Antibody therapeutic approaches for cancer

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Wie beweert dat iets niet kan, mag degene die ermee bezig is absoluut niet storen
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Chapter 1

Introduction

ANTIBODIES AND IMMUNITY

In the early days of biology, it was poorly understood how the body could eliminate pathogenic micro-organisms. In 1796, important insights were gained by Edward Jenner, who successfully induced protective immunity against human smallpox in patients, by vaccinating them with cowpox, the bovine analog of smallpox[1]. In 1890, Emil von Behring and Shibasaburo Kitasato described the presence of anti-toxic effects of serum[2]. They showed that tetanus or diphteriae-exposed animals could be successfully treated with serum of animals that were cured from these infections. Only a few years later, Paul Ehrlich was able to actively vaccinate animals by feeding them small increasing quantities of diphtheria or tetanus toxins, thereby inducing antibody (Ab) responses[3]. In parallel to these studies, William Coley successfully treated cancer patients with an extract of Streptococcus and Serratia (Coley's mixed toxins), suggesting a role for the immune system in the treatment of malignancies[4]. Coley's toxin most likely contained components capable of activating the immune system. The active contents, however, are still not unraveled.

Nowadays, it is clear that infections can activate the adaptive immune system, consisting of B lymphocytes and T lymphocytes. After activation, B lymphocytes can develop into Ab-producing cells. Antibodies are composed of an antigen binding domain (Fab fragment), which specifically recognizes the pathogen, and a constant domain (Fc fragment), which can interact with complement proteins in serum, and Ab (or Fc) receptors on immune cells. Antibodies can be divided into five classes of immunoglobulins (Ig), which differ in their constant domain; IgM and IgD Ab can bind antigens with low affinity and are produced by B cells in the initial phase of an immune response. During secondary immune responses, a class-switch towards the higher affinity IgG, IgE and IgA Ab occurs with help of $CD4^+$ T lymphocytes[5]. Each Ab class can interact with a specific class of Fc receptors, with a unique immune cell distribution[6]. Antibody class-switching, thus, affects immune responses to pathogens by influencing immune cell effector functions.

ANTIBODY THERAPY OF CANCER

In the early 20^{th} century, it was believed that although mouse tumors were shown to be immunogenic, human tumors were non-immunogenic. In the 1960s, however, a human tumor antigen, carcinoembryonic antigen was described for colorectal cancer[7]. The discovery of more tumor antigens followed, which catalyzed the development of anti-tumor Ab for cancer therapy. However, scientists struggled with the production of specific Ab until 1975, in which year Drs. George Köhler and Cesar Milstein developed the so-called hybridoma technology, that allowed the generation of limitless amounts of specific monoclonal Ab (mAb)[8]. Drs. Köhler and Milstein received a Nobel Price for Physiology or Medicine in 1984 for their work.

In the last decades, Ab therapy has been acknowledged as a successful strategy to treat malignancies[9]. Patients with non-Hodgkin's lymphoma respond effectively to the anti-CD20 mAb rituximab, which was approved in 1997 as the first cancer therapeutic mAb by the United States FDA[10]. Furthermore, the anti-Her-2/neu mAb trastuzumab, the first FDA-approved mAb for therapy of a solid tumor, is used as therapy against breast cancer, leading to significant benefit in disease-free survival[11]. At present, a total of 18 mAb (all of the IgG class) have been approved by the FDA, of which 8 for the treatment of cancer[9]. The modes of action of anti-tumor Ab include crosslinking of targets on tumor cells, which can lead to anti-proliferative or pro-apoptotic effects. Binding of mAb to their targets may interfere with binding of natural ligands, as well[12]. Furthermore, the mAb Fc tail may bind to complement components, resulting in activation of the complement pathway[13]. In addition, anti-tumor mAb may bind via their constant regions to Fc receptors, thereby activating immune cell effector functions[14].

RECEPTORS FOR IgG

In humans, 3 classes of leukocyte IgG receptors (FcγR) have been described[14]. Activation of FcγR can initiate effector functions such as endocytosis, phagocytosis, Ab-dependent cellular cytotoxicity (ADCC), release of oxygen radicals, cytokine production and antigen presentation[15-17].

FcγRI (CD64), which binds IgG with high affinity, is expressed on cells of the myeloid lineage, including monocytes, macrophages, and dendritic cells. FcγRI expression can furthermore be induced on neutrophils by treatment with granulocyte-colony stimulating factor or interferon-γ[18,19]. FcγRI is associated with a common FcR γ chain, which contains immunoreceptor tyrosine-based activation motifs (or ITAM)[20,21]. The murine homologue of this receptor exerts similar functions as human FcγRI, but is only expressed on monocytes, macrophages and dendritic cells.

FcγRII (CD32) molecules can interact only with IgG complexes and are broadly expressed on myeloid cells, lymphocytes, platelets and endothelial cells[14]. The FcγRII class of receptors contain two categories of FcγR; activatory FcγRIIa molecules, which bear an ITAM signaling motif within their cytoplasmic tail, and inhibitory FcγRIIb molecules, which contain an ITIM (or immunoreceptor tyrosine-based inhibitory motif)[22,23]. Inhibitory FcγRIIb molecules can down-modulate signals of activatory, ITAM-bearing, receptors[14]. Consequently, these inhibitory receptors can down-regulate Ab-induced anti-tumor responses, as well[24]. Mice express a murine homologue for FcγRIIb, but not for FcγRIIa.

FcγRIII (CD16) molecules primarily bind complexed of aggregated IgG and consist of 2 isoforms. FcγRIIIa is expressed on monocytes, macrophages, natural killer cells, and (some) T cells, and is expressed on cells in association with ITAM-containing signaling chains, such

as the FcR γ, β, or ζ chains[14]. Human FcγRIIIb represents a glycosyl-phosphatidylinositol (GPI)-linked molecule, and is expressed on neutrophils. Mouse FcγRIII is homologous to human FcγRIIIa[17]. Recently, an FcγRIV molecule has been described in mice, which has high homology with human FcγRIIIa[17,25]. The cell distribution of mouse FcγRIV differs from human FcγRIIIa, as mouse FcγRIV is expressed on all myeloid cells, including neutrophils, and is not expressed on lymphocytes.

Fc receptors are now known to be important for Ab therapeutic approaches of cancer. This was shown in murine models, in which the therapeutic effect of anti-tumor Ab was abolished in mice that lack activatory FcγR[24]. A role for Fc receptors in Ab therapy has also been observed in patients, where the therapeutic efficacy of rituximab was linked to inter-individual differences (polymorphisms) in FcγRIIa and FcγRIIIa[26,27].

IgA RECEPTORS

IgA is produced in higher quantities per day than all other Ab classes combined[28]. IgA constitutes the most prominent Ab class in mucosal secretions, where it is found as secretory IgA (SIgA), and the second most prevalent Ab class in blood, where it is known as serum IgA[29]. SIgA does not trigger inflammatory responses after binding to antigens, which is beneficial for the body, as immune responses in the gut against food antigens would lead to chronic inflammation[29]. The main functions of SIgA include prevention of invasion of micro-organisms from the gut towards the blood, neutralisation of bacterial toxins, and inhibition of bacterial adherence to the mucosal wall[30].

Binding of pathogens to serum IgA, however, induces activation of immune cells via triggering of an IgA receptor (FcαRI, CD89)[29]. Activation of FcαRI induces a plethora of functions, including endocytosis, phagocytosis, ADCC, release of oxygen radicals, cytokine production, and antigen presentation (for a detailed review, see **Chapter 2**). FcαRI is selectively expressed on myeloid cells, including monocytes, macrophages, neutrophils, eosinophiles, and dendritic cells[31]. A murine homologue for FcαRI has not been identified, but human FcαRI-transgenic mice have been made, in which FcαRI expression mirrors the cell distribution in humans[32]. These transgenic mice enabled the study of FcαRI function *in vivo*, and showed that FcαRI cell surface expression and function were dependent on association of the receptor with the common ITAM-bearing FcR γ chain[32]. The role of FcαRI in tumor therapy has been studied, as well. Triggering of neutrophil FcαRI effectively induced tumor cell lysis, *in vitro*[33,34]. Furthermore, although anti-CD20 IgG mAb were unable to trigger neutrophil-mediated lysis of CD20-positive B cell lymphoma cells, targeting of neutrophil FcαRI was effective[35]. FcαRI has thus been suggested to represent an attractive candidate target for Ab-therapy.

SCOPE OF THESIS

A review of the biological function of FcαRI is included in **Chapter 2**. In **Chapter 3,** FcαRI and FcγRI-mediated functions on neutrophils were compared. The differences between both receptors were addressed in more detail in **Chapters 4 and 5**. The capacity of FcαRI to present antigens and to serve as target for dendritic cell-based immunotherapy was evaluated in **Chapter 6**. Limitations of mouse tumor models are discussed in **Chapter 7**. In **Chapter 8**, the potential of anti-tumor Ab to prevent development of liver metastasis, and the underlying therapeutic mechanisms were assessed in various knockout mouse strains. **Chapter 9** contains a summarizing discussion, and presents a perspective for future Ab treatments of cancer.

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Chapter 2

The Fc receptor for IgA (FcαRI, CD89)

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ABSTRACT

Traditionally IgA has been regarded as a non-inflammatory antibody, which inhibits adhesion of micro-organisms to the mucosal wall without initiation of inflammatory responses. Recently, however, a dichotomy has been suggested between the actions of secretory IgA (SIgA), which is present at mucosal sites, and serum IgA. SIgA exerts its function as first line of defense by limiting invasion of pathogens. Serum IgA in turn may be engaged in inflammatory responses after breaching of mucosal wall integrity. Several receptors for IgA have been described. However, the - as yet - best characterized prototypic Fc receptor for IgA, FcαRI (CD89), is the most likely candidate for initiation of inflammatory responses, as it binds poorly to SIgA, but vigorously triggers potent effector functions upon binding to serum IgA. Here, new insights in IgA-FcαRI binding are described and the functional implications of these interactions are discussed.

INTRODUCTION

Immunoglobulins (Ig) can - upon recognition of an antigen - bind via their Fc tail to Fc receptors, which are expressed on many different cell types including immune cells, hereby initiating potent effector mechanisms. Fc receptors for IgG (FcγR) and IgE (FcεR) have been studied extensively, but interest for IgA Fc receptors has only been emerging recently, even though IgA constitutes the main antibody (Ab) class at mucosal surfaces and is the second prevalent Ig in serum[1-3]. This seemingly lack of interest may be due to the traditional view of IgA as a non-inflammatory Ab. The functions of IgA at the mucosa - where it is expressed as secretory IgA (SIgA) - include inhibition of bacterial adherence to the mucosal wall, prevention of invasion of micro-organisms from the gut towards the blood and neutralization of bacterial toxins[4]. Binding of SIgA to antigens, however, does not trigger inflammatory responses, which is beneficial because immune responses against the abundance of food antigens and commensal bacteria at mucosal sites would put an individual at risk for development of chronic inflammation.

In recent years, however, it has become clear that IgA Fc receptors can initiate potent activatory responses, such as endocytosis, phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), antigen presentation and release of inflammatory mediators, which challenges the view of IgA as a non-inflammatory Ab. Several receptors for IgA - including the Fcα/μ receptor, asialoglycoprotein-receptor (ASGP-R), transferrin receptor (TfR, CD71), secretory component (SC) receptor, M cell receptor, and polymeric Ig receptor - have been described, which can bind to the Fc tail, IgA carbohydrate side chains or to accessory molecules such as the J-chain and SC[5-7]. However, the myeloid IgA Fc receptor, FcαRI (CD89), is the most thoroughly characterized receptor and the focus of this review.

IgA AND FcαRI

IgA

IgA is the most abundantly produced Ig (66 mg/kg/day) and constitutes the main Ab class at mucosal surfaces[8]. In serum, IgA represents the second prevalent Ig, even though levels are only a fifth of total Ab serum levels, which may be due to its short half-life (3-6 days) compared to IgG. In contrast to other Ig - which have Y-shaped structures - IgA exists as a T-shaped structure[9]. Two subclasses of IgA, IgA1 and IgA2, have been identified that differ by an additional 13 amino-acid sequence with O-linked glycosylation sites in the hinge region of IgA1[3,8]. This region is a target for at least two families of IgA1 bacterial proteases, whereas IgA2 is not susceptible to proteolysis by such proteases. No functional implications of this heterogeneity have yet been shown, although it might explain the predominance of IgA2 molecules at mucosal surfaces. Interestingly, *N. Meningitidis* proteinases were recently shown

to cleave off Fc fragments of IgA1, hereby impairing interactions of IgA to FcαR, which prevents killing of bacteria by effector cells[10]. In human serum, IgA molecules are mainly of the IgA1 subclass. Furthermore, here it consists mostly as monomeric form, which is in contrast with IgA in mucosal secretions where it is produced as dimer (dIgA) by local B-cells in the lamina propria. Dimerization is stimulated by linkage with an adjoining peptide, called J-chain[8]. Transport towards the apical surface of the epithelial cells, occurs through binding of dIgA to the polymeric Ig receptor (pIgR), which binds dIgA on the basolateral surface[5]. After trafficking through the cell, pIgR is proteolytically cleaved off on the apical surface, resulting in release of dIgA, which is still associated with the cleaved pIgR ectodomain called SC. The complex of dIgA and SC is referred to as secretory IgA (SIgA).

FcαRI

FcαRI is a transmembrane receptor expressed on cells of the myeloid lineage including neutrophils, monocytes, tonsilar, splenic, alveolar and liver macrophages (the latter also known as Kupffer cells), eosinophils, and subpopulations of dendritic cells (DC)[6,7,11-14]. A recent survey of FcαRI distribution revealed that most FcαRI-positive cells in tissues are neutrophils with high expression of FcαRI, whereas recently migrated monocytes show lower FcαRI expression[15]. Receptor size varies between 55-75 kDa on monocytes and neutrophils and 70-100 kDa on eosinophils due to differences in glycosylation, and expression was shown to be modulated by a number of substances[6,7]. Formyl-methionyl-leucyl-phenylalanine (FMLP), tumour necrosis factor-α (TNF-α) and interleukin (IL)-8 were shown to upregulate FcαRI expression on neutrophils, whereas receptor upregulation on monocytes was induced amongst others by lipopolysaccharide, TNF-α, IL-1β or granulocyte-macrophage colony stimulating factor (GM-CSF)[6,7]. Conversely, transforming growth factor-β, interferon-γ, suramin and the ligand IgA itself were shown to downregulate FcαRI expression[7,16].

FcαRI is composed of two extracellular Ig-like domains (206 amino acids total), which are orientated approximately 90° apart. Furthermore, it consists of a transmembrane region (19 amino acids) and a small cytoplasmic tail (41 amino acids). The receptor is a member of the multi-chain immune recognition receptor family and associates with the signaling FcR γ chain subunit, forming the trimer FcαRI/γγ[17]. FcR γ chain is also a component of IgG and IgE Fc receptor complexes[1,2,18]. However, FcαRI bears a positively charged Arg^{209} in its transmembrane region leading to an electrostatic interaction with a negatively charged amino acid in the transmembrane region of the FcR γ chain[19]. In contrast, IgG and IgE Fc receptors lack such a positively charged amino acid, which possibly results in a weaker association of these receptors with FcR γ chain compared to FcαRI.

The gene encoding FcαRI is located in the so-called leukocyte receptor cluster alongside members of a family of receptors, including natural killer cell inhibitory receptors, leukocyte Ig-like receptors, and LAIR-1 and -2, on chromosome 19q13.4 with whom it shows approximately 35% homology[20,21]. Although FcαRI is also closely related to a bovine IgG Fc recep-

tor (Fcγ2R), it is only distantly related to human FcγR and FcεR, which map on chromosome 1q32.3 (~20% homology)[22,23]. Up until now, no murine FcαRI equivalent has been found, but recently a bovine FcαRI was described, which shares a great degree of homology with human FcαRI and can bind both human as well as bovine IgA[24].

FcαRI is encoded by five exons[25]. The first exon S1 encodes the 5' untranslated region, the initiation codon and most of the signal peptide. S2 contains the second part of the signal peptide, whereas the extracellular domains are encoded by EC1 and EC2 (also referred to as D1 and D2). The last exon TM/C encodes the transmembrane region and the short cytoplasmic tail. cDNAs for several alternative splice variants have been described *in vitro*[26,27]. A shorter version of the full-length transcript lacking 66 base pairs from D2 has also been demonstrated *in vivo*, and is exclusively expressed on alveolar macrophages, although its function remains unclear[28]. A recent study, in which a D2-deficient splice variant to full length FcαRI ratio was compared in inflammatory responses showed differences in ratios in pneumonia patients compared to healthy individuals[29]. However, the biological relevance of this observation is not yet understood.

IgA binding to FcαRI

FcαRI is a low / medium affinity receptor for IgA (Ka ~10^6 M^{-1})[30]. Monomeric IgA binds poorly and rapid dissociation between IgA and receptor (t ½ ~ 25 seconds) suggests that monomeric IgA interacts only transiently. In contrast, polymeric IgA (pIgA) and IgA complexes bind avidly to FcαRI[30].

Even though FcαRI shares structural similarities with FcγRIII and FcεRI, binding of IgA to FcαRI is strikingly different compared to interactions of the abovementioned Fc receptors with their ligands, which bind their ligand via the membrane proximal extracellular Ig-like domain (D2) (Fig.1)[31,32]. In contrast, the ligand-binding site on FcαRI is located on the membrane distal D1 domain[30,33]. Additionally, binding of IgA to FcαRI occurs at the IgA C_H2-C_H3 interface, which differs from binding of IgG to FcγR (hinge-proximal site at the top of C_H2 domains) and IgE to FcεRI (top of C_H3)[34,35]. The FcαRI:IgA interface is composed of a central hydrophobic core flanked by charged residues. The basic nature of several residues on FcαRI, which can putatively interact with acidic residues on IgA, suggested that charge matching may play a role in binding. Mutation analyses of charged residues on IgA, however, showed little effect on the ability of IgA to interact with FcαRI, implying that electrostatic interactions are unlikely to play an important role in receptor-ligand binding[36,37].

Remarkably, IgA binds to FcαRI in an 'upright' orientation in contrast to IgG and IgE which are bound to FcγRIII and FcεRI respectively in an 'upside-down' orientation[31,38,39]. The observed difference is presumably a result of a more rigid conformation of IgA1 - due to heavily O-linked glycosylation in its hinge region -, which would render IgA1 insufficiently flexible to allow binding in the aforementioned 'upside-down' position[38].

Moreover, recent X-ray crystallographic studies confirmed the previous observed FcαRI:IgA 2:1 stoichiometry (Fig. 1)[37,38]. This is in contrast with FcγRIII:IgG and FcεRI:IgE binding, which have a 1:1 stoichiometry[31,39]. IgA molecules can bind either one or two FcαRI in solution[37]. Although IgA can bind two FcαRI simultaneously, binding of one IgA molecule to two FcαRI is presumably not sufficient to induce efficient cross-linking required for downstream signaling. A number of possible explanations have been offered[38]. Firstly, the distance between the C-termini of two FcαRI may be too far apart (~124Å) to register as a receptor dimerization event for triggering a signaling cascade. The latter may require close clustering of multiple receptors. Secondly, under physiological conditions the concentration of IgA in serum is 50- to 100- fold above the Kd for the FcαRI:IgA binding interaction, which would drive the equilibrium towards 1:1 complexes of FcαRI with IgA. Thirdly, FcαRI may be tethered to cytoskeletal elements, which may not be triggered to undergo the essential rearrangements compatible with receptor clustering.

