1, TNF-α, GM-CSF or LPS and GM-CSF, IL-5 or IL-4, respectively. In addition to phagocytosis, release of neutrophil extracellular traps (NETs) has been described as an extra tool in antimicrobial strategies of neutrophils. NETs are web-like structures, consisting of DNA and nuclear as well as cytoplasmic proteins, which are released by neutrophils and trap and kill bacteria and fungi. Recently, we demonstrated that phagocytosis of serum IgA opsonized particles (bacteria and beads) enhanced the release of NETs, which was due to increased reactive oxygen species (ROS) production. It was also described that FcαRI can induce different forms of neutrophil death, depending
Crosslinking of FcαRI on neutrophils induces different proinflammatory functions, including the release of cytokines and inflammatory mediators, phagocytosis and ADCC. Interestingly, we have demonstrated that crosslinking of FcαRI by complexed monomeric and dIgA induced neutrophil recruitment and release of the chemoattractant leukotriene B4 (LTB4), leading to amplification of neutrophil migration. This seems to be specific for IgA, as crosslinking of FcγR by IgG did not induce the essential LTB4 release and consequently did not mediate neutrophil migration. Crosslinking of FcαRI by complexed IgA thus induces robust inflammatory responses as protective mechanism against invading pathogens.

IgA and FcαRI in mucosal immunity

Functions of IgA

SIgA is important as first line of defense in mucosal areas, by preventing penetration of the mucosal wall by pathogenic microorganisms or foreign antigens (Figure 3). The predominant presence of hydrophilic amino acids in IgA Fc and the abundant glycosylation of both IgA and SC makes SIgA a hydrophilic, negatively charged molecule. In this way, SIgA can surround microorganisms with a ‘hydrophilic shell’ that is repelled by mucosal surfaces. Furthermore, SIgA can agglutinate microbes and interfere with bacterial motility by interacting with their flagella. Additionally, SIgA is also able to interact with and neutralize bacterial products such as enzymes and toxins. The opsonic activity of SIgA is, however, poor compared with dIgA or serum IgA, which is presumably due to (partial) blockage of FcαRI binding site on IgA by SC (Figure 1). Additionally, SIgA is unable to trigger efficient phagocytosis by neutrophils or Kupffer cells, supporting its anti-inflammatory role. However, SIgA can initiate respiratory burst activity by neutrophils, albeit less efficient compared to serum IgA. Moreover, this process is dependent on expression of Mac-1 (CD11b/CD18), suggesting that SIgA needs this integrin co-receptor to bind or activate FcαRI.

The human and mouse IgA systems have several dissimilarities, most notably the absence of FcαRI in mice. Functions of SIgA are comparable, and its importance in epithelial barrier function for sustained mucosal homeostasis was demonstrated in pIgR/SC-deficient mice, since these mice lack IgA transport over the mucosal wall. In homeostatic conditions, dIgA is produced by local plasma cells in the lamina propria and is transported through epithelial cells via pIgR (Figure 3; 1). It is then released as SIgA into the lumen. pIgR/SC-deficient mice developed increased serum IgG levels, including antibodies directed against Escherichia coli, suggesting excessive activation of the systemic immune system. Additionally, the epithelial barrier function in these mice is defective, as elevated levels of albumin were found in saliva and feces, supporting leakage of serum proteins. It was hypothesized that this was due to increased bacterial colonization resulting in irritation of the epithelium. Thus, pIgR and SIgA may play an important role in regulating mucosal homeostasis.

Because of the abundant presence of microbial flora and food components in the intestinal tract, antigens continuously reach the lamina propria through diffusion or transcytosis.
Figure 2. Signaling pathways of FcαRI after ligand binding. (A) Inside-out signaling or priming of FcαRI. Resting FcαRI is serine phosphorylated (P), and has low capacity to bind IgA. Priming with cytokines leads to signaling via PI3-K and PKC, which leads to dephosphorylation of FcαRI by PP2A, switching inactive FcαRI into an active, ligand binding receptor. (B) Crosslinking of FcαRI IgA-opsonized bacterium induces redistribution of FcαRI to plasma membrane rafts. Src kinase Lyn phosphorylates the tyrosines in the ITAMs in the cytoplasmic tail of associated FcRγ-chains. Phosphorylated ITAMs subsequently act as docking platforms for several signaling molecules. Syk plays a pivotal role, and activates 1) the PI3-K signaling route 2) PLCγ, and 3) Shc. This results in proinflammatory cellular functions such as phagocytosis, antibody dependent-cellular cytotoxicity, respiratory burst, degranulation, antigen-presentation and release of cytokines and inflammatory mediators. (C) Binding of monomeric IgA to FcαRI (in absence of FcαRI crosslinking) leads to partial phosphorylation of ITAMs. This results in recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) to FcαRI. Subsequently, inhibisome clusters are formed that impair phosphorylation of Syk, LAT and ERK, and hereby inhibit ITAM signaling via other activating Fc receptors.
Dimeric IgA can interact with these antigens, and antigen-dIgA immune complexes are subsequently transported back to lumen via the pIgR route (Figure 3; 2). This might be an effective way to clear the mucosa of undesired excessive immune complexes that otherwise may trigger unwarranted adaptive immune responses. Most IgA-deficient patients do not suffer from serious complications, but are susceptible to develop allergies and autoimmune diseases\(^4\), which suggests that the diminished epithelial barrier function may lead to inappropriate immune responses against food components or indigenous bacterial flora. This may also play a role in several mucosal disorders, such as gluten-sensitive enteropathy and inflammatory bowel disease (IBD), as frequencies of these diseases are increased in selective IgA-deficient patients\(^4\). Another feature of dIgA is its ability to neutralize intracellular viruses. \textit{In vitro} experiments have demonstrated that addition of specific IgA to the basolateral surface of cultured polarized epithelial cells resulted in intracellular co-localization of IgA and Sendai virus and decreased virus titers\(^65\). This neutralization has also been shown for influenza\(^65\). In addition, intravenous administration of IgA anti-outer capsid viral proteins protected mice against rotavirus infection, while administration via the lumen or intestinal tract was ineffective\(^66\). This has also been shown for rotavirus\(^67\). This finding supports that transcytosis of IgA via epithelial cells is required for viral inactivation in animal models, and potentially also in humans.

\textit{In vitro} studies with polarized epithelial cells demonstrated that IgA has an inhibitory effect on transepithelial entry of HIV\(^68\). Additionally, the potential of anti-HIV polymeric IgA antibody to effectively inhibit mucosal transmission was recently shown \textit{in vivo} in humanized mice\(^69\). A trial in Southern Africa demonstrated that HIV uninfected women had HIV-1 specific IgA antibodies in their vaginal secretions\(^70\). Interestingly, a recent \textit{in vivo} study in mice showed the potential of SIgA to serve as vaccine carrier for a HIV antigen via mucosal administration to target the gastrointestinal environment\(^71\). In these mice, chemically bound HIV antigen to SIgA was delivered into the intestinal mucosal via oral administration, where SIgA interacted with mucosal microfold cells present in gut-associated lymphoid tissues. These complexes were subsequently selectively captured by dendritic cells and this elicited both humoral and cellular immune responses at systemic and mucosal levels\(^71\).

In conclusion, SIgA serves mainly as antiseptic barrier by preventing entry of pathogens, but can also act as vaccine carrier for delivery of (HIV) antigens to induce mucosal and systemic immune responses. During trans-epithelial cell transport, dIgA can intercept virus particles and interfere with virus replication or assembly when transported through an infected epithelial cell. Systemic passive vaccination with dIgA may therefore be utilized as therapy to neutralize intracellular viruses in the mucosa\(^72\).