Priming with cytokines may modulate interaction with the cytoskeleton. GM-CSF was able to stimulate rapid increase in the phagocytic capacity and affinity of FcαRI for IgA on neutrophils, without effecting FcαRI expression level[40]. Similarly, in eosinophils, stimulation with IL-4 and IL-5 led to increased ligand binding without affecting FcαRI expression level, suggesting that cytokine stimulation leads to altered receptor affinity or avidity[41]. Ser263 in the FcαRI α chain was demonstrated to be responsible for receptor modulation upon cytokine stimulation, as substition of this amino acid into alanin resulted in constitutive high affinity IgA binding[41]. Dephosphorilation of ser263 was postulated to result in disruption of cytoskeletal interaction allowing sufficient mobility of two FcαRI to enable binding to the same IgA

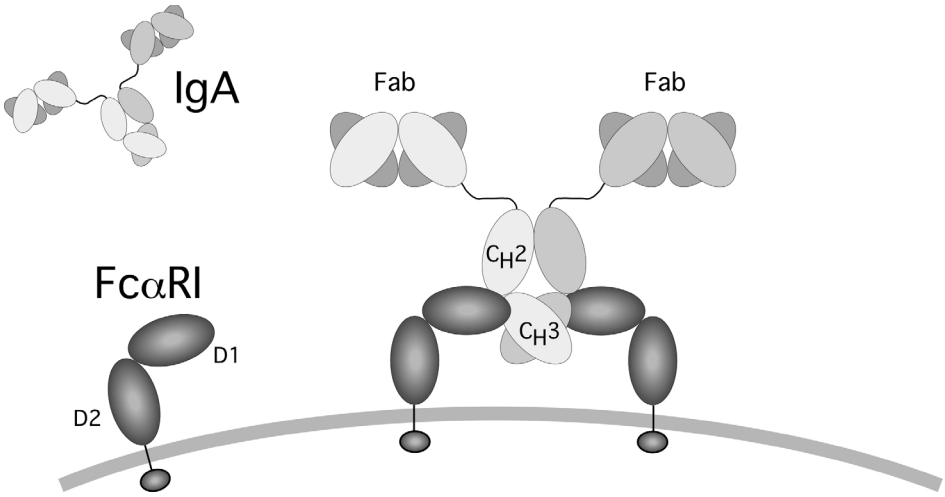

Figure 1. IgA binding to FcαRI. The two extracellular domains of FcαRI (D1 and D2) are orientated approximately 90° degrees apart. D1 contains the IgA- binding site, whereas the FcαRI binding site on IgA is located at the interspace of the constant region heavy chain 2 (C_H2) and C_H3 domains in the IgA Fc tail. IgA can bind to two FcαRI in an 'upright' position. *Figure provided by courtesy of Dr. A.B. Herr.*

molecule, hereby generating higher avidity interactions. The ligand binding α chain is thus not only involved in passive IgA binding, but inside-out signaling via the α chain is required for modulation of interaction between FcαRI and the cytoskeleton.

BIOLOGICAL FUNCTIONS OF FcαRI

FcαRI signaling

The cytoplasmic tail of FcαRI does not bear any known signaling motifs. Consequently, FcαRI is dependent on association with the FcR γ chain for signaling and function (Fig. 2)[17]. FcR γ chains bear an immunoreceptor tyrosine (Tyr)-based activation motif (ITAM) in their cytoplas-

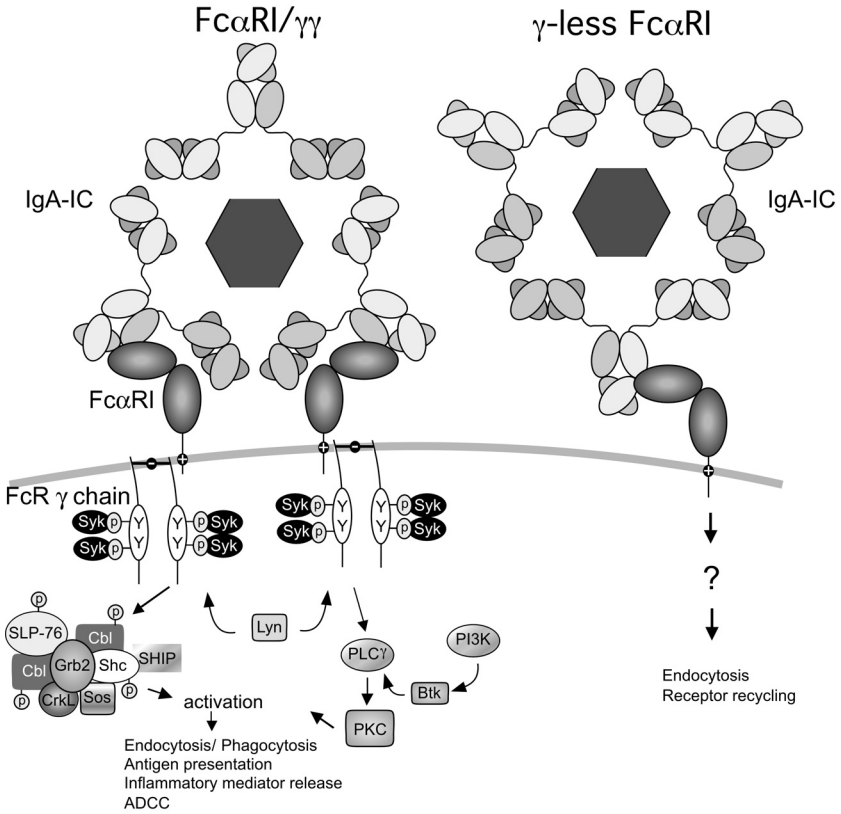

Figure 2. Signaling of FcαRI upon ligand binding. IgA immune complexes (IgA-IC) can cross-link FcαRI, hereby triggering signaling events via FcR γ chains, which are in complex with FcαRI via an electrostatic interaction. The tyrosines (Y) of ITAM motifs in the cytoplasmic tail of FcR γ chains are phosphorylated (p) by $p56^{lyn}$ (Lyn). Next, $p72^{syk}$ (Syk) associates with phosphorylated Y, followed by modulation of a multimolecular adaptor complex (Cbl, SLP-76, Grb2, CrkL, Shc, Sos, SHIP). In addition, phospholipase Cγ2 (PLCγ) and phosphoinositide 3-kinase (PI3K) are recruited, Btk is phosporylated, and activation of serine/threonine kinases like protein kinase C (PKC) is observed. Activation via FcαRI leads to effector functions, such as endocytosis, phagocytosis, antigen presentation, release of inflammatory mediators and ADCC. In the absence of FcR γ chain, IgA-IC binding to FcαRI leads to initiation of an unknown signaling cascade, resulting in endocytosis and recycling of the FcαRI-IgA complex.

mic regions - consisting of two Tyr-containing 'YxxL' boxes interspaced by 7 amino acids. The ITAM is essential for initiation of activatory signals as mutation of either of the Tyr was shown to reduce or abrogate signaling[42]. Cross-linking of FcαRI was shown to induce relocation of FcαRI into lipid rafts, which are rich in signaling molecules - such as the Src protein Tyr kinase (PTK) $p56^{lyn}$ - and are proposed to function as signaling platforms on the cell membrane[43]. Upon FcαRI triggering, the Tyr in the FcR γ chain ITAM are phosphorylated by $p56^{lyn}$, followed by $p72^{syk}$ association with phosphorylated ITAM and modulation of a multimolecular adapter complex (containing Cbl, Shc, SHIP, Grb2, SOS, SLP-72, CrkL)[44-46]. Additionally, both phosphoinositide kinases - such as phosphoinositide 3-kinase (PI 3-kinase) and phospholipase Cγ2 -, and serine/threonine kinases like protein kinase C (PKC) α, PKCε, and protein kinase B α were recruited, suggesting that lipid rafts recruit multiple classes of signaling molecules initiated by FcαRI cross-linking[47]. FcαRI cross-linking triggers calcium release from intracellular stores in neutrophils, and induction of NADPH oxidase activity that is sensitive to inhibition by PI 3-kinase inhibitors[48,49].

FcαRI expression on monocytes and neutrophils has also been observed in absence of FcR γ chain association, the so-called γ-less FcαRI, which suggests that FcαRI may associate with another - yet uncharacterized - molecule[50,51]. γ-less FcαRI can endocytose and recycle bound IgA, which may prolong IgA half-life (Fig. 2). As binding of IgA to FcαRI is pH dependent, trafficking towards the late endosome (pH ~ 5.0) leads to a 60-70 fold reduction in the affinity of IgA for FcαRI/ γγ, and IgA dissociates from the receptor leading to degradation. In contrast, γ-less FcαRI traffics towards the early sorting endosomes (pH ~ 6.0), where affinity is only four to five fold reduced. Therefore, a higher proportion of IgA remains bound to FcαRI, which is subsequently recycled back to the cell surface[37]. Notably, expression of FcαRI was completely absent in FcαRI transgenic (Tg) mice that were crossed with FcR γ chain-deficient mice, supporting the importance of FcR γ chain for FcαRI function[52]. Species-specific differences may underlie the observed discrepancy between these observations.

On monocytes, cross-linking of FcαRI can result in shedding of FcαRI. Two soluble forms of FcαRI (sFcαRI) have been identified. One form is generated by FcR γ-chain dependent proteolytic cleavage, leading to the formation of a 30 kDa soluble product[53]. A second soluble receptor, with a size of 50-70 kDa, was identified in IgA nephropathy (IgAN) patients[54]. sFcαRI were shown to associate with pIgA, thereby creating IgA-FcαRI complexes in the circulation[55].

A role for the β_2 integrin Mac-1 (CR3; CD11b/CD18) in FcαRI function has been implicated as well[52]. Mac-1 deficient ($Mac\text{-}1^{-/-}$) neutrophils were unable to induce ADCC via FcαRI, which is presumably a result of inability to spread on antibody-coated tumor targets, leading to impaired immunological synapse formation between FcαRI/$Mac\text{-}1^{-/-}$ neutrophils and tumor cells. Extracellular lysis may, therefore, be absent due to abnormal interactions between $Mac\text{-}1^{-/-}$ effector cells and target cells[56]. Mac-1 was furthermore required for binding of SIgA to neutrophil FcαRI and as such functions as a novel accessory protein[57].

Effector functions of FcαRI

The putative function of IgA receptors has been a subject of debate in literature. Earlier data demonstrating suppression of neutrophil chemotaxis by human myeloma IgA, and failure of IgA to induce phagocytosis by human neutrophils suggested an anti-inflammatory function of FcαRI[58]. This was accentuated by the poor ability of IgA in complement activation[8]. Conversely, IgA anti-yeast Ab proved more efficient than IgG Ab at triggering phagocytosis, and ability of IgA to induce phagocytosis has now been confirmed by several laboratories[12,40,52,59,60]. In recent years, data supporting the capacity of FcαRI to trigger a plethora of inflammatory functions - including ADCC, endocytosis, phagocytosis, generation of superoxide radicals, cytokine production and release of inflammatory mediators - has been accumulating, pointing to an activatory role (for review, see refs. 6, 7).

Most cellular functions triggered via FcαRI depend on tyrosine kinase activity of FcR γ chain – with the exception of endocytosis since no difference in endocytosis rate between FcαRI/γγ complexes or γ-less FcαRI was observed[19,50,52]. A recent study describing antigen presenting capacity of FcαRI confirmed that FcR γ chain was not required for endocytosis of IgA-ovalbumin complexes[61]. However transport of ligated FcαRI to lamp-1$^+$ late endocytic compartments, remodeling and activation of these compartments, as well as degradation of IgA complexes and efficient antigen presentation proved dependent on association with FcR γ chain[61].

Although FcαRI mediates phagocytosis of beads, bacteria, and yeast particles by unstimulated neutrophils, phagocytic capacity of neutrophils or monocytes is enhanced by priming with cytokines like GM-CSF and IL-8, or IL-1, TNF-α and GM-CSF, respectively[6,40,59,62]. For induction of eosinophil-mediated phagocytosis of IgA-coated beads, priming with GM-CSF, IL-4, or IL-5 is essential[63]. In contrast to serum IgA, SIgA was shown incapable of triggering phagocytosis by either neutrophils *in vitro* or Kupffer cells *in vivo*, which is presumably due to (partial) blockage of the FcαRI-binding site on IgA by SC[12,35,38].

SIgA is, however, capable of initiating respiratory burst activity by neutrophils - albeit less efficient than serum IgA -, which was shown dependent on Mac-1, suggesting that SIgA is unable to bind or activate FcαRI alone without the integrin co-receptor[12,57,64]. SIgA is, furthermore, the most potent stimulus for induction of eosinophil degranulation, which is presumably also linked to expression of a unique receptor for SC on eosinophils[65,66].

FcαRI in immunity

Recently, low levels of FcαRI expression were observed on immature monocyte-derived dendritic cells (MoDC). Because *in vitro* cultured MoDC resemble interstitial DC - present beneath the epithelium, within the parenchyma of organs, and in blood -, these observations may be relevant for the *in vivo* situation, although functional implications are not yet completely clear[13,14]. One study reported internalization of serum IgA after binding to FcαRI, which was accompanied by maturation of MoDC and production of IL-10, thus suggesting a

role in mounting specific immune responses against mucosa-derived antigens[13]. A second study, which confirmed low level expression of FcαRI on immature MoDC, however, reported more efficient uptake of SIgA, compared to serum IgA[14]. Importantly, uptake of SIgA was not accompanied by signs of DC maturation. Moreover, binding of SIgA by MoDC was partially blocked by anti-mannose receptor Ab, but could not be blocked by anti-FcαRI blocking Abs, which indicate that SIgA does not interact with MoDC via FcαRI, but via carbohydrate-recognizing receptors like the mannose receptor. Mucosal DC were shown to open tight junctions between epithelial cells and send their dendrites outside the epithelium to sample the gut lumen[67]. Interactions with SIgA might contribute to this sampling process and suggests that internalization of SIgA by DC may play a role in maintaining self-tolerance against commensal bacteria[14].

A similar dichotomy between the actions of serum IgA and SIgA was previously described (Fig. 3). SIgA, - although important in maintaining mucosal integrity by inhibiting adherence of pathogenic micro-organisms to the mucosal wall - is unable to initiate phagocytosis via FcαRI, which is consistent with its proposed 'anti-inflammatory' character[5]. This is supported by the fact that intestinal macrophages lack FcαRI expression[68]. Nonetheless, FcαRI has been implicated in mucosal defense as well. Kupffer cells, which are the macrophages of the liver and essential for elimination of invasive bacteria that have entered via the gut, were shown

Figure 3A

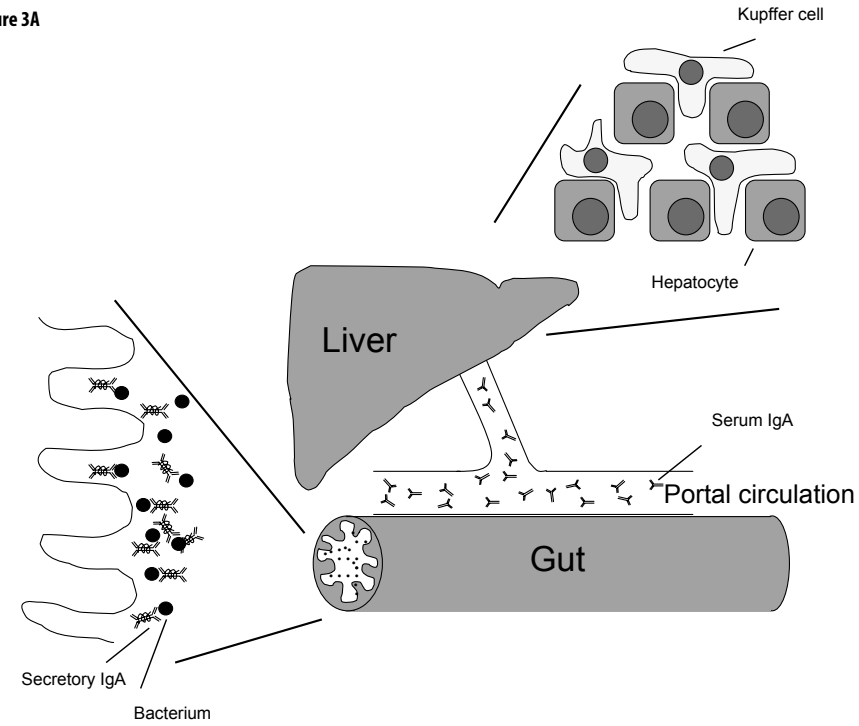

Figure 3B

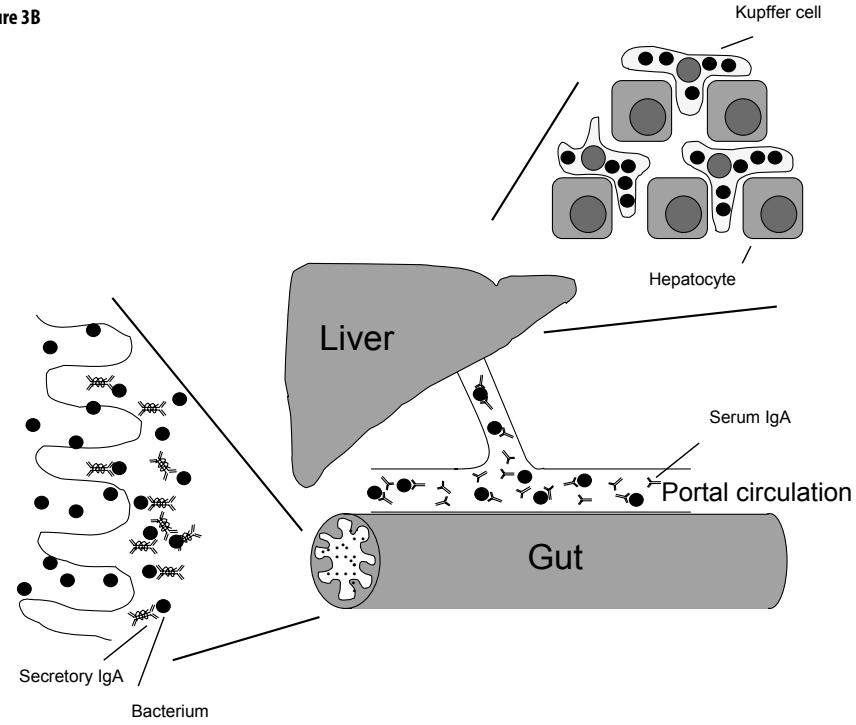

Figure 3. The putative role of IgA and FcαRI in mucosal immunity. A) Under physiological conditions, secretory IgA inhibits invasion of pathogens into the mucosal wall as first line of defense, without activating inflammatory responses. B) Under pathological conditions pathogens can invade the portal circulation due to disruption of the mucosal barrier. Concomitantly produced inflammatory cytokines induce FcαRI expression on Kupffer cells in the liver. FcαRI-positive Kupffer cells can subsequently phagocytose pathogens that have entered the circulation – which are thus exposed to serum IgA- hereby preventing septicaemia and disease.

to express FcαRI after stimulation with inflammatory mediators[12]. Additionally, Kupffer cells of FcαRI Tg mice vigorously ingested serum IgA-, but not SIgA- opsonized *E. coli* bacteria upon injection in the bloodstream. These results suggest that in the absence of inflammatory processes, SIgA serves as an anti-septic coat, thereby preventing invasion of micro-organisms (Fig. 3). However, under pathological circumstances - characterized by a disruption of the barrier in the gastro-intestinal tract and concomitantly produced cytokines - Kupffer cells express FcαRI. Bacteria that have invaded from the gastro-intestinal tract into the portal circulation (and are thus exposed to serum IgA), are subsequently phagocytosed by FcαRI-positive Kupffer cells, thereby preventing septicaemia and disease. It is thus proposed that FcαRI-serum IgA interactions on Kupffer cells provide a second line of defense at the interface of mucosal and systemic immunity, which designates serum IgA as inflammatory antibody (Fig. 3). Additionally, targeting FcαRI with reagents recognizing *B. Pertussis* was shown to result in enhanced bacterial clearance in lungs of Tg mice after challenge with *B. Pertussis*[69].

FcαRI AND DISEASE

Involvement of FcαRI in disease

IgA-mediated diseases are usually associated with higher IgA serum levels, which can lead to deposition of IgA complexes in tissues with concomitant tissue damage. Because impairment in endocytic capacity of FcαRI may lead to diminished removal of IgA complexes from the blood, a role for FcαRI has been implicated[70]. It was also observed that increased serum IgA levels can result in shedding of FcαRI from immune cells leading to deposition of FcαRI-IgA complexes into tissues[54]. Furthermore, polymorphisms in the promoter region of the FcαRI gene have been identified in patients with IgAN[71].

IgAN represents one of the most commonly known diseases in which IgA immune complex deposits are responsible for kidney damage, although the origin of the disease is still incompletely understood. FcαRI Tg mice were shown to spontaneously develop IgAN due to interaction of the human FcαRI with mouse pIgA, hereby implying a role for FcαRI[72]. Development of IgAN was transferable by infusion of sFcαRI-IgA complexes from FcαRI Tg mice to wild-type recipients[54]. If FcαRI Tg mice were crossed with SCID mice (which lack IgA), disease did not develop unless IgA from IgAN patients was injected. Importantly, infusion of IgA from healthy donors did not induce disease, suggesting that altered IgA composition in patients contributed to disease as well. This notion is strengthened by the observation that IgAN patients show altered O-glycosylation in the hinge region of IgA compared to healthy donors[73]. Quantity of sFcαRI-IgA complexes, however, was not found to differ between IgAN patients and healthy individuals[74]. Recently, overexpression of CD71 has been shown in IgAN patients, which may contribute to pathophysiology of disease as well[75].

Allergic patients and patients with gram-negative bacteremia displayed increased expression levels of FcαRI, but it is not yet understood whether higher FcαRI expression has a harmful or protective role in these diseases[76,77].

FcαRI and antibody immunotherapy

Although antibody-based immunotherapeutic approaches generally involve recruitment of IgG Fc receptors, IgA Ab have also been identified as potential candidates for immunotherapy of malignant diseases, as FcαRI was shown to efficiently induce ADCC against tumor cells by neutrophils and macrophages[78-80]. In fact, FcαRI proved to represent the most effective Fc receptor on neutrophils for the induction of tumor cell killing[81-83]. Addition of IgA anti-tumor Ab or bispecific antibodies (BsAb) targeting FcαRI and tumor antigens to a 3-dimensional tumor cell culture in collagen gel, resulted in massive neutrophil infiltration into tumor colonies with concomitant destruction of tumor cells. Addition of IgG anti-tumor Ab led to minimal neutrophil accumulation, whereas BsAb targeting FcγRI and tumor cells were completely inefficacious. Moreover, release of inflammatory mediators such as IL-8, TNF-α and IL-1β was observed only after targeting to FcαRI, but not to FcγR (own unpublished data).

Recently, a receptor for IgA on natural killer (NK) cells was described as well[84]. Because NK cells are considered one of the most potent effector cell populations for the induction of tumor cell killing, this observation may have important implications for devising IgA-based immunotherapeutic approaches[85]. Since MY43 - a blocking anti-FcαRI Ab - was unable to inhibit IgA binding to NK cells, the aforementioned IgA receptor presumably does not represent FcαRI, although it cannot yet be excluded that it is a FcαRI splice variant. Furthermore, higher binding of SIgA than serum IgA was observed, again supporting the expression of an alternative IgA receptor. The presence of Fcα/μ receptor, SC receptor, CD71, and ASGP-R has not yet been addressed, but carbohydrate-recognizing receptors - like the mannose receptor -, were excluded as possibility[84]. Taken together, although the identity of the IgA receptor on NK is presently not characterized, it appears unlikely that it represents FcαRI. With respect to immunotherapy, treatment with IgA Ab – which can bind both FcαRI and the NK IgA receptor – may thus be more effective than FcαRI BsAb therapy.

CONCLUDING REMARKS

Although the function of IgA in immunological responses is not yet completely elucidated, it has become clear that its role should be considered as a dual concept. SIgA has more anti-inflammatory properties, which include prevention of pathogen invasion but may also involve induction of tolerance via interactions with DC. Conversely, serum IgA should be defined as inflammatory Ab through interactions with FcαRI on effector immune cells. The recently solved crystal structure of the IgA-FcαRI complex emphasizes the unique characteristics of FcαRI within the Fc receptor family, and warrants further study to the complicated role of IgA and FcαRI in immunity.

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Chapter 3

Immature neutrophils mediate tumor cell killing via IgA- but not IgG Fc receptors

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ABSTRACT

Anti-tumor antibodies (Ab) are promising therapeutics for cancer. Currently, most Ab-based therapies focus on IgG Ab, which interact with IgG Fc receptors (FcγR) on effector cells. In this study, we examined human and mouse neutrophil-mediated tumor cell lysis via targeting the IgA Fc receptor, FcαRI (CD89), in more detail. FcαRI was the most effective Fc receptor in triggering tumor cell killing, and initiated enhanced migration of neutrophils into tumor colonies. Importantly, immature neutrophils that are mobilized from the bone marrow upon G-CSF treatment efficiently triggered tumor cell lysis via FcαRI, but proved incapable of initiating tumor cell killing via FcγR. This may provide a rationale for the disappointing results observed in some earlier clinical trials in which patients were treated with G-CSF and anti-tumor Ab targeting FcγR.

INTRODUCTION

Over the last few years, therapeutic monoclonal Ab (mAb) have been acknowledged as promising drugs for cancer treatment[1]. In this approach, tumor cells are linked via anti-tumor mAb to Fc receptors on immune cells, which leads to tumor cell killing. Clinical studies demonstrated encouraging results in the treatment of malignancies, provided mAb were directed at appropriate tumor antigens, and a number of anti-tumor mAb have now been approved for cancer therapy by the Food and Drug Administration[2].

As yet, it remains unclear how mAb exert their anti-tumor properties. Therapeutic mAb may cross-link antigens on tumor cells, leading to pro-apoptotic, or anti-proliferative effects[1]. Additionally, Fc receptor mediated effector functions by immune cells, like phagocytosis, enhanced presentation of tumor antigens, and antibody-dependent cellular cytotoxicity (ADCC), may contribute to therapeutic efficacy of mAb, as protection against tumor growth was abrogated in FcR γ chain-deficient mice[3,4]. ADCC has been well-documented for monocytes/macrophages, as well as for natural killer cells[5,6]. Furthermore, neutrophilic granulocytes (neutrophils) have also been shown to exert ADCC[7].

Until now, neutrophils received relatively little attention as effector cells for Ab-therapy, despite their well-documented anti-tumor properties[8]. *In vitro*, neutrophils have been shown to exert potent cytolytic capacity against a variety of tumor cells in the presence of anti-tumor mAb, and *in vivo* studies support a role for neutrophils in tumor rejection[7,9,10]. It has been furthermore demonstrated that neutrophils can induce Ab-dependent apoptosis in human breast cancer cells[11]. Additionally, neutrophils represent the most populous FcγR-expressing leukocyte subset within the blood, and their numbers can be increased by treatment with granulocyte-colony stimulating factor (G-CSF)[12]. Moreover, activated neutrophils can secrete a plethora of inflammatory mediators and chemokines, like macrophage inflammatory proteins, monocyte chemoattractant proteins, and interleukin-8 (IL-8), hereby attracting other immune cells such as monocytes, dendritic cells and T cells, which may lead to generalized anti-tumor immune responses[13,14]. Neutrophils may thus represent an attractive effector cell population for antibody therapy.

All approved therapeutic mAb are of the IgG isotype, which can interact with IgG Fc receptors (FcγR)[2]. FcγR are widely expressed on a number of cells, including non-cytotoxic cells such as platelets, B cells, and endothelial cells. Binding of IgG to such cells may act as an antibody "sink". In addition, binding of IgG to the inhibitory Fc receptor, FcγRIIb, might lead to down-regulation of immune responses[3,15]. To overcome some of these problems, attempts have been made to selectively target activatory Fc receptors via bispecific antibodies (BsAb), recognizing both the Fc receptor and tumor antigen of interest. Neutrophils constitutively express the low affinity FcγRIIa (CD32) and FcγRIIIb (CD16) subclasses[15]. Additionally, stimulation of neutrophils with G-CSF (or interferon-γ; IFNγ) induces expression of the high affinity FcγRI (CD64), which represents the predominant cytotoxic FcγR on neutrophils[16]. These

data stimulated the evaluation of a combined therapy of G-CSF and FcγRI-specific BsAb in a number of clinical trials[17-19]. These trials, however, only showed limited therapeutic effects, indicating that improvement of neutrophil-mediated Ab-therapy is required.

Recently, the IgA Fc receptor (FcαRI, CD89) has been identified as candidate target for tumor therapy[20,21]. FcαRI is constitutively expressed on myeloid effector cells, including neutrophils, monocytes, macrophages, eosinophils and dendritic cells, but not on non-cytolytic cell populations. Furthermore, FcαRI can potently trigger effector functions like oxidative burst, cytokine release and phagocytosis, and has been documented as a potent trigger molecule on neutrophils for tumor cell lysis[22,23]. Notably, targeting FcαRI was able to overcome the "antigen restriction" observed in neutrophil-mediated ADCC with IgG mAb against the B cell lymphoma tumor antigen CD20[24]. FcαRI may thus represent an attractive alternative for neutrophil-mediated Ab-therapy. Its potential as target molecule for the initiation of tumor cell killing was therefore addressed in detail in the present study.

MATERIALS AND METHODS

Blood and bone marrow donors.

Studies were approved by the Medical Ethical Committee of Utrecht University (The Netherlands), in accordance with the Declaration of Helsinki. A peripheral blood sample (30 ml) was drawn from healthy untreated volunteers or healthy donors receiving rhG-CSF (Neupogen, 5 μg/kg of body weight, twice daily for five days, Amgen Inc., Thousand Oaks, CA), respectively. Bone marrow samples were obtained from cardiac patients undergoing surgery. All donors gave informed consent.

Transgenic mice.

Generation of FcαRIxFcγRI double transgenic (Tg) mice was described earlier[25]. To induce FcγRI expression on neutrophils and increase neutrophil counts in blood, mice were injected subcutaneous with 15 μg pegylated G-CSF (kindly provided by Amgen Inc., Thousand Oaks, CA) in 150 μl PBS three days before blood collection. Mice were bred and maintained at the Central Animal Facility of the Utrecht University, The Netherlands. All experiments were performed according to institutional and national guidelines.

Cell lines.

The breast carcinoma cell line SK-BR-3, which over-expresses the proto-oncogene product HER-2/*neu*, and the malignant B-cell lymphoma RAJI (Burkitt's lymphoma), were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated FCS and antibiotics (RPMI/10%). SK-BR-3 cells were harvested using trypsin-EDTA (Gibco BRL).

Antibodies and flow cytometry.

Antibodies A77 (mIgG1 anti-FcαRI), m22 (mIgG1 anti-FcγRI) and 520C9 (mIgG1 anti-Her-2/*neu*) were produced from hybridomas (Medarex, Bloomsbury, NJ). F3.3 (mIgG1 anti-HLA class II) producing hybridomas were obtained from the Tenovus Research Laboratory (University of Southampton, UK). Chimeric human/mouse antibodies were generated as previously described[26].

FcγRIxHER-2/*neu* BsAb (22x520C9; MDX-H210) was obtained from Medarex. FcαRIxHER-2/*neu* BsAb (A77x520C9) and FcαRIxHLA class II BsAb (A77xF3.3) were produced by chemically cross-linking F(ab') fragments of 520C9 or F3.3 mAb with F(ab') fragments of FcαRI-specific mAb A77 as described[27].

Surface expression of FcαRI and FcγRI on neutrophils (2 x 10^5 cells or 25 μl blood) was determined with mAb A77 (FcαRI) or m22 (FcγRI), respectively (10 μg/ml), followed by incubation with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG Ab (Southern Biotech Association; SBA, Birmingham, AL). Percentage of neutrophils in blood and bone marrow was determined with PE-conjugated anti-mouse GR-1 mAb (Becton Dickinson Biosciences, Franklin Lakes, NJ) or FITC-conjugated anti-human CD66b mAb (Serotec, Oxford, UK). Maturation status of isolated human bone marrow cells was measured with FITC-conjugated anti-CD11b mAb (Immunotech, Marseille, France) and PE-conjugated anti-CD16 mAb (Becton Dickinson Biosciences,) as described previously[28]. Cells were analyzed on a FACScan (Becton Dickinson Biosciences).

Isolation of neutrophil effector cells.

Neutrophils were isolated from heparin anti-coagulated peripheral blood samples by standard Ficoll-Histopaque (Sigma-Aldrich, Steinheim, Germany) density gradient centrifugation. Neutrophils isolated from G-CSF treated donors were used directly after isolation, whereas neutrophils from healthy untreated volunteers were cultured overnight at 37°C with IFNγ (300 units/ml; Boehringer Ingelheim, Alkmaar, The Netherlands) to induce FcγRI expression.

Bone marrow neutrophils were isolated as described previously[29]. Bone marrow samples were incubated on ice with a lysis solution of pH 7.4 (0.16M ammonium-chloride, 0.01M potassium bicarbonate and 0.1mM sodium-edetate) for 5 minutes to remove erythrocytes, after which cells were incubated for 1 hour in RPMI/10% at 37°C. Non-adherent cells were harvested and neutrophils were separated by discontinuous percoll gradient centrifugation (successively 81%, 62%, 55%, 50% and 45% of percoll). Percoll layer 1 and 5 in the gradient contained non-myeloid cells, lipids, cellular debris and erythrocytes, respectively. Percoll layer 2, 3 and 4 (hereafter labeled as P2, P3 and P4) comprised different neutrophil maturation stages. Neutrophils from bone marrow samples were used directly after isolation.

Chromium release assay.

^{51}Cr release assays were performed as described earlier[30]. Briefly, 1 x 10^6 target cells were incubated with 100 μCi of ^{51}Cr (Amersham, Little Chalfont, UK) for 2 hours at 37°C and washed three times. ^{51}Cr-labeled target cells were plated in 96-well round-bottom microtiter plates (5 x 10^3 cells/well) in the absence or presence of different concentrations of BsAb or mAb and RPMI/10% containing 4 x 10^5 or 2 x 10^5 neutrophils (E:T ratio of 80:1 or 40:1) per well, respectively. After 4 hours at 37°C, ^{51}Cr release in the supernatant was measured as counts per minute (cpm). Percentage of lysis of tumor cells was calculated as follows: (experimental cpm–basal cpm)/(maximal cpm-basal cpm)x100%.

Real time video recording assay.

SK-BR-3 cells (5 x 10^4) were cultured for 2 days in RPMI/10% in a 24-wells plate. Next, neutrophils (5 x 10^5) were added together with BsAb (1 μg/ml) and video recording was performed for 2 hours with an inverted phase-contrast microscope (Nikon Eclipse TE300) in a humidified, 7% CO_2 gassed, temperature-controlled (37°C) chamber. A randomly selected field of 220 x 200 μm was recorded at a speed of 168 images per second using a color video camera (SONY, including a CMAD2 adapter) coupled to a time-lapse video recorder (SONY, SVT S3050P). Percentage of SK-BR-3 cells that had detached after 2 hours (indicative of cell death) was determined.

Collagen culture assay.

Collagen was isolated from rat tails and dissolved in 96% acetic acid (2 mg/ml). MilliQ, 0.34M NaOH and DMEM (10X) (Sigma, Taufkirchen, Germany) were mixed (1:1:1), after which 2.3 ml was added to 10 ml collagen and 1.3 ml SK-BR-3 cells (5 x 10^5/ml) on ice. This final mixture was plated in 24 wells plates (1 ml/well) and allowed to coagulate, after which 1ml RPMI/10% was added. Cultures were grown for two weeks to allow tumor colony formation, followed by addition of neutrophils in absence or presence of BsAb (0.5 or 1 μg/ml). After 24 hours, collagen gels were washed with 150mM NaAc pH 5.0 containing Indian ink (1:100) (30 minutes), fixed overnight at room temperature with zinc salts-based fixative (0.5 g/L calcium acetate, 5.0 g/L zinc acetate, 5.0 g/L zinc chloride in 0.1M Tris buffer)[31] and embedded in paraffin.

Immunohistochemistry.

Paraffin slides were de-paraffinised in ethanol and endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol (30 min, room temp). Non-specific binding was blocked by incubation with 10% normal rabbit serum (15 min, room temp). Neutrophils were stained with a mouse anti-human CD66b mAb (Pharmingen), followed by biotinylated rabbit anti-mouse IgM Ab (Zymed, San Francisco, CA) and horseradish peroxidase (HRP)-labeled Streptavidin (Zymed). 3.3-diaminobenzidine (DAB) was used as substrate (Sigma-Aldrich), resulting in a brown staining. Slides were counterstained with Mayers' haematoxyline (Klinipath, Duiven,

The Netherlands), after which they were embedded in Entallan (Merck, NJ). The number of neutrophils that migrated into tumor colonies, and the percentage of tumor colonies that contained more than one neutrophil, was quantified. Cytospins were stained with Diff-Quick according to manufacturers instructions (Dade Behring, Marburg, Germany).

Binding assay.

Neutrophils and SK-BR-3 cells were labeled with PKH-67, a FITC-fluorescent membrane marker, or PKH-26, a PE-fluorescent membrane marker, respectively, according to manufacturers instructions (Sigma-Aldrich). Labeled neutrophils and SK-BR-3 tumor cells were incubated with or without BsAb (1 μg/ml) for 30 min at 4°C in RPMI/10% at different Effector:Target (E:T) ratios. Binding was analyzed on a FACScan (Becton Dickinson Biosciences).

Calcium mobilization assay.

Neutrophils were labeled with SNARF-1 (2.8 μM) and Fluo-3 (1.4 μM) (Molecular Probes, OR) for 30 min at 37°C, after which cells were washed and incubated with anti-FcαRI (A77) or anti-FcγRI (m22) mAb (10 μg/ml) for 30 min at 4°C. Cells were washed twice and resuspended in calcium mobilizing buffer. Intracellular free calcium levels after cross-linking Fc receptors with $F(ab)'_2$ fragments of goat anti-mouse IgG1 Ab (SBA) were analyzed on a FACScan. The first 20 seconds of each run - prior to cross-linking - were used to establish baseline intracellular calcium levels.

MAPK phosphorylation assay.

Neutrophils were labeled with anti-FcαRI (A77) or anti-FcγRI (m22) mAb (10 μg/ml) for 30 min at 4°C. After washing, Fc receptors were cross-linked with $F(ab)'_2$ fragments of goat anti-mouse IgG1 Ab (SBA) at 37°C for different time points (varying between 0 to 60 seconds). Ice-cold PBS was added to stop reactions, after which samples were boiled in reducing sample buffer, run on 10% SDS-PAGE gels, and electro-transferred to nitrocellulose membranes (0.45 μm, Millipore Corporation, MA). Membranes were blocked with 5% bovine serum albumin (Roche Diagnostics, Mannheim, Germany) and probed with anti-phospho-p44/42 MAPK, or anti-total MAPK Ab for 2 hours (Cell Signaling Technology, MA). Following washing, membranes were further incubated for 1 hour with peroxidase-conjugated goat anti-rabbit Ab (Pierce Biotechnology, IL). Staining was visualized using the ECL detection system (Amersham).

Statistical analysis.

Data are shown as mean ± standard deviation (SD). Group data are shown as mean ± standard error of the mean (SEM). Statistical differences were determined using the two–tailed unpaired Student's *t*-test or ANOVA. Significance was accepted when $p < 0.05$.

RESULTS

Neutrophil ADCC.

Mature neutrophils express FcαRI, but only low levels of FcγRI (Ref. 16 and Fig. 1A). To compare FcαRI and FcγRI mediated Ab-therapy, neutrophils were therefore stimulated with IFNγ (IFNγ neutrophils), which enhanced FcγRI expression (Fig. 1B). Additionally, neutrophils from G-CSF stimulated healthy donors (G-CSF neutrophils) were collected, and FcαRI and FcγRI expression was assessed (Fig. 1C). IFNγ neutrophils, as well as G-CSF neutrophils from 64% of the donors (Fig. 1C, left plot), showed similar FcαRI and FcγRI expression levels. Neutrophils from 36% of G-CSF treated donors had higher FcγRI expression levels (Fig. 1C, right plot), resulting in a slight increase in overall FcγRI expression compared to FcαRI (Fig. 1D).

Levels of neutrophil-mediated tumor cell lysis varied greatly between donors. However, triggering of FcαRI on IFNγ neutrophils via FcαRIxHER-2/*neu* (A77x520C9) BsAb consistently resulted in higher SK-BR-3 tumor cell lysis, compared to FcγRI targeting (MDX-H210) (Fig. 1E). Mean tumor cell lysis in the presence of 1 μg/ml FcαRI or FcγRI BsAb was 94% ± 16% and 38% ± 11%, respectively (n=4). In ~75% of G-CSF donors, ADCC of SK-BR-3 tumor cells, mediated by G-CSF neutrophils, was higher via triggering FcαRI compared to FcγRI (Fig. 1F). Mean tumor cell lysis in the presence of 1 μg/ml BsAb was 52% ± 13 % and 26% ± 11% for targeting FcαRI and FcγRI, respectively (n=8). No difference in tumor cell lysis was observed using neutrophils from ~25% of G-CSF donors (targeting FcαRI or FcγRI resulted in 42% ± 17% or 46% ± 28% tumor cell lysis at 1 μg/ml BsAb, respectively; n=3). In the presence of low BsAb concentrations (0.08 μg/ml), neutrophil-mediated tumor cell killing was more efficient via FcγRI BsAb. Maximal FcγRI-mediated tumor cell lysis, however, never reached the levels that were obtained upon engagement of FcαRI. A further increase of BsAb concentration did not lead to higher levels of tumor cell killing, which is likely due to saturation of both neutrophils and tumor cells at high BsAb concentrations, hereby interfering with efficient neutrophils - tumor cell interactions. At low E:T ratios, neutrophils from all donors were less efficient in initiating tumor cell killing via FcγRI BsAb compared to FcαRI BsAb, including at low BsAb concentrations (data not shown). Cross-linking of neither FcαRI nor FcγRI by anti-Fc receptor mAb resulted in tumor cell killing, hereby ruling out any "bystander" killing of tumor cells as a result of neutrophil degranulation (data not shown)

We next examined whether the observed differences in tumor cell killing could be reproduced when other tumor antigens were targeted. We therefore examined FcαRI and FcγR mediated tumor cell killing of RAJI B-cell lymphoma cells that express HLA class II. Both anti-HLA class II (F3.3) IgA1 and IgA2 mAb triggered lysis of RAJI cells more efficiently than anti-HLA class II (F3.3) IgG1 mAb (Fig. 1G). Furthermore, FcαRIxHLA class II BsAb (A77xF3.3) was equally effective in mediating tumor cell killing as IgA mAb.