\textit{Contribution of Fc\textsubscript{α}RI in mucosal immunity}

In homeostatic conditions, mucosal areas contain only a few Fc\textsubscript{α}RI-positive cells. For example, intestinal macrophages lack Fc\textsubscript{α}RI expression\(^13\), which is consistent with the proposed anti-inflammatory role of IgA to maintain mucosal integrity during homeostasis. Neutrophils, however, are the first cells to arrive during infection. They are able to
efficiently phagocytose dIgA opsonized bacteria \cite{73}. Interestingly, crosslinking of FcαRI on neutrophils by dIgA leads to neutrophil recruitment due to release of the potent neutrophil chemoattractant LTB4 \cite{62}. Thus, once pathogens have been able to breach the epithelial barrier, and are opsonized with dIgA in the lamina propria, a self-contained positive feedback loop is initiated, which results in enhanced recruitment of neutrophils until clearance of invading pathogens has been achieved (Figure 3; 4). Furthermore, this might also result in enhanced NET formation, which can prevent systemic dissemination. The ability of dIgA to recruit neutrophils therefore functions as a second line of defense in mucosal areas.

Interactions between serum IgA and FcαRI and their role in immunity are more complicated and still incompletely understood. On the one hand, monomeric IgA can down-regulate activation via other Fc receptors through ITAMi signaling (Figure 2C) \cite{58}. This likely represents an anti-inflammatory mechanism to prevent uncontrolled release of inflammatory cytokines during acute and/or chronic inflammation. By contrast, Kupffer cells in the liver express FcαRI, and these cells efficiently eliminated serum IgA-opsonized *Escherichia coli* bacteria from portal circulation \cite{14}. Serum IgA interactions with FcαRI on Kupffer cells therefore represent a third line of defense by removing pathogens that have invaded via the gut and have infiltrated the portal circulation (Figure 3; 5).

It was also demonstrated that opsonization of *Escherichia coli*, *Streptococcus aureus*, *Bordetella pertussis*, *Neisseria meningitides* and *Salmonella typhimurium* with dIgA or serum IgA resulted in enhanced uptake by neutrophils \cite{8,14,34,60,62,73}. Investigating the *in vivo* role of FcαRI has, however, been seriously hampered by the absence of an orthologue in experimental mouse models. To overcome this limitation, two transgenic mouse models have been created. The CD11b promoter was used in one model, which resulted in high human FcαRI expression on monocytes and macrophages \cite{74}. A cosmid clone bearing the human FcαRI gene, including the regulatory elements, has been used to create a second model. The latter transgenic mice express FcαRI on cells of the myeloid lineage (mostly on neutrophils), which corresponds to the human situation more closely \cite{38,75}. The important role of FcαRI in mucosal infections is supported by *in vivo* studies in human FcαRI transgenic mice that express human FcαRI on myeloid cells. Infection of mice with human IgA-opsonized *Bordetella pertussis* led to enhanced bacterial clearance in the lungs of human FcαRI transgenic mice compared to non-transgenic littermates \cite{76}. In addition, passive transfer of human IgA anti-*Mycobacterium tuberculosis* resulted in protection of human FcαRI transgenic mice against infection, but not of FcαRI-negative control mice \cite{77}.

Taken together, naturally occurring serum IgA (not complexed with an antigen) induces inhibitory signals via ITAMi through FcαRI to dampen excessive immune responses (initiated by other Ig-immune complexes). However, crosslinking of FcαRI during infection with dIgA- or serum IgA-opsonized pathogens results in potent proinflammatory responses by neutrophils and Kupffer cells in order to clear the infection.

**Targeting FcαRI in disease**

Because crosslinking of FcαRI by IgA immune complexes potently recruits and activates
neutrophils, the presence of aberrant IgA might result in aggravated proinflammatory responses, leading to tissue damage. This might play a role in different inflammatory diseases that are characterized by an increase in serum (auto-) IgA levels, such as IgA nephropathy, Henoch-Schönlein purpura, ankylosing spondylitis, Sjögren’s syndrome, alcoholic liver cirrhosis, celiac disease, asthma, inflammatory bowel disease and dermatitis herpetiformis.

Figure 3. Schematic model of the proposed functions of IgA and FcαRI in mucosal immunity.