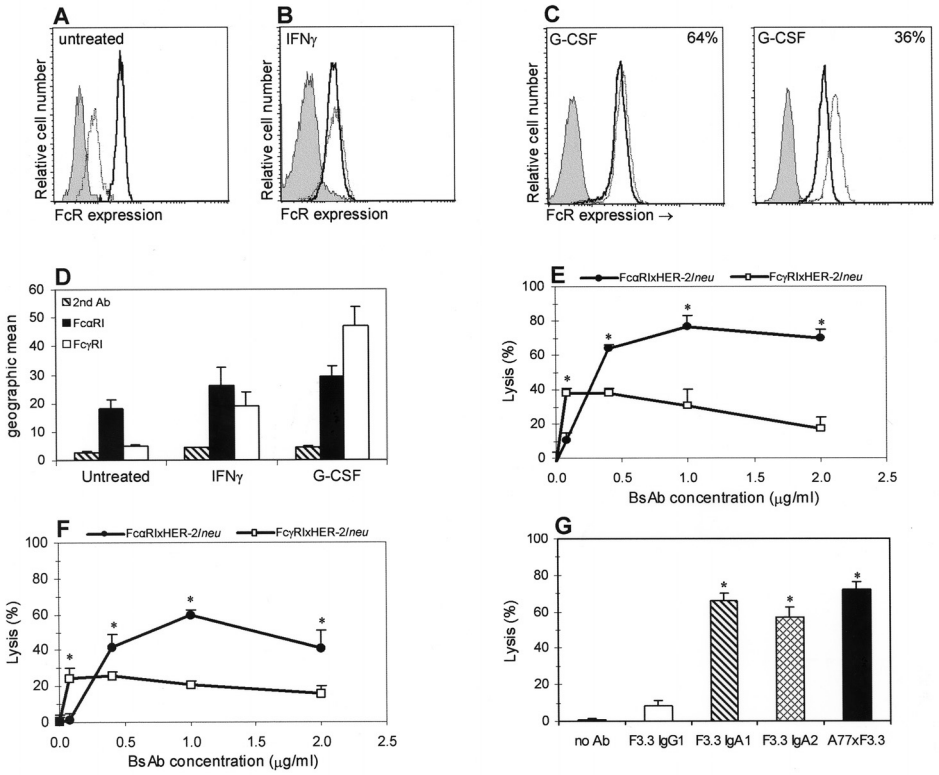

Figure 1. Neutrophil mediated ADCC of SK-BR-3 and RAJI cells. Surface expression of FcαRI (bold line) and FcγRI (thin line) determined by flow cytometry on isolated untreated (A), IFNγ (B), and G-CSF neutrophils (C). FcαRI and FcγRI expression level on G-CSF neutrophils was similar in 64% of donors (C, left plot). Neutrophils from 36% of G-CSF treated donors had slightly higher FcγRI expression compared to FcαRI expression (C, right plot). Neutrophils were stained with A77 (FcαRI) or m22 (FcγRI), followed by incubation with FITC-labeled goat anti-mouse IgG. The filled area represents secondary Ab only. (D) Mean Fluorescent Indexes (geographic mean ± SEM) of secondary Ab (hatched bar), FcαRI (black bar), and FcγRI (white bar) expression on untreated-, IFNγ-, and G-CSF- neutrophils. Lysis of SK-BR-3 tumor cells by IFNγ (E) or G-CSF neutrophils (F) in the presence of increasing concentrations of FcαRIxHER2/*neu* (A77x520C9, •), or FcγRIxHER2/*neu* (22x520C9, □) BsAb. Chromium release from triplicates was measured and data are expressed as mean ± SD. One representative experiment out of four or out of eleven is shown, respectively. (G) Lysis of RAJI cells by G-CSF neutrophils in the presence of anti-HLA class II (F3.3) IgG1 (white bar), IgA1 (hatched bar), IgA2 (cross-hatched bar) mAb, or FcαRIxHLA class II BsAb (A77xF3.3; black bar) (2 μg/ml). Data are presented as mean percentage lysis ± SEM from 6 individual experiments. * $p < 0.05$ compared to FcγR.

Real time video recording of neutrophil mediated tumor cell killing.

To visualize differences in neutrophil-mediated ADCC between FcαRI and FcγRI in time, a real time video recording assay was established. In the absence of BsAb, G-CSF neutrophils accumulated around SK-BR-3 cells, but were not activated (characterized by round shaped neutrophils). Furthermore, no tumor cell killing was observed (Fig. 2A, D, G). In the presence of FcγRIxHER-2/*neu* BsAb, as well as FcαRIxHER-2/*neu* BsAb, irregularly shaped neutrophils (reflecting activation) bound to SK-BR-3 cells. Minimal detachment of SK-BR-3 cells - indica-

tive of cell death[32] - was observed in the presence of FcγRIxHER-2/*neu* BsAb (Fig. 2B, E, G). Addition of FcαRIxHER-2/*neu* BsAb, however, resulted in significantly higher numbers of detached SK-BR-3 cells (Fig. 2C, F, G).

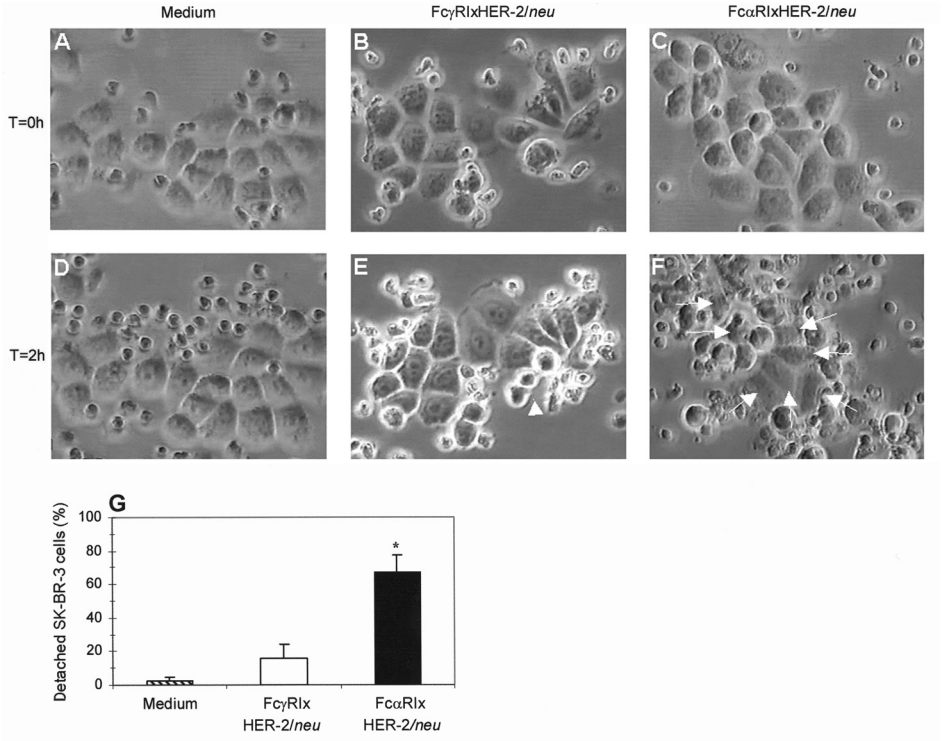

Figure 2. Real time video recording of BsAb-induced SK-BR-3 killing by neutrophils. G-CSF neutrophils (smaller cells) were added to adherently growing SK-BR-3 cells (larger cells) in the absence (A, D), or presence of 1 μg/ml FcγRIxHER-2/*neu* (B, E) or FcαRIxHER-2/*neu* (C, F) BsAb. Interactions between cells were recorded for 2 hours. Time-points 0 (A, B, C), and 2 hours (D, E, F) are shown. (E) SK-BR-3 cells that were detached in presence of FcγRIxHER-2/*neu* BsAb are indicated by arrowheads. (F) In the presence of FcαRIxHER-2/*neu* BsAb, high numbers of SK-BR-3 cells were detached. SK-BR-3 cells that were unaffected by neutrophils are indicated by arrows. (G) Percentage of SK-BR-3 cells that were detached by neutrophils in the absence (hatched bar), or presence of FcγRIxHER-2/*neu* (white bar) or FcαRIxHER-2/*neu* (black bar) BsAb after 2h. A representative experiment out of three is shown. Data represent mean ± SD. * $p < 0.05$ compared to FcγRIxHER-2/*neu* BsAb.

Neutrophil migration towards tumor colonies.

A 3-dimensional (3D) collagen culture assay was set up to study migration of G-CSF neutrophils in the presence of BsAb towards tumor cell colonies. Random neutrophil migration into collagen was present in the absence of BsAb, but no noticeable interactions with tumor cells were found (Fig. 3A, D). Although migration into tumor colonies was observed in presence of different concentrations of FcγRIxHER-2/*neu* BsAb (Fig. 3B, D, and data not shown), addition of FcαRIxHER-2/*neu* BsAb resulted in significantly higher neutrophil numbers that accumulated in and around SK-BR-3 tumor colonies (Fig. 3C, D). In addition, the percentage

of positive tumor colonies (containing more than one neutrophil) was also higher in presence of FcαRIxHER-2/*neu* BsAb, compared to FcγRIxHER-2/*neu* BsAb (89 ± 10%, compared to 45 ± 7%, respectively; n=3). Furthermore, only FcαRIxHER-2/*neu*, but not FcγRIxHER-2/*neu* BsAb induced tumor colony destruction (Fig. 3C).

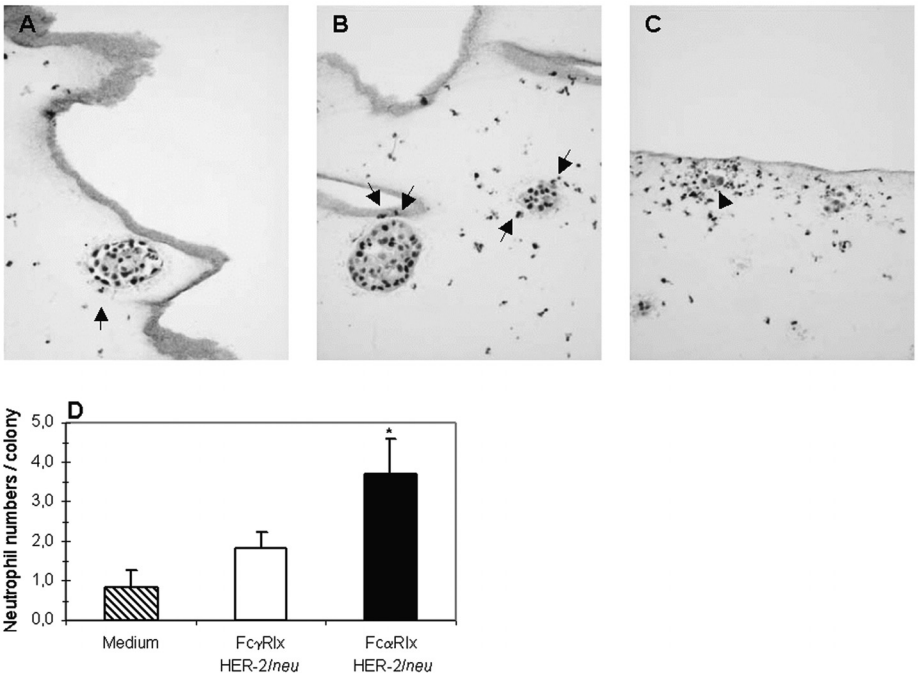

Figure 3. BsAb-induced neutrophil migration towards tumor colonies. For a full-color picture, see Appendix page 169. G-CSF neutrophils were added to SK-BR-3 tumor colonies in collagen, either in the absence (A), or presence of 0.5 μg/ml FcγRIxHER-2/*neu* (B) or FcαRIxHER-2/*neu* (C) BsAb. Collagen was fixed and slides were stained for CD66b (neutrophils, brown). Neutrophils attached to tumor colonies are indicated in A and B by arrows. In (C) remnants of a SK-BR-3 tumor colony are marked by an arrowhead. (D) Numbers of neutrophils per colony in the absence (hatched bar), or presence of FcγRIxHER-2/*neu* (white bar) or FcαRIxHER2/*neu* (black bar) BsAb. Results represent mean ± SEM from three individual experiments. * $p < 0.05$, compared to FcγRIxHER-2/*neu* BsAb.

FcαRI and FcγRI mediated signaling in neutrophils.

We studied binding of neutrophils to tumor cells in the presence of BsAb in order to exclude poorer binding via FcγRI as a factor of importance in the observed difference in tumor cell killing. Therefore, fluorescent cells were incubated at 4°C, and binding of neutrophils to SK-BR-3 tumor cells in the presence of BsAb was studied. However, higher levels of neutrophil - tumor cell interactions were observed at varying E:T ratios in the presence of FcγRIxHER-2/*neu* BsAb, compared to FcαRIxHER-2/*neu* BsAb (Fig. 4A and 4B). Similar results were obtained when longer times of incubation (up to 3 hours), or other BsAb concentrations were used (0.5 – 2 μg/ml) (data not shown).

Next, signaling via both Fc receptors was studied in calcium mobilization and MAPK phosphorylation assays. After cross-linking either FcαRI or FcγRI, levels of intracellular calcium started rising after 10 seconds (Fig. 4C). However, cross-linking of FcαRI resulted in maximal intracellular calcium mobilization after 25 seconds, whereas calcium mobilization peaked 55 seconds after FcγRI cross-linking. Furthermore, cross-linking of FcαRI or FcγRI led to rapid MAPK phophorylation, which was detected within 15 seconds after cross-linking of either FcαRI or FcγRI (Fig. 4D). The quantity of phosphorylation, however, was consistently higher upon triggering FcαRI, compared to FcγRI. Thus, triggering of FcαRI resulted in faster and more robust signaling compared to FcγRI.

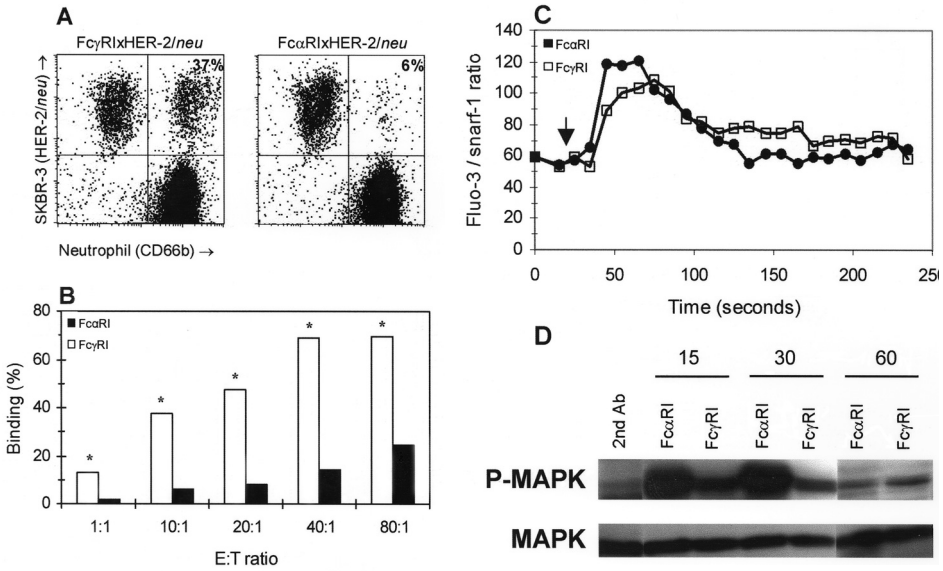

Figure 4. FcαRI and FcγRI mediated signaling in neutrophils. (A) SK-BR-3 cells and G-CSF neutrophils were stained with red (PKH26) and green (PKH67) fluorescent labels, respectively, and binding (double positive cells) was analyzed after incubation at 4°C for 30 min with 1 μg/ml FcγRIxHER-2/*neu* (left panel), or FcαRIxHER-2/*neu* (right panel) BsAb. (B) Percentage binding in presence of FcγRIxHER-2/*neu* (white bars), or FcαRIxHER-2/*neu* (black bars) BsAb was determined at varying E:T ratios. Experiments were repeated four times, yielding essentially similar results. * $p < 0.05$. (C) Intracellular free calcium levels were measured after cross-linking FcαRI (•) or FcγRI (□). Neutrophils were incubated with anti-FcαRI (A77) or anti-FcγRI (m22) mAb, and baseline calcium levels (Fluo-3/snarf-1 ratio) were established for 20 seconds, after which a cross-linking Ab was added (arrow). Calcium mobilization assays were repeated three times, yielding similar results. (D) After incubation of neutrophils with anti-FcαRI (A77) or anti-FcγRI (m22) mAb, Fc receptors were cross-linked with a secondary Ab for 15, 30 or 60 seconds. As a negative control, unlabeled neutrophils were incubated with secondary Ab only. Samples were boiled with reducing sample buffer and analyzed on a western blot with anti-phospho-p44/42 MAPK Ab. The membrane was stripped, and re-probed with an anti-total MAPK Ab as an indicator of protein loading. MAPK phosphorylation assays were repeated three times, yielding similar results.

Cytolytic capacity of bone marrow neutrophils.

G-CSF is frequently used in cancer patients to enhance blood neutrophil numbers as it mobilizes neutrophils from the bone marrow[12]. Therefore, we next assessed the capacity of immature bone marrow neutrophils to initiate ADCC. Neutrophils were isolated from human bone marrow using a percoll discontinuous density gradient, resulting in separation of neutrophil precursors into 3 distinct populations (Fig. 5A-I). Neutrophils from the second percoll layer (P2 neutrophils) contained early neutrophil precursors, which are defined by intermediate CD11b, and low CD16 expression (Fig. 5B), as well as round to kidney-shaped nuclei (Fig. 5C). P3 neutrophils were immature band neutrophils, with intermediate CD11b and heterogeneous CD16 expression (Fig. 5E). Nuclei were horseshoe-shaped (Fig. 5F). Percoll layer P4 was mainly composed of mature neutrophils (P4 neutrophils), which had high CD16 expression levels (Fig. 5H) and a segmented nucleus (Fig. 5I). FcαRI expression was low on P2 neutrophils, but expression levels increased during neutrophil maturation, whereas P2 neutrophils had high FcγRI expression, which decreased during development (Fig. 5A, D, G).

The cytolytic capacity of these neutrophil populations was evaluated with standard chromium release assays. P2 neutrophils were unable to initiate SK-BR-3 killing (data not shown, n=3), which is presumably due to low granule levels. P4 neutrophils exhibited efficient cytolytic capacity, but had low FcγRI expression levels, hereby excluding them from further studies in which FcαRI and FcγRI function was compared. P3 neutrophils showed similar expression levels of FcαRI and FcγRI, and were used as effector cells (Fig. 5J). Targeting FcαRI resulted in efficient lysis of SK-BR-3 cells. Targeting of P3 neutrophils via FcγRIxHER-2/*neu* BsAb, however, did not lead to tumor cell killing (mean tumor cell lysis was 34% ± 25% (1 μg/ml) or 74% ± 39% (2 μg/ml) after targeting to FcαRI, and 0% ± 1% or 0% ± 2% (in the presence of either 1 or 2 μg/ml) on targeting FcγRI, n=4). Moreover, the anti-HER-2/*neu* mAb Herceptin was not able to initiate P3 neutrophil-mediated SK-BR-3 cell lysis either, indicating that all FcγR, which are expressed on immature neutrophils were ineffective in mediating ADCC (data not shown, n=8). Similar data were observed in collagen culture assays, in which P3 neutrophils migrated efficiently into SK-BR-3 tumor colonies in the presence of FcαRIxHER-2/*neu* BsAb, whereas targeting to FcγRI did not result in interactions between neutrophils and tumor colonies (Fig. 5K).

Ex vivo triggering of FcαRI and FcγRI on mouse blood and bone marrow cells.

Syngeneic animal models provide important tools for unraveling mechanisms of Ab-therapy, provided they mirror the human situation. FcαRIxFcγRI double Tg mice were previously described[25] and express human FcαRI constitutively on mature neutrophils, whereas human FcγRI expression can be induced by treatment with G-CSF, which is comparable to humans. To study whether the observed differences between FcαRI- and FcγRI- mediated ADCC could be extrapolated to FcαRIxFcγRI double Tg mice, mouse blood and bone marrow cells were collected and evaluated in functional studies.

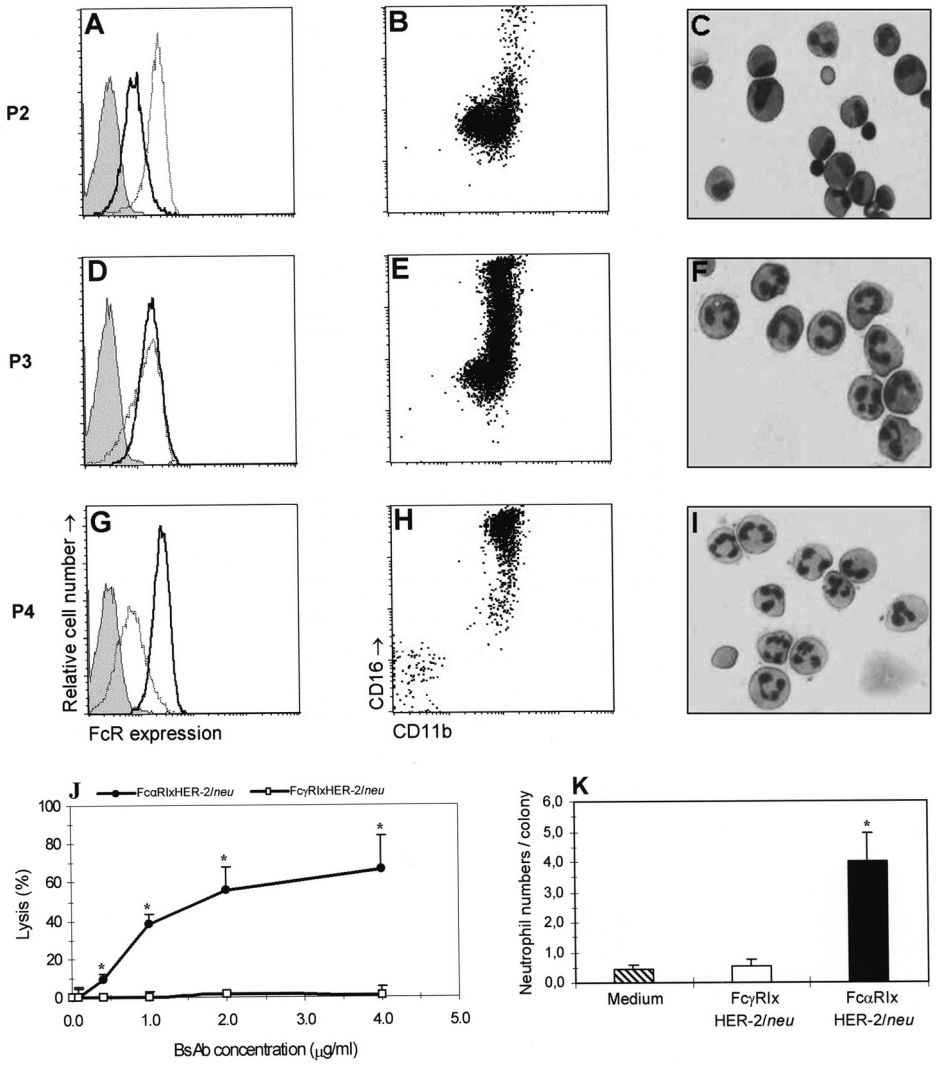

Figure 5. Bone marrow neutrophils as effector cells for tumor cell killing. Bone marrow neutrophils were separated into 3 neutrophil maturation stages, labeled immature P2 (A, B, C), intermediate P3 (D, E, F) and more mature P4 (G, H, I) neutrophils. FcαRI (bold lines) and FcγRI (thin lines) expression levels (A, D, G) were measured by flow cytometry (filled areas represent secondary Ab only). Expression of CD11b (FITC) and CD16 (PE) (B, E, H) and cell morphology (C, F, I) were used to confirm maturation state of bone marrow neutrophils. (J) Lysis of SK-BR-3 cells by P3 neutrophils (E:T ratio 80:1) in the presence of increasing amounts of FcαRIxHER-2/*neu* (•), or FcγRIxHER-2/*neu* (□) BsAb. Data represent mean ± SD of triplicate samples. One representative experiment out of four is shown. (K) Numbers of P3 neutrophils per SK-BR-3 tumor colony in the absence (hatched bar), or presence of FcγRIxHER-2/*neu* (white bar) or FcαRIxHER-2/*neu* (black bar) (1 μg/ml). Results represent mean ± SEM from three individual experiments. * $p < 0.05$.

After G-CSF treatment, FcαRI expression level on blood neutrophils was slightly higher compared to FcγRI (Fig. 6A). Bone marrow neutrophils from untreated mice showed no difference in expression levels (Fig. 6B). Similar to human blood neutrophils, SK-BR-3 tumor cell lysis by mouse blood cells was higher in the presence of FcαRIxHER-2/*neu* BsAb, compared to FcγRIxHER-2/*neu* BsAb (Fig. 6C). Mean tumor cell lysis in the presence of 1 μg/ml BsAb was 84% ± 16% or 36% ± 4% after targeting to FcαRI or FcγRI, respectively (n=3). Additionally, efficient killing of SK-BR-3 cells was observed upon engagement of FcαRI on mouse bone marrow cells, whereas SK-BR-3 cell lysis was absent in the presence of FcγRIxHER-2/*neu* BsAb or anti-HER-2/*neu* mAb, which is identical to results obtained with human cells (Fig. 6D and data not shown). Mean tumor cell lysis was 34% ± 11% (1 μg/ml) or 53% ± 18% (2 μg/ml) on targeting FcαRI, and 0% ± 2% or 0% ± 1% (in the presence of either 1 or 2 μg/ml) after targeting to FcγRI (n=3).

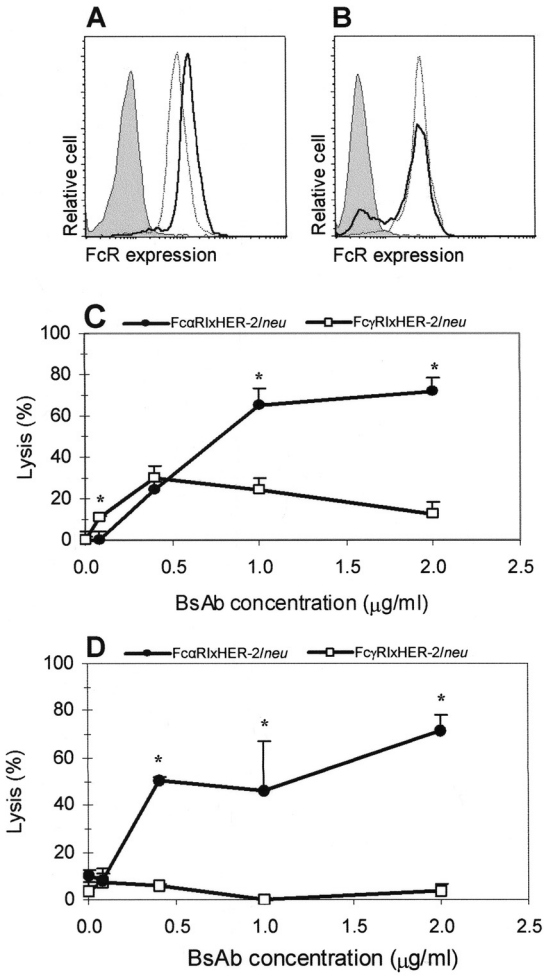

Figure 6. *Ex vivo* triggering of FcαRI and FcγRI on mouse blood and bone marrow cells. Expression levels of FcαRI (bold line), and FcγRI (thin line) were determined on neutrophils from G-CSF treated mice (A), and bone marrow cells from untreated mice (B) (filled area represents secondary Ab only). Lysis of SK-BR-3 cells by mouse blood cells (C), or mouse bone marrow cells (D) in the presence of FcαRIxHER-2/*neu* (•), or FcγRIxHER-2/*neu* (□) BsAb. Data are expressed as mean ± SD of triplicates. Experiments were repeated three times, yielding similar results. * $p < 0.05$.

DISCUSSION

Neutrophils have previously been proposed as attractive effector cell population for Ab-therapy, since they represent the most populous Fc receptor-expressing leukocyte subset in blood and their numbers can be easily increased. It was demonstrated that FcαRI represents the most potent Fc receptor on neutrophils for induction of ADCC, which has been shown for a variety of tumor antigens, including the EGF receptor, HLA class II, CD20, CD30, HER-2/*neu* and EpCAM[24,26,33-37]. In our studies, maximal tumor cell killing was higher upon targeting FcαRI, both in ^{51}Cr release assays and real-time video recordings, although FcγRI mediated tumor cell killing was somewhat higher in the presence of low BsAb concentrations. This is presumably due to the higher number of neutrophil – tumor cell interactions in the presence of FcγRI BsAb compared to FcαRI BsAb. At low BsAb concentrations, the number of FcαRI mediated neutrophil - tumor cell interactions may be insufficient to reach the threshold necessary for induction of tumor cell killing, as tumor cell lysis was absent at E:T ratios lower than 10:1 (data not shown). The difference in Fc receptor-mediated binding of neutrophils and tumor cells is presently unclear as receptor expression levels, as well as affinities of the used anti-Fc receptor mAb were similar. Differences in Fc receptor distributions within the cell membrane of neutrophils might represent a possible explanation, as FcγRI has recently been observed to constitutively reside in so-called lipid rafts, whereas membrane FcαRI is only partially raft-localized[38-40]. This dissimilarity in cell membrane distribution may influence the accessibility for BsAb and tumor cells. We furthermore observed that effectiveness of IgG mAb differed greatly between donors (data not shown), which is likely linked to a polymorphism in the extracellular domain of FcγRIIa, located at amino acid position 131, as it was shown that neutrophils from FcγRIIa-H/H131 donors were significantly less effective in triggering antibody-dependent apoptosis than neutrophils isolated form FcγRIIa-R/R131 donors[11,30].

Importantly, FcαRI was the only Fc receptor that consistently induced neutrophil migration towards tumor cells in 3D collagen culture assays, which led to destruction of tumor colonies. This was observed after targeting Her-2/*neu* on SK-BR-3 mamma-carcinoma cells as well as targeting EpCAM on colon carcinoma SW948 tumor cells with anti-EpCAM mAb (Fig. 3, and data not shown). FcγRI BsAb proved ineffective in inducing neutrophil migration in the 3D collagen. Furthermore, release of IL-8, which is the prototypic neutrophil chemokine was only observed in the presence of FcαRI BsAb, which may explain the higher migration of neutrophils towards tumor colonies (data not shown).

Interestingly, signaling via FcαRI is believed to be mediated via similar signaling routes that are also used by other Fc receptors, and requires association with the common Fc receptor γ chain (FcR γ chain). Earlier work showed that effector functions such as ADCC by either FcγRI or FcαRI were dependent on the ITAM signaling motifs within the FcR γ chain[41,42]. Several phenomena might explain the observed differences between FcαRI and FcγRI mediated tumor cell killing. First, FcαRI and FcγRI may initiate different killing mechanisms by neutrophils.

"Boiling" of cytoplasm and membrane "blebbing" of tumor cells - indicative of apoptosis[32] - was observed in our real-time video recording experiments after addition of neutrophils and FcαRIxHer-2/*neu* BsAb, which is in concordance with earlier data in which neutrophil-mediated apoptosis of human breast cancer cells was demonstrated after targeting FcαRI[11]. Second, FcαRI strongly associates with the FcR γ chain, based on an additional electrostatic interaction within the transmembrane regions, which may trigger enhanced neutrophil activation[433]. Third, FcαRI may initiate additional signaling pathways, as it has been shown that a subpopulation of FcαRI is expressed on neutrophils without associated FcR γ chain[444]. FcαRI might thus interact with an - up till now - unidentified molecule. Our observation that FcαRI cross-linking results in a more rapid rise in intracellular free calcium, and higher levels of MAPK phosphorylation (Fig. 4C and 4D), supports the notion that FcαRI initiates more efficient signaling pathways.

Because G-CSF mobilizes immature neutrophils from the bone marrow[12], we also investigated ADCC capacity of bone marrow neutrophils. Only FcαRI BsAb proved capable of triggering tumor cell killing, whereas FcγRI BsAb were ineffective. It was previously shown that maximal simultaneous triggering of FcαRI and FcγRI in IFNγ neutrophils led to decreased FcγRI mediated tumor cell killing[25]. This suggests that receptors may compete for available FcR γ chain, a phenomenon that has also been observed for FcεRI and FcγRIII in mast cells[455]. It was furthermore demonstrated that FcR γ chain is required for stable FcγRI expression in IIA1.6 transfectants, as expression was lost over time in the absence of FcR γ chain[41]. The observation that FcγRI expression decreases during neutrophil maturation suggests that FcγRI is not associated with FcR γ chain. Neutrophils were reported to express relatively low FcR γ chain levels, compared to monocytes[466]. It is therefore possible that limited availability of FcR γ chain in immature neutrophils results in a favorable association with FcαRI due to a stronger electrostatic interaction, explaining the inability to induce tumor cell killing via FcγRI. Additionally, bone marrow neutrophils proved unable to induce ADCC via IgG Ab. FcγRIIa, which is involved in neutrophil-mediated ADCC, bears an ITAM signaling motif within its cytoplasmic tail, and as such can convey its own signaling irrespective of the FcR γ chain. It has been demonstrated though, that association of FcγRIIa with FcR γ chain was required for initiation of antigen presentation and cytokine production[477]. It is therefore conceivable that FcγRIIa-mediated ADCC depends on interaction with the FcR γ chain, as well.

Another explanation for the differences in neutrophil-mediated tumor cell killing might be due to interactions of FcαRI or FcγRI with other interacting proteins, as Beekman *et al.* recently described that periplakin was involved in FcγRI-mediated ligand binding and function[48]. Differences in periplakin expression in immature neutrophils might therefore affect FcγRI-mediated ADCC. Alternatively, as FcαRI can be expressed without the FcR γ chain[44], other proteins might additionally interact with this Fc receptor in immature neutrophils, which may circumvent the FcR γ chain dependency for its effector functions. Further research

on this topic is necessary to clarify differences between FcαRI and FcγR mediated tumor cell killing.

ACKNOWLEDGEMENTS

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Chapter 4

FcR γ chain dependent signaling via IgA but not IgG Fc receptors in immature neutrophils

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ABSTRACT

Neutrophil-mediated tumor cell lysis is more efficiently triggered by FcαRI (CD89), than by FcγRI (CD64). This difference is most evident in immature neutrophils in which FcγRI-mediated tumor cell lysis is absent. Here, we show that in immature neutrophils FcR γ chain-dependent functions (such as antibody-dependent cellular cytotoxicity and respiratory burst), as well as signaling (calcium mobilization and MAPK phosphorylation) were potently triggered via FcαRI, but not FcγRI. Internalization, which is an FcR γ chain-independent function, was however effectively initiated by both receptors. These data suggest an impaired functional association of FcγRI with the FcR γ chain, which prompted us to perform co-immuno-precipitation experiments. As a weaker association was observed between FcγRI and FcR γ chain, compared to FcαRI and FcR γ chain, our data support that differences between FcαRI- and FcγRI-mediated functions are attributable to dissimilarities in association with the FcR γ chain.

INTRODUCTION

Immune cell signaling can be initiated by binding of antibodies (Ab) to Fc receptors. After activation, Fc receptors trigger effector functions such as antibody-dependent cellular cytotoxicity (ADCC), release of oxygen radicals, endocytosis, phagocytosis, degranulation, antigen presentation, and release of inflammatory mediators (for review [1]). In humans, three classes of leukocyte IgG Fc receptors, namely FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and one class of IgA Fc receptor (FcαRI, CD89) have been described[2,3]. FcγRIIIb is a glycosyl-phosphatidylinositol (GPI)-linked protein[4]. FcγRIIa bears a so-called activatory ITAM (Immunoreceptor Tyrosine-based Activation Motif) signaling motif in its cytoplasmic tail, which is required for induction of effector functions, whereas FcγRIIb bears an inhibitory ITIM signaling motif[5,6]. The α chains of FcαRI, FcγRI, and FcγRIIIa lack such signaling motifs. Therefore, these latter receptors associate with the common ITAM-containing FcR γ chain in order to mediate effector functions[7,8].

Of all FcγR, cell distribution of FcγRI most closely resembles FcαRI expression. Both receptors are selectively expressed on cells of the myeloid lineage, including monocytes, macrophages and dendritic cells[2,9]. Furthermore, neutrophils constitutively express FcαRI, whereas FcγRI expression can be induced on these cells by addition of either interferon-γ (IFNγ) or granulocyte-colony stimulating factor (G-CSF)[10,11]. Neutrophils represent the most populous cytotoxic effector cell subset within the blood, which numbers can be increased by treatment with G-CSF[12]. They play a prominent role in bacterial infections[13], but exert well-documented anti-tumor properties, as well, since neutrophils have been shown to play a role in tumor rejection in the presence of anti-tumor Ab, both *in vitro* and *in vivo*[14-17]. Therefore, we - and others - studied the potential of FcαRI[18,19] and FcγR[20,21] on these immune cells to induce tumor cell lysis.

FcγRI was reported to represent the most potent neutrophil FcγR for induction of ADCC[20,21]. However, compared to FcγRI, tumor cell lysis, as well as tumor-directed neutrophil migration, was more efficient via targeting of neutrophil FcαRI[18,19]. This difference between both receptors was most evident in bone marrow neutrophils, in which FcγRI-initiated tumor cell killing was hampered[19]. However, cell surface expression of either receptor depends on association with the FcR γ chain, which was shown in FcαRI and FcγRI transgenic mice, in which surface expression was lost when these mice were crossed with FcR γ chain-deficient mice[22,23]. Moreover, for initiation of most immune effector functions, both receptors depend on signaling via the FcR γ chain, as well[24,25]. In the present work, we therefore studied the underlying mechanisms behind the discrepancies between FcαRI and FcγRI-mediated effector functions in more detail.

MATERIALS AND METHODS

Antibodies.

Antibodies A77 (mIgG1 anti-FcαRI), m22 (mIgG1 anti-FcγRI) and 520C9 (mIgG1 anti-Her-2/neu) were produced from hybridomas (Medarex, Bloomsbury, NJ). FcγRIxHer-2/neu bispecific Ab (BsAb) (22x520C9; MDX-H210) was also obtained from Medarex. FcαRIxHer-2/neu BsAb (A77x520C9) was produced by chemically cross-linking F(ab') fragments of monoclonal Ab (mAb) 520C9 with F(ab') fragments of mAb A77 as described[26]. For neutrophil staining, FITC-conjugated anti-CD11b mAb (Immunotech, Marseille, France), PE-conjugated anti-CD16 mAb (Becton Dickinson Biosciences (BD), San Jose, CA), and FITC-conjugated anti-human CD66b mAb (Serotec, Oxford, UK) were used. FITC-conjugated and unconjugated $F(ab')_2$ fragments of goat anti-mouse IgG1 mAb were purchased from Southern Biotech Association (SBA; Birmingham, AL). $F(ab)'_2$ fragments of FITC-labeled rabbit anti-goat IgG (H+L) Ab were obtained from Jackson immuno research (West Grove, PA). For western blot analyses, rabbit anti-FcR γ chain Ab (Upstate, Dundee, UK), rabbit anti-phospho-p44/42 MAPK, or rabbit anti-total MAPK Ab (Cell Signaling Technology, Danvers, MA), and peroxidase (PO)-conjugated goat anti-rabbit Ab (Pierce Biotechnology, Rockford, IL), were used.