In homeostatic conditions, plasma cells in the lamina propria produce dIgA that binds to the pIgR, which is expressed on the basolateral side of epithelial cells. (1) This complex is transported through epithelial cells, where pIgR is cleaved, releasing SlgA into the lumen. SlgA protects the mucosa from bacterial penetration, agglutinates bacteria, interferes with their motility and neutralizes bacterial products. (2) When antigens reach the lamina propria they will be opsonized by dIgA and can be transported back to the lumen via the same route as free dIgA, ridding the mucosa of superfluous antigens. (3) During transcytosis IgA can intercept viruses, interfere with viral synthesis and/or assembly and thus neutralize viruses intracellularly. (4) When pathogens have breached the epithelial barrier, they are opsonized by dIgA. Neutrophils that express FcαRI will phagocytose dIgA-opsonized microorganisms and release LTB4, which will recruit more neutrophils. This can lead to a self-controlled positive feedback loop, until pathogens have been eliminated and infection has been cleared. (5) When pathogens enter the portal circulation, they will be opsonized by serum IgA and subsequently phagocytosed by FcαRI-positive Kupffer cells, preventing septicemia and disease.

IgA nephropathy

IgA nephropathy (IgAN) is the most prevalent form of primary glomerulonephritis that often results in end-stage kidney failure. IgAN is characterized by the accumulation of aberrant glycosylated IgA1 immune complexes in the glomerular mesangium. It has been shown that different IgA receptors are involved in the pathogenesis of this disease. FcαRI can be shed from the cell membrane, and is referred to as soluble FcαRI (sFcαRI). IgA-sFcαRI complexes were observed in patients with IgAN. Moreover, mesangial deposition of sFcαRI-IgA complexes induced glomerular and interstitial macrophage infiltration, mesangial matrix expansion, hematuria, and mild proteinuria in FcαRI transgenic mice.
Complexed sFcaRI-IgA also induced kidney inflammation in FcαRI and human IgA transgenic/knock-in mice through interaction with the mesangial IgA1 receptor transferrin receptor 1 (TfR1) \(^{82}\). Release of proinflammatory cytokines by mesangial cells was increased by sFcaRI-TfR1 interactions, which suggests that IgA1-sFcaRI might be causing local mesangial cell activation. Furthermore, sFcaRI-TfR1 interactions induced the expression of transglutaminase 2 (TGase2), which resulted in up-regulation of TfR1 expression. Thus for IgAN development, co-operation between IgA1, sFcaRI, TfR1 and TGase2 on mesangial cells is necessary \(^{82}\). TGase2 is responsible for the induction of a pathogenic amplification loop that facilitates IgA1-sFcaRI deposition and the activation of mesangial cells, which makes TGase2 an interesting therapeutic target.

Ingestion of gluten, a major food component and a target antigen in celiac disease, exacerbated intestinal IgA secretion and inflammation in these FcαRI and human IgA transgenic/knock-in mice \(^{83}\). Furthermore, increased serum IgA anti-gliadin antibodies were found, which correlated with proteinuria in mice and patients. This suggests an important role of gluten in IgAN, as uptake might result in formation of IgA immune complexes containing sFcaRI, gliadin and anti-gliadin IgA. These complexes might subsequently deposit in the mesangium leading to renal inflammation. It was proposed that alimentary antigens, such as gliadin, can induce increased mucosal immune responses and intestinal alterations, resulting in breakdown of oral tolerance and in this way contribute to IgAN \(^{83}\). This also suggests the possibility of a gluten-free diet as therapeutic approach in these patients.

Alternatively, it was investigated in a recent study whether targeting FcαRI-ITAMI can halt the progression of pristane (natural hydrocarbon oil) induced lupus nephritis (LN) \(^{84}\). This is a well-established model of murine lupus, as mice injected with pristane develop lupus specific autoantibodies, hemolytic anemia, proteinuria and glomerulonephritis, which resembles human systemic lupus erythematosus. LN was induced in transgenic mice that express a human FcαRI \(^{R209L}/FcR-\gamma\) chimeric receptor. Targeting FcαRI with the mAb MIP8a halted disease progression and lupus activation through inhibition of cytokine production, recruitment of leukocytes and renal inflammation. This implies that anti-FcαRI mAb can be used to induce inhibitory signals through FcαRI to control LN.