Cell lines.

The breast carcinoma cell line SK-BR-3, which over-expresses the tumor-associated antigen Her-2/neu was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated FCS and antibiotics (RPMI/10%). SK-BR-3 cells were harvested using trypsin-EDTA (Gibco).

Blood and bone marrow donors.

A peripheral blood sample (30 ml) was drawn from healthy donors receiving rhG-CSF (Neupogen, 5 μg/kg of body weight, twice daily for five days, Amgen Inc., Thousand Oaks, CA). Bone marrow samples were obtained from cardiac patients undergoing surgery. Studies were approved by the Medical Ethical Committee of the Utrecht University (The Netherlands), in accordance with the Declaration of Helsinki. All donors gave informed consent.

Neutrophil isolation.

Neutrophils from healthy G-CSF stimulated donors (G-CSF neutrophils) were used as positive control for FcγRI-mediated effector functions, and were isolated from heparin anti-coagulated peripheral blood samples by standard Ficoll-Histopaque (Sigma-Aldrich, Steinheim, Germany) density gradient centrifugation.

Bone marrow neutrophils were isolated as described previously[19]. In short, erythrocytes were removed by incubation for 5-10 minutes at 4°C with a lysis solution of pH 7.4 (0.16M ammonium-chloride, 0.01M potassium bicarbonate and 0.1mM sodium-edetate), after which

cells were separated by discontinuous percoll gradient centrifugation (successively 81%, 62%, 55%, 50% and 45% of percoll). Percoll layer 1 and 5 in the gradient contained non-myeloid cells, lipids, cellular debris and remaining erythrocytes, respectively. Percoll layer 2, 3 and 4 comprised different neutrophil maturation stages.

Maturation status and purity of isolated human bone marrow cells was confirmed by cytospin preparations and staining with FITC-conjugated anti-CD11b mAb and PE-conjugated anti-CD16 mAb, as described previously[19,27]. Purity of isolated blood neutrophils was confirmed by cytospin preparation, and staining with FITC-conjugated anti-human CD66b mAb. Neutrophil surface expression of FcαRI and FcγRI was determined with mAb A77 (FcαRI) or m22 (FcγRI), respectively (10 μg/ml), followed by incubation with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG1 mAb. Cells were analyzed on a FACScan (BD). In all experiments, neutrophil purity exceeded 95% and cell viability was more than 98%, as determined by trypan blue exclusion.

Ab-dependent cellular cytotoxicity (ADCC).

^{51}Cr release assays were performed as described earlier[21]. Briefly, SK-BR-3 target cells were incubated for 2 hours at 37°C with ^{51}Cr (100 μCi / $1x10^6$ cells; Amersham, Little Chalfont, UK), washed, and plated in 96-well round-bottom microtiter plates (5×10^3 cells/well), together with 4×10^5 neutrophils/well (E:T ratio of 80:1) in presence of 2 μg/ml BsAb. After a 4 hour incubation at 37°C, ^{51}Cr release in the supernatant was measured as counts per minute (cpm). The percentage of tumor cell lysis was calculated as follows: (experimental cpm–basal cpm)/(maximal cpm-basal cpm)x100%.

Respiratory burst assay.

Neutrophils were incubated with anti-FcαRI (A77), anti-FcγRI (m22), or, as an isotype control, irrelevant (520C9) mAb (10 μg/ml) for 30 min at 4°C. After washing, $F(ab')_2$ fragments of goat anti-mouse IgG1 mAb were added to crosslink neutrophil Fc receptors, and tubes were placed in a 953 LB Biolumat (Berthold, Wildbad, Germany). Luminol (150 mM) was injected in all tubes, and light emission was recorded continuously for 30 min at 37°C. Addition of formyl-methionyl-leucyl-phenylalanine (fMLP) to neutrophils was used as a positive control.

Calcium mobilization assay.

Neutrophils were labeled with SNARF-1 (2.8 μM) and Fluo-3 (1.4 μM) (Invitrogen, Breda, The Netherlands) for 30 min at 37°C. After washing, cells were incubated with anti-FcαRI (A77), or anti-FcγRI (m22) mAb (10 μg/ml) for 30 min at 4°C. Cells were washed twice and resuspended in calcium mobilizing buffer. Fc receptors were crosslinked with $F(ab')_2$ fragments of goat anti-mouse IgG1 mAb, and intracellular free calcium levels were measured by FACScan. The first 20 seconds of each run - prior to cross-linking - were used to establish baseline intracellular calcium levels. fMLP was added to neutrophils as a positive control.

Internalization assay.

Neutrophils were preincubated with 20% pooled human serum to prevent aspecific binding to IgG Fc receptors (30 min at 4°C). Thereafter, cells were incubated for 30 min at 4°C with anti-FcαRI (A77), anti-FcγRI (m22) or, as an isotype control, irrelevant (520C9) mAb. After that, cells were washed and incubated with $F(ab)'_2$ fragments of goat anti-mouse IgG1 mAb (30 min at 4°C). Samples were then split. One sample was kept at 4°C in order to measure total surface expression of the Fc receptors. The other sample was put at 37°C for the indicated time points to allow internalization, which was stopped by adding ice-cold FACS-buffer. Remaining FcαRI and FcγRI surface expression was visualized by staining for 30 min at 4°C with $F(ab)'_2$ fragments of FITC-labeled rabbit anti-goat IgG (H+L) Ab. Internalization of Fc receptors at 37°C was calculated as percentage of initial Fc receptor surface expression, which was determined in 4°C samples.

MAPK phosphorylation assay.

Neutrophils were incubated with anti-FcαRI (A77), or anti-FcγRI (m22) mAb (10 μg/ml) for 30 min at 4°C. After washing, Fc receptors were cross-linked with $F(ab)'_2$ fragments of goat anti-mouse IgG1 mAb at 37°C for the indicated time points. Ice-cold PBS was added to stop reactions, after which samples were boiled in reducing Laemmli sample buffer, run on 10% SDS-PAGE gels, and electro-transferred to nitrocellulose membranes (0.45 μm, Millipore Corporation, Billerica, MA). Membranes were blocked with 5% bovine serum albumin (Roche Diagnostics, Mannheim, Germany) and probed with rabbit anti-phospho-p44/42 MAPK, or rabbit anti-total MAPK Ab for 2 hours. Following washing, membranes were incubated for an additional hour with PO-conjugated goat anti-rabbit Ab. Staining was visualized using the ECL detection system (Amersham).

Co-Immunoprecipitation assay.

Interaction of FcαRI and FcγRI with the FcR γ chain was measured by lysing neutrophils (5 x 10^7) with RIPA buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% DOC, and 0.1% SDS) or NP40 buffer (10 mM Tris pH 7.4, 137 mM NaCl, 13.5 mM NaPyrophosphate, 51.9 mM FNa, 10% glycerin, 0.5% NP40), both supplemented with 1 mM phenylmethylsulfonyl fluoride, 250 μM sodium orthovanadate, 10 mM dithiothreitol, and a protease inhibitor cocktail (Roche Diagnostics) for 30 min at 4°C. Homogenates were spun for 20 min at 13.000 rpm. Supernatants were pre-cleared head over head for 30 min at 4°C with prot A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA). After that, beads were removed, and anti-FcαRI (A77), anti-FcγRI (m22) or, as an isotype control, irrelevant (520C9) mAb was added overnight (4°C, head over head). Prot A/G beads were added for 3 additional hours (4°C, head over head), after which beads were washed 3 times with lysisbuffer, and boiled in reducing Laemmli sample buffer. Samples were run on 15% SDS-PAGE gels, electro-transferred to nitrocellulose membranes (0.45 μm, Millipore Corporation), and membranes were blocked with 5% low fat

milk powder in PBS. Membranes were probed with rabbit anti-FcR γ chain Ab, followed by incubated with PO-conjugated goat anti-rabbit Ab. Staining was visualized using the ECL detection system (Amersham).

Statistical analysis.

Data are shown as mean ± standard deviation (SD). Group data are shown as mean ± standard error of the mean (SEM). Statistical differences were determined using the two–tailed unpaired Student's *t*-test or ANOVA. Significance was accepted when $p < 0.05$.

RESULTS

Fc receptor expression and neutrophil-mediated tumor cell killing.

To study the mechanisms underlying the differences in FcαRI and FcγRI-mediated effector functions in neutrophil precursor subsets, cells were isolated from bone marrow, and separated into 3 populations as described[19]. In short, percoll layer 2 (P2 neutrophil precursors) comprised the earliest neutrophil precursors (round to kidney-shaped nucleus), percoll layer 3 (P3 neutrophil precursors) contained "intermediate" neutrophil precursors (horseshoe-shaped nucleus), and percoll layer 4 (P4 neutrophil precursors) consisted of the most mature neutrophil precursors (segmented nucleus).

First, FcαRI and FcγRI expression levels were determined (Fig. 1A, and B). In bone marrow, P2 neutrophil precursors expressed a high level of FcγRI, which was downregulated during differentiation. On mature blood neutrophils, the level of FcγRI was low to absent (data not shown, n=5), but could be upregulated by G-CSF treatment. As such, experiments were only preformed with G-CSF stimulated blood neutrophils. FcαRI expression levels were low on P2 neutrophil precursors, and were upregulated during differentiation. In blood, unstimulated as well as G-CSF neutrophils expressed high FcαRI levels.

The capacity of bone marrow neutrophils to lyse tumor cells via triggering of FcαRI or FcγRI was determined in ^{51}Cr release assays, in which SK-BR-3 tumor cells were used as targets. G-CSF neutrophils were able to lyse tumor cells via triggering of FcγRI, but lysis was more efficient by targeting of FcαRI (Fig. 1C). P2 neutrophil precursors were unable to mediate tumor cell killing via either Fc receptor. P3 neutrophil precursors, which co-expressed FcαRI and FcγRI, could effectively trigger tumor cell killing via FcαRI, but not via FcγRI. P4 neutrophil precursors, with low expression of FcγRI, did also not induce tumor cell lysis by targeting to FcγRI either, whereas FcαRI-mediated lysis was efficient.

Respiratory burst and Fc receptor internalization on bone marrow neutrophils.

We next investigated whether Fc receptor-mediated tumor cell lysis was the sole effector function in which FcγRI activation was absent, or whether other neutrophil functions were

impaired as well. Therefore, Fc receptor-mediated respiratory burst activity was investigated. Crosslinking of FcαRI or FcγRI on G-CSF neutrophils induced respiratory burst activity, albeit that the activity mediated via FcγRI was consistently lower, compared to FcαRI (Fig. 2A). FcαRI triggering on P2, P3, and P4 neutrophil precursors activated a respiratory burst as well, although the maximal burst activities and durations were lower, compared to respiratory bursts observed in G-CSF neutrophils (Fig. 2B-D). In P2 neutrophil precursors, which expressed the highest FcγRI level, crosslinking of FcγRI induced some respiratory burst activity. This burst level, however, was minimal and reached only 0.5 x 10^6 cpm (Fig. 2B). Crosslinking of FcγRI in P3 and P4 neutrophil precursors did not induce any respiratory burst activity (Fig. 2C, and 2D).

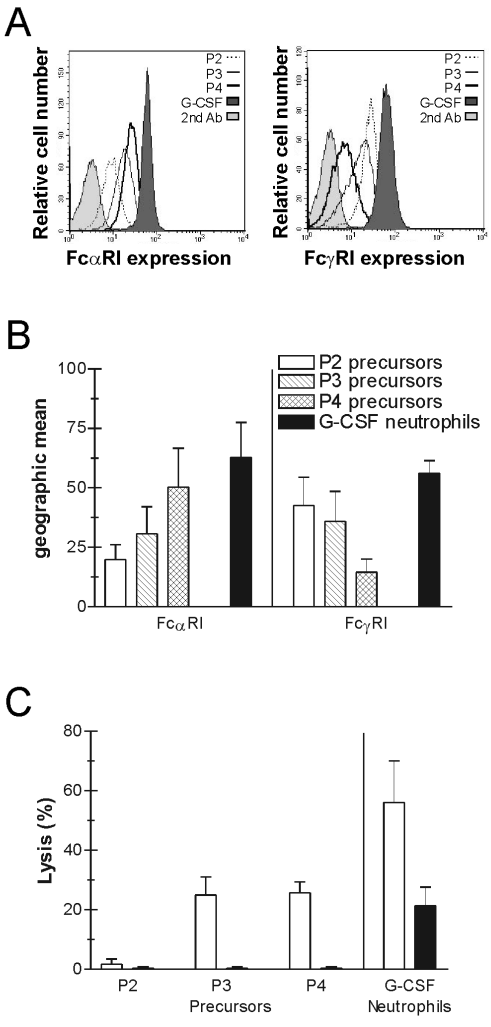

Figure 1. FcαRI and FcγRI expression levels and neutrophil-mediated tumor cell killing. (A) Surface expression of FcαRI (left panel), or FcγRI (right panel) in P2 (dotted line), P3 (thin line), and P4 (thick line) neutrophil precursors, as well as G-CSF neutrophils (dark filled area). Filled light gray area represents secondary Ab only. A representative example out-of-eight is shown. (B) Quantification of FcαRI and FcγRI expression on P2 (white bars), P3 (hatched bars) and P4 (cross-hatched bars) neutrophil precursors, and on G-CSF neutrophils (black bars). Mean fluorescent intensities (MFI) ± SEM of 4 experiments are shown. (C) Lysis of SK-BR-3 tumor cells by P2, P3, or P4 neutrophil precursors and G-CSF neutrophils in the presence of 2 μg/ml FcαRIxHer-2/neu BsAb (white bars) or 2 μg/ml FcγRIxHer-2/neu BsAb (black bars) measured in a ^{51}Cr release assay. One representative example out-of-three experiments is shown.

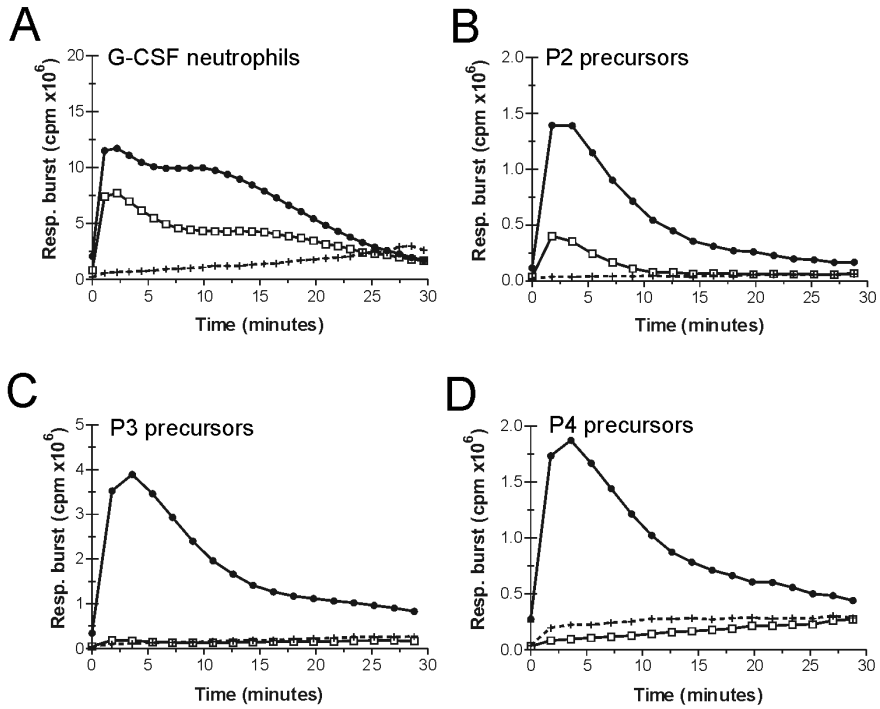

Figure 2. FcαRI- and FcγRI-induced respiratory burst by neutrophils. Respiratory burst triggered after crosslinking FcαRI (•), or FcγRI (□) measured with a luminometer for 30 minutes in G-CSF neutrophils (A), and in P2 neutrophil precursors (B), P3 neutrophil precursors (C), or P4 neutrophil precursors (D). As a negative control, respiratory burst was measured in the presence of a control isotype Ab (+, dotted lines). Experiments were repeated 3 times, yielding similar results.

Impaired FcR γ chain-mediated signaling by FcγRI in neutrophil precursors.

Furthermore, FcR γ chain-mediated signaling was investigated by triggering either receptor classes. As P2 neutrophil precursors express low FcαRI levels, and P4 neutrophil precursors express low FcγRI levels, we used P3 precursors, which co-express both Fc receptors, for further experiments. Fc receptor crosslinking induces tyrosine phophorylation of ITAM, which triggers activation of the PI-3K pathway, leading to calcium mobilization[28]. In G-CSF neutrophils, crosslinking of either FcαRI or FcγRI induced an increase in intracellular free calcium levels, but FcγRI-mediated calcium mobilization was consistently slower compared to FcαRI-mediated mobilization (Fig. 3A). In P3 neutrophil precursors, however, a rise in intracellular free calcium was only observed after crosslinking FcαRI, but not FcγRI (Fig. 3B).

Fc receptor crosslinking furthermore activates the RAS-ERK pathway, thereby triggering phosphorylation of MAPK (P-MAPK)[29]. We previously showed that crosslinking of either FcαRI or FcγRI on G-CSF neutrophils induced MAPK phosphorylation, albeit that P-MAPK levels were higher following triggering of FcαRI, compared to FcγRI[19]. In P3 neutrophil precursors,

FcαRI crosslinking induced MAPK phosphorylation (Fig. 4). In contrast, crosslinking of FcγRI on these precursors did not result in phosphorylation of MAPK.

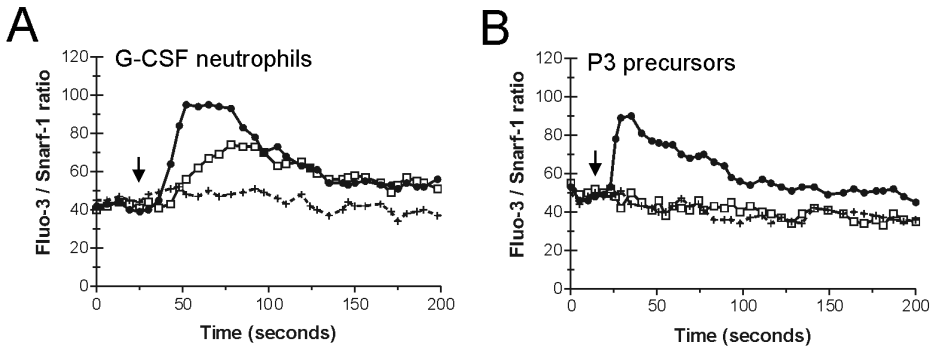

Figure 3. Effect of Fc receptor crosslinking on intracellular free calcium levels. Intracellular free calcium levels were measured after cross-linking FcαRI (•) or FcγRI (□) in G-CSF neutrophils (A), or P3 neutrophil precursors (B). Baseline calcium levels were established for 20 seconds, after which a crosslinking Ab was added (arrows). As a negative control, intracellular free levels were measured in the presence of crosslinking Ab only (+, dotted lines). Calcium mobilization assays were repeated 4 times, yielding similar results.

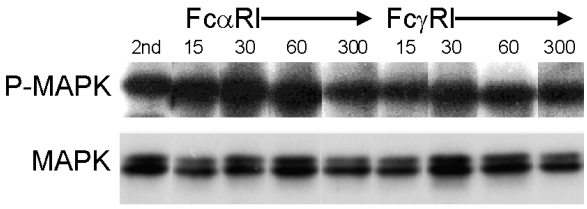

Figure 4. Effect of Fc receptor crosslinking on MAPK phosphorylation. P3 neutrophil precursors were incubated with anti-FcαRI, or anti-FcγRI mAb, and crosslinked with a secondary Ab at 37°C for the indicated times (depicted in seconds). As a negative control, unlabeled neutrophils were incubated with crosslinking Ab only (indicated by "2nd"). Western blots were stained for phosphorylated MAPK (P-MAPK), stripped, and re-stained for total MAPK to assess protein loading.

Efficient internalization of both FcαRI and FcγRI in neutrophil precursors.

As all abovementioned FcR γ chain-dependent functions were abrogated via FcγRI, we next performed an internalization assay, which is a FcR γ chain-independent function[24,30]. Crosslinking of either FcαRI or FcγRI on P3 neutrophil precursors induced efficient receptor internalization (Fig. 5A, and B). Within 5 minutes, both FcαRI and FcγRI levels decreased for 50%-70% (Fig. 5C). These data therefore suggest that the discrepancy between FcαRI and FcγRI-mediated functions was located within the FcR γ chain pathway.