### Rheumatoid arthritis

It was furthermore investigated whether targeting FcαRI with monomeric IgA (ITAMI signaling) is a promising therapy for rheumatoid arthritis (RA) \(^{54,55,58,85}\). This is a systemic and chronic autoimmune disease, and patients suffering from RA experience long-term joint damage resulting in chronic pain and disability. RA is characterized by the presence of autoantibodies, such as rheumatoid factor \(^{86}\). These are IgM, IgG or IgA antibodies directed against the Fc domain of IgG, leading to the formation of antibody immune complexes \(^{87}\). The second type of RA-related autoantibodies react to citrullinated proteins and peptides and are referred to as anti-citrullinated protein antibodies (ACPA) \(^{88}\).

Plasma-derived human monomeric IgA inhibited activation via other receptors (e.g. FcγRI) on FcαRI-expressing transfectants, blood phagocytes from healthy individuals
and synovial cells from RA patients in a FcαRI-FcR γ-chain dependent manner. FcαRI transgenic mice (expressing FcαRI on monocytes/macrophages) developed arthritis when they were injected with a cocktail of IgG antibodies against collagen II. Treatment with either monomeric IgA or anti-FcαRI Fabs effectively reduced or prevented the induction of arthritis. This indicates that human monomeric IgA can shift activating IgG-induced ITAM signaling into the inhibitory FcαRI-ITAMi pathway, suggesting potential future use of IgA to treat autoimmune diseases when patients have IgG autoantibodies.

Interestingly, several studies reported a correlation between the presence of IgA RF or IgA ACPA in blood or synovial fluid and worse disease prognosis as well as severe bone erosions. This raises the hypothesis that IgA can contribute to pathology, as crosslinking of FcαRI by immune complexes triggers neutrophil-induced inflammation. Presence of RF has previously been used as a predictive value for progression of disease. The importance of IgA RF was supported by a recent study in which the amount of IgA autoantibody was significantly associated with severity of disease in RA patients that were IgM RF negative. No significant correlation was found between IgG levels and the severity of RA. The involvement of IgA RF in extra-articular manifestations in RA was also reported. In a large patient cohort study, a positive correlation was found between the increase of serum IgA and the grade of cartilage destruction in active RA. Furthermore, clinical studies showed that anti-TNF-α treatment of RA patients reduced IgM, IgG and IgA RF levels. Interestingly, high pre-treatment IgA RF levels were associated with poor clinical response to TNF-α inhibitors. These studies collectively support that IgA autoantibodies are involved in mediating joint cartilage erosions in active RA. The mechanisms of IgA-induced involvement in RA, however, are not yet resolved.

Skin blistering diseases
The hypothesis that IgA-FcαRI interactions play a harmful role in autoimmune diseases is supported by pathophysiology of the rare skin blistering disease Linear IgA Bullous Disease (LABD). Patients with this disease have IgA autoantibodies directed against...
collegen XVII as well as neutrophil accumulation in the skin, which ultimately leads to extensive skin damage and blister formation. We previously demonstrated that neutrophil activation and concomitant tissue damage is a direct consequence of FcαRI triggering through IgA autoantibodies. Aberrant IgA autoantibodies are found in several chronic autoimmune skin diseases, including LABD, dermatitis herpetiformis and IgA pemphigus. IgA autoantibodies in these diseases are directed against structural proteins of the dermal-epidermal junction or proteins involved in maintaining cell-cell adhesion in the epidermis. For example, anti-epidermal transglutaminase 3 IgA auto-antibodies are present in DH patients, whereas IgA autoantibodies against collagen XVII or desmocollin-1 are present in LABD or IgA pemphigus, respectively. Collagen XVII is also known as the bullous pemphigoid antigen of 180 kDa (BP180), which is a transmembrane hemidesmosomal protein involved in maintaining cell-matrix adhesion in the skin.

Considering the high neutrophil influx in the skin of LABD patients, we investigated the role of IgA autoantibodies in the recruitment of neutrophils and the involvement of FcαRI in inducing tissue damage. When normal skin cryosections were incubated with neutrophils and serum of LABD patients (which contained anti-collagen XVII IgA autoantibodies), separation of the dermis and epidermis was observed, which reflects blister formation. Tissue damage was induced by activated neutrophils secreting elastase and ROS via respiratory burst, since inhibition of either pathway reduced dermal-epidermal separation. Importantly, IgA-induced tissue damage was prevented by blocking FcαRI using an anti-FcαRI mAb.

Furthermore, an influx of eosinophils in the skin of LABD patients has also been observed, but the association with disease pathology is incompletely understood. Eosinophils can express FcαRI as well. Previously, it was shown that crosslinking of FcαRI on eosinophils led to respiratory burst activity, although less than neutrophils. We demonstrated that eosinophils migrated towards IgA-coated beads, supporting that eosinophils might contribute to IgA-induced pathogenesis in LABD as well.

Dermatitis herpetiformis is clinically similar to LABD, as it is characterized by the presence of autoimmune IgA deposits and neutrophil influx in the skin. However, DH is associated with celiac disease (gluten-sensitive enteropathy). The ingestion of gluten in these patients leads to inflammation and damage in the small intestine. Patients with DH that have ongoing, albeit controlled disease show a partial activation of their circulating neutrophils. Interestingly, these cells have also increased ability to bind IgA despite similar FcαRI expression, which is consistent with a pattern of neutrophil priming. Thus, in both LABD and DH activation of neutrophils by IgA autoantibodies through FcαRI triggering results in accumulation of immune cells in the skin, in particular neutrophils and eosinophils, and formation of subepidermal blisters.

**Inflammatory Bowel Disease**

The above mentioned mechanism of neutrophil activation by IgA autoantibodies may play a role in other diseases as well. Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastro-intestinal tract, and is sub-divided into ulcerative colitis...
and Crohn’s disease \textsuperscript{112,113}. These diseases are associated with a strong inflammatory response to a yet unidentified source, but which likely includes aberrant responses against the commensal microflora \textsuperscript{114}. Furthermore, IBD is also associated with mucosal damage and increased epithelial permeability. Loss of barrier function allows the invasion of commensal bacteria in the subepithelial space or lamina propria. Crohn’s disease is not a neutrophil mediated disease, as it is characterized by compact aggregations of mononuclear cells \textsuperscript{112,113}. In contrast, massive influx of neutrophils is observed in ulcerative colitis, leading to extensive tissue damage \textsuperscript{112,114}. It was shown that specific composition of the intestinal microbiota affects the development of IBD. It was recently demonstrated that the administration of highly IgA-opsonized intestinal bacteria of IBD patients increased susceptibility to colitis in germ-free mice \textsuperscript{115}.

Because IgA is the most abundant antibody in mucosal tissues, invading bacteria are opsonized with dIgA, which can subsequently activate Fc\alpha RI on neutrophils \textsuperscript{62}. As such, we hypothesize that also in ulcerative colitis IgA immune complexes are able to crosslink Fc\alpha RI, resulting in activation and migration of neutrophils. As newly recruited neutrophils will be activated by IgA opsonized microorganisms as well, a neutrophil recruitment loop is initiated. This hypothesis is supported by Fc\alpha RI-expressing neutrophil accumulations that are found in colon tissue slides of patients with ulcerative colitis \textsuperscript{62}. Furthermore, intracellular staining of IgA was observed in neutrophils, suggesting that IgA-antigen complexes had been taken up, leading to neutrophil activation.