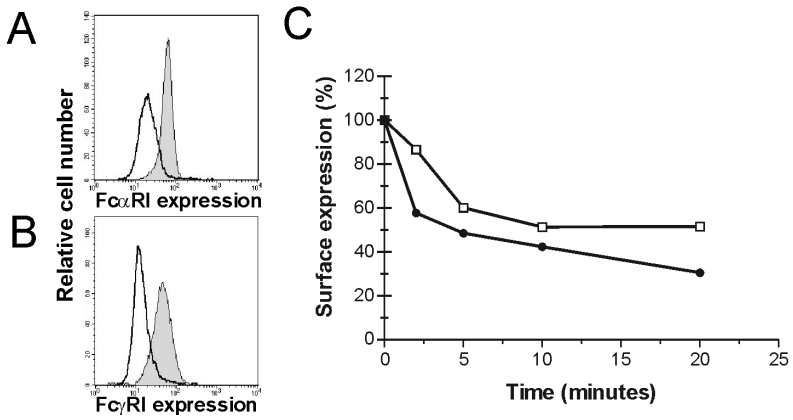

Figure 5. Fc receptor internalization in immature neutrophils. (A) FcαRI, or (B) FcγRI surface expression levels before Fc receptor crosslinking (filled area), and 20 minutes after Fc receptor crosslinking (thick line) at 37°C. (C) Receptor expression before crosslinking was set at 100%. FcαRI (•) or FcγRI (□) were crosslinked for the indicated time periods, and remaining surface expression levels were determined, as described in the materials and methods section. Representative examples out-of-two individual experiments are shown.

Co-immunoprecipitation of FcR γ chain with FcαRI or FcγRI in neutrophil precursors.

To evaluate association of both receptors with the FcR γ chain, we set-up a co-immuno-precipitation assay. When P3 and P4 neutrophil precursors were lysed using mild conditions with RIPA buffer, FcR γ chain was pulled down via FcαRI as well as FcγRI, indicating that both Fc receptors were associated with FcR γ chain (Fig. 6A). However, in a NP40 lysis detergent, FcR γ chain was co-immunoprecipitated via FcαRI, but not via FcγRI, indicating that association of FcγRI with FcR γ chain was abrogated using this detergent (Fig. 6B). In parallel experiments, similar results were observed with mature neutrophils (data not shown).

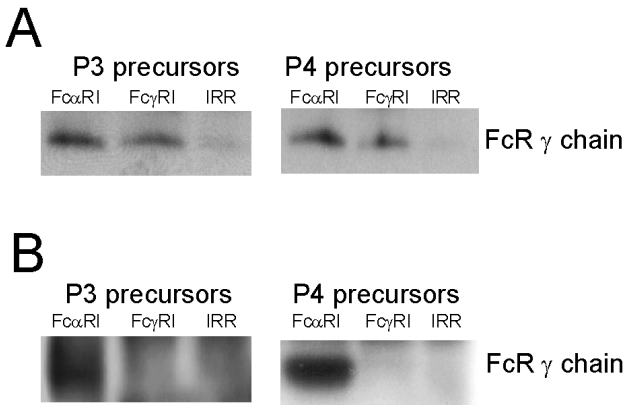

Figure 6. Interaction of FcαRI and FcγRI with FcR γ chain in immature neutrophils. P3, and P4 neutrophil precursors were lysed with either RIPA buffer (A), or with a NP40 detergent (B), and immuno-precipitated with anti-FcαRI, anti-FcγRI, or irrelevant isotype control mAb (indicated by 'IRR'), after which samples were stained on western blots for FcR γ chain. One experiment out-of-two is shown, yielding similar results.

DISCUSSION

Triggering of neutrophil FcαRI induces more efficient tumor cell killing, compared to FcγRI[18,19]. Furthermore, immature neutrophils effectively lyse tumor cells via FcαRI, but not via FcγRI[19]. In the present study, we evaluated the mechanisms underlying the dissimilarities in Fc receptor-mediated effector functions in different subsets of neutrophil precursors. We observed that ADCC, respiratory burst, and signaling functions like calcium mobilization and MAPK phosphorylation, were effectively triggered by FcαRI, but not by FcγRI. Fc receptor internalization, however, was readily induced by crosslinking either FcαRI or FcγRI.

Interestingly, FcαRI signaling is believed to be mediated through signaling routes that are also used by FcγRI, requiring the common FcR γ chain[7,8,22,23]. Earlier work showed that most effector functions, such as phagocytosis and cytokine production, by either FcγRI or FcαRI were dependent on the ITAM motifs within this signaling chain[24,31]. However, a few FcR γ chain independent functions have been described. Both FcαRI and FcγRI can mediate endocytosis without the FcR γ chain. This was shown for FγRI in transfection studies with COS cells, and for FcαRI in colostral neutrophils[24,32,33], which express a population of FcαRI that is not associated with the FcR γ chain, but can mediate IgA endocytosis[30]. Because Fc receptor internalization was the only effector function that was not hampered via FcγRI, our data suggest that the observed discrepancy between FcαRI and FcγRI-mediated function is the result of a difference between both Fc receptors in the FcR γ chain signaling pathway. Furthermore, as early signaling events, like calcium mobilization and MAPK phosphorylation, were not initiated by FcγRI, our data suggest that the interaction with the FcR γ chain was affected.

This hypothesis was supported by co-immunoprecipitation studies, in which we found a less stable interaction between FcγRI and FcR γ chain, compared to FcαRI. This correlated well with earlier data in which FcαRI was shown to bear a positively charged amino acid residue on position 209, which associates with a negatively charged amino acid of the FcR γ chain, resulting in an electrostatic interaction within the transmembrane region[25]. The position of the positively charged amino acid residue proved important, as changing its position within the transmembrane region abrogated signaling and effector functions, except for internalization, due to disturbance of the physical association between FcαRI and FcR γ chain[34]. FcγRI lacks such a positively charged amino acid in its transmembrane region, which might explain the weaker association with the FcR γ chain.

The FcR γ chain is required for stable FcγRI expression, as expression was lost in the absence of the FcR γ chain[23]. Furthermore, FcεRI and FcγRIII were shown to compete for available FcR γ chain in mast cells[35], a phenomenon that was also suggested to occur in neutrophils for FcαRI and FcγRI, when both Fc receptors were maximally engaged[36]. In addition, neutrophils express relatively low levels of FcR γ chain, compared to monocytes[37]. Therefore, we postulate that, due to a stronger association of FcαRI with the FcR γ chain and limited availability of FcR γ chain in immature neutrophils, FcαRI competes with FcγRI for available FcR γ chain. This

consequently leads to an inability of immature neutrophils to signal via FcγRI, and loss of FcγRI expression during maturation. As FcγRI triggering on P2 neutrophil precursors induced a small respiratory burst, some FcR γ chain might have been available for FcγRI due to the low FcαRI expression level on these early precursors.

In G-CSF neutrophils, FcR γ chain protein levels, measured by western blot, were considerably increased compared to the levels in bone marrow- and unstimulated blood neutrophils (data not shown, n=3), indicating that more FcR γ chain is availably after G-CSF stimulation. This might lead to the observed upregulation of FcγRI expression and function in G-CSF neutrophils (Fig. 1 and ref [20]). This was furthermore supported in studies with FcαRIxFcγRI double transgenic mice; similar to human neutrophil precursors, mouse bone marrow neutrophils were hindered to mediate ADCC via triggering of FcγRI, but not FcαRI[19]. However, bone marrow neutrophils from G-CSF treated mice could mediate ADCC via triggering of FcγRI, as well (data not shown, n=4).

Taken together, our data suggest that inefficient signaling and effector functions via FcγRI on neutrophils is most likely induced by differences in interaction between FcαRI or FcγRI with the common FcR γ chain. This may lead to competition between both receptors for available FcR γ chain in favor of FcαRI, hereby hindering function via FcγRI.

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Chapter 5

Antibody targeting to FcαRI triggers neutrophil migration towards tumor colonies

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Submitted for publication

ABSTRACT

Neutrophils can potently kill tumor cells in the presence of anti-tumor antibodies (Ab). For *in vivo* targeting of neutrophils, however, cells need to migrate from the circulation, through the endothelial blood barrier, towards tumors. Therefore, to study Ab-induced neutrophil migration, we established a 3-dimensional collagen culture assay, in which either SK-BR-3 or SW-948 tumor colonies were grown. Minimal neutrophil migration towards tumor colonies was observed in the presence of IgG anti-HER-2/neu, or IgG anti-Ep-CAM Ab. Addition of human umbilical vein endothelial cells (HUVEC), hereby mimicking the endothelial blood barrier, did not affect FcγR-induced migration. In contrast, the IgA Fc receptor (FcαRI, CD89), which represents the most potent neutrophil trigger molecule for induction of Ab-mediated cell killing, triggered massive neutrophil migration and tumor colony destruction. In addition, release of chemotactic factors and inflammatory cytokines interleukin-1β and tumor necrosis factor-α were increased, as well. Here, the presence of HUVEC further enhanced production of chemotactic stimuli and neutrophil accumulation into tumor colonies. These data thus support that stimulation of neutrophil FcαRI, but not FcγR, initiates an amplification loop between neutrophils and endothelial cells, leading to enhanced neutrophil migration towards tumor colonies, and tumor kill.

INTRODUCTION

Neutrophils represent the most populous type of cytotoxic effector cells within the blood. They can play a role in tumor rejection *in vivo,* as depletion of these cells results in increased tumor outgrowth in rats and mice[1-3]. Their numbers can, furthermore, be augmented by treatment with granulocyte-colony stimulating factor (G-CSF)[4]. In addition, neutrophils can secrete a plethora of cytokines and chemokines, including macrophage inflammatory proteins, monocyte chemoattractant proteins, and interleukin-8 (IL-8)[5,6], which can attract other immune cells, like monocytes, dendritic cells, and T cells, resulting in more generalized anti-tumor immune responses. Importantly, anti-tumor antibodies (Ab) or bispecific Ab (BsAb) - which establish a link between Fc receptors on immune cells and tumor-associated antigens (TAA) on tumor cells - enhance neutrophil-mediated tumor cell lysis[7,8]. Moreover, blood from G-CSF stimulated donors induces more efficient Ab-mediated tumor cell killing compared to blood from unstimulated donors, which was attributable to the increased neutrophil numbers[9]. Neutrophils might thus represent potent effector cells for Ab therapy for cancer.

Neutrophils constitutively express the IgA Fc receptor FcαRI (CD89), and IgG Fc receptors FcγRIIa (CD32), and FcγRIIIb (CD16)[10,11]. Expression of FcγRI (CD64) can furthermore be induced by interferon-γ (IFNγ) or G-CSF treatment[7]. FcγRI has been documented as the most potent FcγR on neutrophils for initiation of Ab-mediated tumor cell lysis[7,9]. In recent years, however, FcαRI received significant attention as target for Ab therapy as neutrophils effectively lysed tumor cells after FcαRI triggering[12]. FcαRI represents a medium affinity receptor, which poorly interacts with monomeric IgA, but binds polymeric IgA and IgA immune-complexes more avidly[13]. This Fc receptor is selectively expressed on myeloid cells, and initiates effector functions such as Ab-dependent cellular cytotoxicity (ADCC), endocytosis, phagocytosis, and release of inflammatory mediators[14,15]. FcαRI-induced tumor cell killing has been studied for TAA, such as CD20, HLA class II, EGF-receptor, EpCAM, CD30, and Her-2/neu[12,16-18]. Furthermore, targeting of neutrophil FcαRI generally results in more efficient tumor cell lysis compared to FcγRI[17,19,20].

In order to achieve Ab-mediated lysis of solid tumors, neutrophils need to extravasate from the bloodstream. Recently, we showed in 3-dimensional collagen cultures, in which tumor colonies were grown, that FcαRI crosslinking on neutrophils triggered effective tumor-specific migration[20]. In the present study, we addressed the mechanism of Ab-induced neutrophil migration towards tumor colonies in more detail.

MATERIALS AND METHODS

Antibodies.

Antibodies A77 (mIgG1 anti-FcαRI), and 520C9 (mIgG1 anti-Her-2/neu) were produced from hybridomas (Medarex, Bloomsbury, NJ). FcαRIxHer-2/neu BsAb (A77x520C9) were produced by chemically cross-linking F(ab') fragments of 520C9 with F(ab') fragments of A77 as described[21]. FcγRIxHer-2/neu BsAb (22x520C9; MDX-H210) was also obtained from Medarex. Anti-EpCAM IgG and IgA Ab were kindly provided by Prof Dr. A.M. Kruisbeek (Crucell, Leiden, The Netherlands).

Cell lines.

The breast carcinoma cell line SK-BR-3, which over-expresses the TAA Her-2/neu, and the colon carcinoma cell line SW-948, which over-expresses the TAA EpCAM, were obtained from the American Type Culture Collection (Manassas, VA). SK-BR-3 cells and SW-948 cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) or DMEM medium (Gibco BRL), respectively, supplemented with 10% heat-inactivated FCS and antibiotics (RPMI/10% or DMEM/10%). Both SK-BR-3 cells and SW-948 cells were harvested using trypsin-EDTA (Gibco BRL).

Blood donors.

Peripheral blood samples (30 ml) were drawn from healthy untreated volunteers or healthy donors receiving rhG-CSF (Neupogen, 5 μg/kg of body weight, twice daily for five days, Amgen Inc., Thousand Oaks, CA). Studies were approved by the Medical Ethical Committee of Utrecht University (The Netherlands), in accordance with the Declaration of Helsinki. All donors gave informed consent.

Isolation of human neutrophils.

Standard Lymphoprep (Axis-Shield, Rodelokka, Oslo, Norway) density gradient centrifugation was used to isolate neutrophils from heparin anti-coagulated peripheral blood samples. Neutrophils were harvested and erythrocytes were removed by hypotonic lysis (containing 155 mM NH4CL, 10 mM KHCO3, 0.1 mM EDTA). During collagen culture, neutrophils were supplemented with IFNγ (300 units/ml; Boehringer Ingelheim, Alkmaar, The Netherlands) to prevent early apoptosis, and to induce FcγRI expression. Purity of isolated neutrophils was confirmed by cytospin preparations. In all experiments, neutrophil purity exceeded 95% and cell viability was more than 98%, as determined by trypan blue exclusion.

Isolation of human umbilical vein endothelial cells (HUVEC).

Blood was flushed out of umbilical cords with cordbuffer (containing 0.298 g/L KCL, 8.182 g/L NaCl, 2.621 g/L HEPES and 2.178 g/L D-glucose), after which they were incubated for

20 minutes at 37°C with 3350 U collagenase (diluted in M199 medium, Gibco BRL) to isolate endothelial cells. Endothelial cells were harvested, spun down, and cultured in endothelial medium (M199 medium supplemented with 10% pooled human serum, 10% newborn calf serum, 5 ng/ml basic fibroblast growth factor (bFGF), 5 U/ml heparin, glutamin, and antibiotics) in 6-wells plates (NUNC GmbH, Wiesbaden, Germany) that had been coated for 30 minutes at 37°C with 1% gelatine. Confluent grown endothelial cell cultures were harvested using trypsin-EDTA.

Collagen culture assay.

Collagen was isolated from rat tails and dissolved in 0.1% acetic acid (2 mg/ml). MilliQ, 0.34M NaOH and DMEM (10X) (Sigma-Aldrich) were mixed (1:1:1), after which 2.3 ml was added to 10 ml collagen on ice. After SK-BR-3 cells (6.5 x 10^5/ml) or SW-948 cells (5 x 10^5/ml) had been added, the final mixture was plated in 24 wells plates (1 ml/well) and allowed to coagulate, after which 1 ml medium (RPMI/10% or DMEM/10% for SK-BR-3 and SW-948 cells, respectively) was added. To allow tumor colony formation, SK-BR-3 cultures were grown for two weeks and SW-948 cultures for one week. Neutrophils were then added in the absence or presence of Ab or BsAb (1 μg/ml) for the indicated time periods. After that, supernatants were collected for analysis of chemotactic potential. Additionally, levels of IL-8, IL-1β, tumor necrosis factor-α (TNF-α) and lactoferrin were determined. Collagen gels were washed with 150mM NaAc pH 5.0 containing Indian ink (1:100, to mark the top of the collagen gels) for 30 min, fixed overnight at room temperature with zinc salts-based fixative (0.5 g/L calcium acetate, 5.0 g/L zinc acetate, 5.0 g/L zinc chloride in 0.1M Tris buffer)[22] and embedded in paraffin.

For experiments with HUVEC, collagen gels were cultured to allow tumor colony formation as described. Collagen gels were then cultured with RPMI/10% supplemented with 10 ng/ml bFGF and 10 U/ml heparin for 24 hours. After that, HUVEC cells were added. A confluent HUVEC layer was formed in 20 hours, after which neutrophils and Ab were added.

Immuno-histochemistry.

Paraffin sections were de-paraffinised in xylene and ethanol, and endogenous peroxidase was blocked at room temperature with 0.3% H_2O_2 in methanol (30 min). Slides were re-hydrated, after which non-specific binding was blocked by incubation with 10% normal rabbit serum (15 min, room temperature) diluted in PBSA (0.5% BSA in PBS). Neutrophils were stained with mouse anti-human CD66b IgM Ab (Pharmingen, San Diego, CA) for 1 hour (room temperature). After washing with 0.05% PBS-Tween (PBST), slides were incubated with biotinylated rabbit anti-mouse IgM Ab (Zymed, San Francisco, CA) for 30 min (room temperature). Slides were washed and further incubated with horseradish peroxidase (HRP)-labeled Streptavidin (Zymed). 3.3-diaminobenzidine (DAB, Sigma-Aldrich) was used as substrate, resulting in a brown staining. Slides were counterstained with Mayers' haematoxyline (Klinipath, Duiven,

The Netherlands), after which they were de-hydrated in ethanol and embedded in Entallan (Merck, Haarlem, The Netherlands).

Chemotaxis assay (Boyden chamber assay).

Freshly isolated neutrophils were labeled with the fluorescent dye calcein (Molecular Probes, Breda, The Netherlands) for 30 min at 37°C. In a chemotactic chamber (Neuroprobe, Gaithersburg, MD), the upper and lower compartments were separated by a 3 μm pore PVP filter (Corning Costar Inc., Brussel, Belgium). To measure chemotaxis (specific neutrophil migration), the lower compartments of a chemotactic chamber were filled with either supernatants of collagen gels, or IL-8 (30 ng/ml) as a positive control. To measure chemokinesis (random neutrophil migration), either supernatants, or IL-8 were added in both the upper and lower compartments. To block IL-8-induced chemotaxis, supernatants were pre-incubated for 20 minutes at 37°C with 10 μg/ml mouse IgG2b anti-human IL-8 mAb (Pharmingen), before supernatants were added to the chemotactic chambers.

Calcein labeled neutrophils (5 x 10^4 cells) were added to the upper compartment, and allowed to migrate for 40 min at 37°C. Thereafter, the supernatant in the lower compartment (containing migrated neutrophils) was diluted in HTAB buffer (containing 1 g/L Tween 20, 2 g/L hexadecyltrimethyl ammonium bromide (CTAB), 2 g/L BSA and 7.44 g/L EDTA in PBS, pH 7.4). Fluorescence was measured in a fluorimeter at an excitation wavelength of 485 nm, and an emission wavelength at 520 nm. As standard measure, a range of 0 to 50.000 calcein labeled neutrophils was used to calculate the number of migrated neutrophils.

Cytokine ELISA.

IL-8, IL-1β and TNF-α levels in supernatants were analysed by coating 96-wells flat-bottom maxisorb plates (NUNC GmbH) with 1 μg/ml mouse anti-human IL-8, IL-1β or TNF-α Ab (Biosource, Camarillo, CA) overnight at 4°C. Plates were washed with PBST, after which aspecific binding was blocked with 0.5% PBST (0.5% BSA dissolved in PBST) for 1 hour (room temperature). Plates were washed again, and supernatants, or as a standard measure recombinant human IL-8 (0-800 pg/ml), IL-1β (0-320 pg/ml), or TNF-α (0-1000 pg/ml) (Biosource) were added for 2 hours. Next, 0.4 μg/ml biotin-labeled mouse anti-human IL-8, IL-1β or TNF-α detecting Ab (Biosource) was added, followed by washing and a 30 min incubation with HRP-labeled streptavidine (Biosource). TetraMethylBenzidine (MP Biomedicals, Heidelberg, Germany) was used as substrate. The enzymatic reaction was stopped by adding 2N H_2SO_4. Optical density (OD) was measured at 450nm.