It was furthermore recently shown that antibody-opsonized bacteria are efficiently taken up by dendritic cells, which resulted in cross-talk between Fc receptors and Toll-like receptors (TLRs) \textsuperscript{116}. This led to synergistic release of several inflammatory cytokines like TNF-\alpha and IL-1\beta as well as induction of Th17 cells, which are known to play a detrimental role in IBD \textsuperscript{112,113}. Cross-talk of Fc receptors and TLRs on dendritic cells resulted in synergistic release of IL-1\beta and LTB4, which may further enhance neutrophil recruitment \textsuperscript{116}. Additionally, co-activation of Fc\alpha RI and TLR4 on neutrophils enhanced release of TNF-\alpha, a potent proinflammatory cytokine that drives intestinal inflammation in IBD. Uncontrolled IgA-mediated neutrophil migration as well as Fc\alpha RI-TLR cross-talk on dendritic cells and neutrophils may thus contribute to inflammation and severe tissue damage in patients with ulcerative colitis.

Thus inhibiting Fc\alpha RI-induced activation might be a novel therapeutic approach to dampen inflammation in patients with IgA-mediated disease.
Thesis aim and outline

IgA is the most abundant antibody class at mucosal surfaces where it plays an important role in mucosal defense. Neutrophils are quick responders and are the first cells that arrive at sites of infection. They recognize and destroy bacteria, and as such play an important role in defense against invading pathogens. Neutrophils express the IgA Fc receptor FcαRI. Previously, we showed that crosslinking of FcαRI on neutrophils by IgA-antigen complexes results in neutrophil activation and release of the potent neutrophil chemoattractant LTB4. Consequently, a self-contained positive feedback loop can be initiated, resulting in enhanced neutrophil recruitment until clearance of invaded pathogens has been achieved. This is a beneficial process to clear the infection. However, neutrophils are filled with toxic components and therefore their activation needs to be controlled tightly. I hypothesize that in different IgA-mediated autoimmune diseases, FcαRI-IgA interactions play a harmful role, as excessive neutrophil activation will result in tissue damage and chronic inflammation. Furthermore, I propose that blocking of FcαRI-IgA interactions may prevent or decrease tissue damage and FcαRI might be a novel therapeutic target for IgA autoantibody-mediated autoimmune diseases.

In chapter 2, we describe enhanced uptake of IgA-coated particles via FcαRI by neutrophils that induces neutrophil extracellular traps (NETs) release. This might be an important process at mucosal surfaces to eliminate microorganisms. In chapter 3, we demonstrate that IgA autoantibodies are present in plasma and synovial fluid of RA patients. IgA immune complexes were able to activate neutrophils, as measured by the production of reactive oxygen species, release of NETs, lactoferrin and chemotactic stimuli. Blocking FcαRI on neutrophils resulted in inhibition of neutrophil activation by IgA immune complexes. In order to investigate the role of IgA and FcαRI interactions in neutrophil activation in experimental arthritis, we tested the susceptibility of our humanized FcαRI/hIgA mice model to collagen induced arthritis in chapter 4. Immunizing mice with collagen II resulted in the production of IgA autoantibodies against collagen II, which bound to the collagen expressed at the cartilage in the joints of mice. Neutrophil number in blood and bone marrow increased over time, but this was not sufficient to induce clinical signs of arthritis, however, we observed loss of joint function. In chapter 5, we develop a novel mice model for Linear IgA Bullous Disease (LABD). Mouse expressing human FcαRI on neutrophils were crossed with mice expressing LysEGFP neutrophils. These mice were used to analyze neutrophil migration using intravital imaging. We demonstrate that injection of anti-mCOL17 hIgA antibodies in the ears of mice induced neutrophil activation as showed by the increased number of neutrophils in the blood vessels and their migration into tissue. Furthermore, we crossed mice expressing human FcαRI with hIgA knock-in mice to test the ability of anti-mCOL17 hIgA antibodies in inducing neutrophil accumulation, inflammation and tissue damage. Blocking FcαRI prevented or decreased IgA-induced neutrophil accumulation and tissue damage in mice.

In chapter 6, we describe differences in cell activation after crosslinking FcαRI or FcγR. IgA stimulation induced stronger proinflammatory responses than IgG. In chapter 7, all previous chapters are summarized and discussed in relation to the current state of the art, suggestions for future research and possible therapeutic implications are provided.
Chapter 1

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