Lactoferrin ELISA.

Lactoferrin levels in supernatants were determined by coating 96-wells flat-bottom maxisorb plated (NUNC GmbH) with 1 μg/ml rabbit anti-lactoferrin Ab (Sigma-Aldrich) overnight at 4°C. Plates were washed with PBST and aspecific binding was blocked with 0.5% PBST. Colla-

gen supernatants, or as a standard measure 0 to 1250 ng/ml lactoferrin (Sigma-Aldrich), were incubated for 1 hour at room temperature, after which Alkalin-Phosphatase (AP)-labeled rabbit anti-human lactoferrin Ab (MP Biomedicals, Heidelberg, Germany) was added for 1 hour at 37°C. p-NitroPhenylPhosphate (pNPP, Sigma-Aldrich) was used as substrate. OD was measured at 405nm.

Statistical analyses.

Data are shown as mean ± standard deviation (SD). Group data are shown as mean ± standard error of the mean (SEM). Statistical differences were determined using the two–tailed unpaired Student's *t*-test or ANOVA. Significance was accepted when $p < 0.05$.

RESULTS

FcαRI-triggered neutrophil migration towards tumor colonies.

To study Ab-induced neutrophil migration towards tumor colonies, we established a 3D-collagen culture model. Because mature neutrophils have a short life-span after isolation[23], IFNγ was added to collagen cultures to limit neutrophil apoptosis. Additionally, IFNγ induces neutrophil FcγRI surface expression[7], which enabled us to study FcγRI-mediated neutrophil migration in parallel. In the presence of anti-Her-2/neu IgG, neutrophil migration towards SK-BR-3 tumor colonies was minimal in most donors, and absent in ~10% of donors (Fig. 1A, and data not shown). Furthermore, negligible migration towards tumor colonies was observed in the presence of FcγRIxHer-2/neu BsAb. As this could have been attributable to insufficient time required for induction of FcγRI surface expression by IFNγ, experiments were repeated with neutrophils from G-CSF stimulated donors, which express high FcγRI levels. This did, however, not lead to enhanced tumor-directed neutrophil migration either (Fig. 1B). In contrast, high numbers of neutrophils migrated towards SK-BR-3 colonies in response to addition of FcαRIxHer-2/neu BsAb, which triggered destruction of tumor colonies (Fig. 1A, and 1B). As G-CSF and IFNγ neutrophils were equally effective, further experiments were performed with IFNγ neutrophils.

Next, we investigated whether anti-tumor IgA Ab, which is the natural ligand for FcαRI, induced tumor-directed neutrophil migration. Because IgA and IgG anti-EpCAM Ab were available, collagen cultures were set up using SW-948 tumor cells that over-express EpCAM. FcγRI triggering via anti-EpCAM IgG induced minimal neutrophil - tumor cell contact, whereas addition of anti-EpCAM IgA resulted in profound neutrophil accumulation and destruction of SW-948 tumor colonies (Fig. 1C). The poorer migration via engagement of FcγR was not attributable to ineffective cell-cell interactions, as higher neutrophil binding to tumor cells was found in the presence of anti-EpCAM IgG Ab, compared to anti-EpCAM IgA (data not shown, and ref. 20). Fixation of collagen gels prior to neutrophil addition did not affect FcαRI-

mediated tumor-directed neutrophil migration, indicating that increased migration was due to release of chemotactic factors from neutrophils, and not from tumor colonies (data not shown).

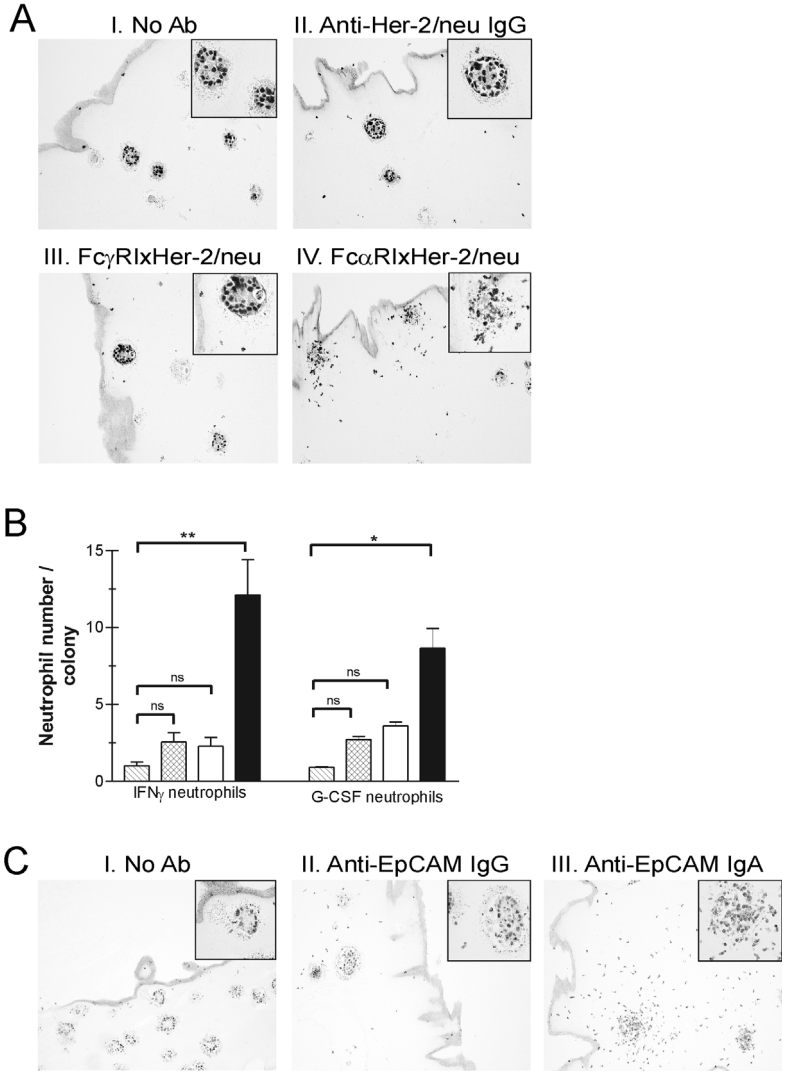

Figure 1. Antibody-induced neutrophil migration towards tumor colonies. For a full-color picture, see Appendix page 170. Collagen cultures were fixed 24 hours after addition of neutrophils and slides were stained for CD66b (neutrophils, brown). (A) IFNγ neutrophils were added to SK-BR-3 tumor colonies (blue) in collagen, either in the absence (panel I), or presence of anti-Her-2/neu IgG Ab (panel II), FcγRIxHer-2/neu BsAb (panel III), or FcαRIxHer-2neu BsAb (panel IV). One representative example out-of-eight is shown. (B) Numbers of IFNγ (left) or G-CSF (right) neutrophils per tumor colony in the absence (hatched bars), or presence of anti-Her-2/neu IgG Ab (cross-hatched bars), FcγRIxHer-2/neu BsAb (white bars) or FcαRIxHer2/neu BsAb (black bars). Results represent mean ± SEM from three experiments. *: $p<0.05$, **: $p<0.01$, ns: non significant. (C) IFNγ neutrophils were added to SW-948 tumor colonies (blue), either in the absence (panel I), or presence of anti-EpCAM IgG (panel II) or IgA (panel III) Ab. One representative example out-of-three is shown.

Antibody-induced release of chemotactic factors by neutrophils.

The presence of chemotactic stimuli was measured in supernatants of SK-BR-3 collagen gels, 24 hours after neutrophil addition. No migration of neutrophils was observed to the lower compartments of Boyden chambers that contained supernatants of either anti-Her-2/neu IgG Ab, or FcγRIxHer-2/neu BsAb stimulated collagen gels. In contrast, supernatants of FcαRIxHer-2/neu BsAb stimulated collagen gels consistently induced increased neutrophil accumulation (Fig. 2A).

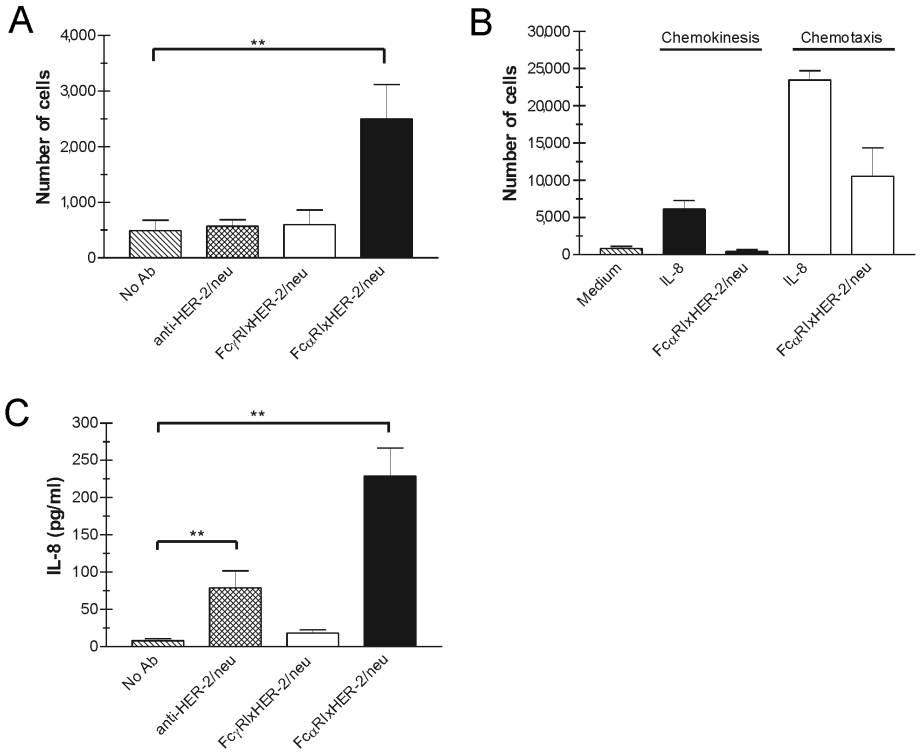

Figure 2. Antibody-induced neutrophil chemotaxis and IL-8 release. (A) Numbers of freshly isolated neutrophils that migrate towards supernatants in the lower compartments of Boyden chambers. Supernatants were collected from SK-BR-3 collagen gels 24 hours after incubation without (hatched bar), or with anti-Her-2/neu IgG Ab (cross-hatched bar), FcγRIxHer-2/neu BsAb (white bar) or FcαRIxHer-2/neu BsAb (black bar). Bars represent mean ± SEM from 5 individual experiments. (B) Chemokinesis (black bars) and chemotaxis (white bars) of neutrophils in response to IL-8 (as a control), or supernatants of FcαRIxHer-2/neu stimulated SK-BR-3 collagen gels. Hatched bar represents negative control (medium only). (C) IL-8 levels in supernatants of SK-BR-3 collagen gels stimulated without (hatched bar), or with anti-Her-2/neu IgG Ab (cross hatched bar), FcγRIxHer-2/neu BsAb (white bar) or FcαRIxHer-2/neu BsAb (black bar). Bars represent mean ± SEM from 10 individual experiments. **: $p<0.01$.

We next determined whether this was attributable to increased random neutrophil movement (chemokinesis), or to specific neutrophil migration (chemotaxis). To measure chemokinesis, both compartments of Boyden chambers were filled with supernatant, or with high levels of IL-8 (as a positive control). The number of neutrophils that migrated randomly into the lower compartments was assessed (Fig. 2B). Neutrophil numbers were somewhat increased if IL-8 was added to both compartments, indicating that neutrophils were activated in the presence of IL-8, leading to enhanced random movement. However, supernatants of FcαRIxHer-2/neu BsAb stimulated collagen gels did not induce increased chemokinesis of neutrophils. To measure neutrophil chemotaxis, a gradient is required, which was accomplished by addition of supernatant (or IL-8) in the lower compartments only. Both IL-8 and supernatant of FcαRIxHer-2/neu BsAb stimulated collagen gels induced elevated accumulation of neutrophils into the lower compartments. These data indicate that triggering of FcαRI specifically evoked chemotaxis, and not random neutrophils migration.

Because IL-8 represents the prototypic neutrophil chemoattractant[6], IL-8 levels in supernatants were also determined. No IL-8 was detected in supernatants of FcγRIxHer-2/neu BsAb stimulated collagen gels (Fig. 2C). IL-8 levels were increased in ~80% of supernatants of collagen gels, which were stimulated with anti-Her-2/neu IgG. However, no IL-8 was detected in 20% of the samples (Fig. 2C, and data not shown). Addition of FcαRIxHer-2/neu BsAb consistently resulted in elevated IL-8 levels. As FcγR neither induced efficient neutrophil migration, nor enhanced chemotactic levels, further experiments focused on FcαRIxHer-2/neu BsAb. The parental anti-Her-2/neu IgG Ab served as a control.

Kinetics of antibody-induced neutrophil migration.

To investigate the kinetics of Ab-induced neutrophil migration, collagen gels were fixed at several time-points after neutrophil addition (Fig. 3A). In the presence of FcαRIxHer-2/neu, neutrophils migrated into collagen gels within 1 hour, followed by neutrophil accumulation around tumor colonies between 2 and 4 hours (Fig. 3A, panel III). Damage to tumor colonies became apparent after 8 hours, and massive destruction (reflected by remnants of tumor colonies between accumulated neutrophils) was observed after 24 hours (Fig. 3A, panel VI). Conversely, anti-Her-2/neu IgG induced minor neutrophil migration into collagen gels between 2 and 4 hours (Fig. 3A, panel II). Some interactions between neutrophils and tumor colonies were observed after 8 and 24 hours, but tumor colony destruction was never detected (Fig. 3A, panel V).

As a measure for neutrophil degranulation, lactoferrin release (from specific granules) was determined. Lactoferrin levels in supernatants of FcαRIxHer-2/neu stimulated collagen gels were raised within 2 hours, and further increased until 8 hours, after which a plateau level was reached (Fig. 3B). In contrast, lactoferrin levels in supernatants of anti-Her-2/neu IgG stimulated collagen gels were only marginally increased in time.

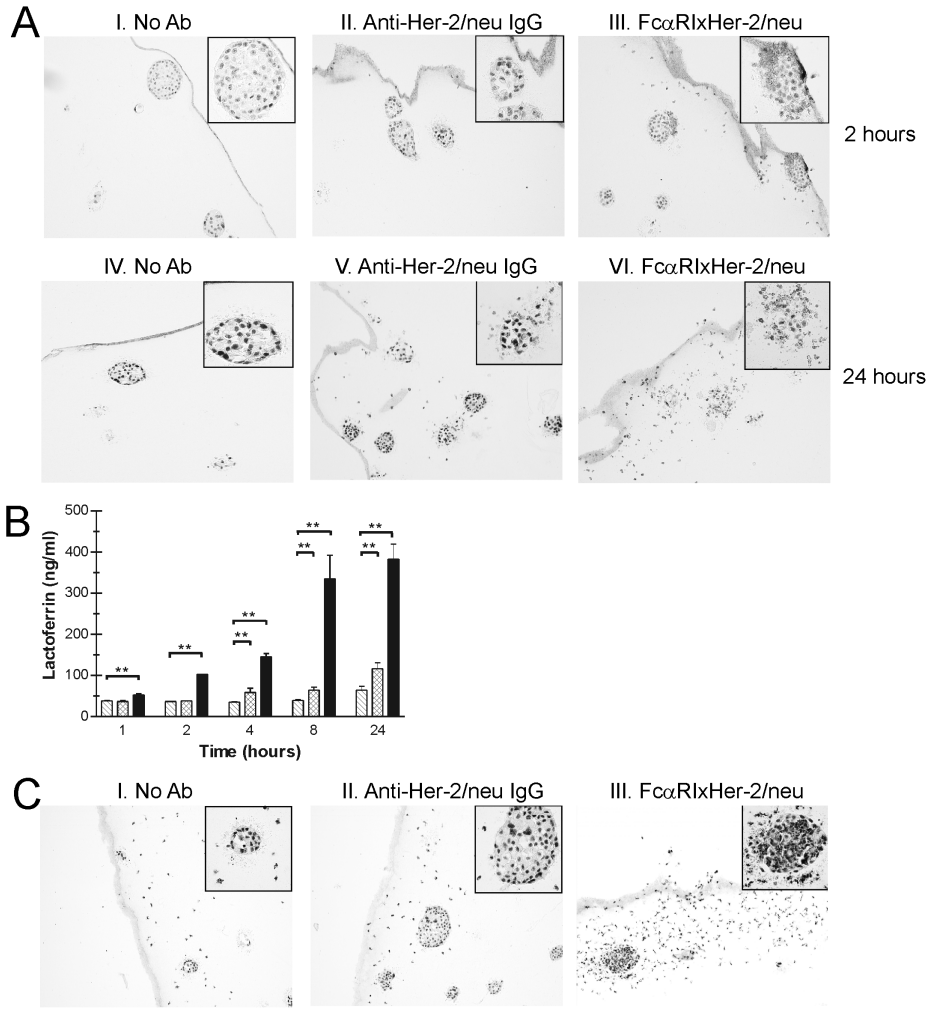

Figure 3. Neutrophil kinetics and responses to HUVEC layered SK-BR-3 colonies in collagen gels. For a full-color picture, see Appendix page 171. (A) SK-BR-3 colonies (blue) in collagen gels, incubated without (panels I, IV), or with anti-Her-2/neu IgG Ab (panels II, V), or FcαRIxHer-2/neu BsAb (panels III, VI), were fixed either 2 hours (panels I-III) or 24 hours (panels IV-VI) after addition of IFNγ neutrophils (brown). One representative experiment out-of-five is shown. (B) Lactoferrin levels were determined in supernatants of SK-BR-3 collagen gels incubated without (hatched bars), or with anti-Her-2/neu IgG Ab (cross-hatched bars) or FcαRIxHer-2/neu BsAb (black bars) for the indicated times. Results represent mean levels ± SEM of 5 separate experiments. (C) IFNγ neutrophils (brown) were added for 24 hours to HUVEC-layered collagen gels containing SK-BR-3 colonies (blue), either in the absence (panel I), or presence of anti-Her-2/neu IgG Ab (panel II), or FcαRIxHer-2/neu BsAb (panel III). Data from one representative experiment out-of-four are shown.

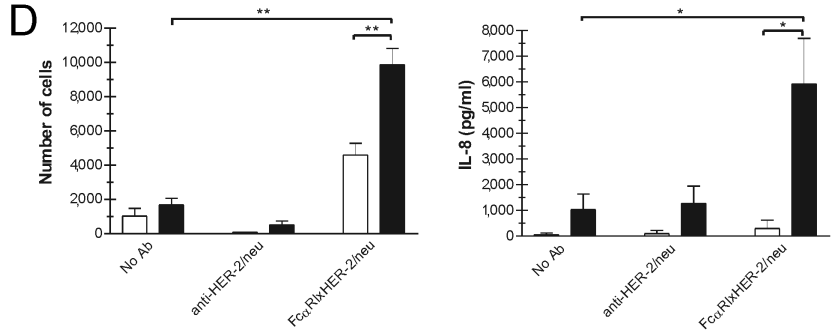

Figure 3. Neutrophil kinetics and responses to HUVEC layered SK-BR-3 colonies in collagen gels. Chemotactic potential (D, left panel), and IL-8 levels (D, right panel) were determined in supernatants of collagen gels, cultured without (white bars) or with (black bars) HUVEC monolayers. Data are presented as mean ± SEM of 3 individual experiments. *: $p<0.05$, **: $p<0.01$.

HUVEC enhances tumor-directed neutrophil migration after FcαRI triggering.

The influence of endothelial cells on Ab-induced neutrophil migration was studied by growing HUVEC as confluent monolayers on top of SK-BR-3 collagen gels. Under all conditions, the presence of HUVEC increased random neutrophil movement into collagen gels (Fig. 3C). However, augmented tumor-directed neutrophil migration and tumor colony destruction was only observed after addition of FcαRIxHer-2/neu. Furthermore, supernatants of collagen gels stimulated with FcαRIxHer-2/neu, but not anti-Her-2/neu IgG, showed an enhanced chemotactic potential in the presence of HUVEC (Fig. 3D, left panel). In addition, the presence of HUVEC profoundly amplified IL-8 levels in supernatants of FcαRIxHer-2/neu-stimulated collagen gels, but not in supernatants of anti-Her-2/neu IgG-stimulated collagen gels (Fig. 3D, right panel).

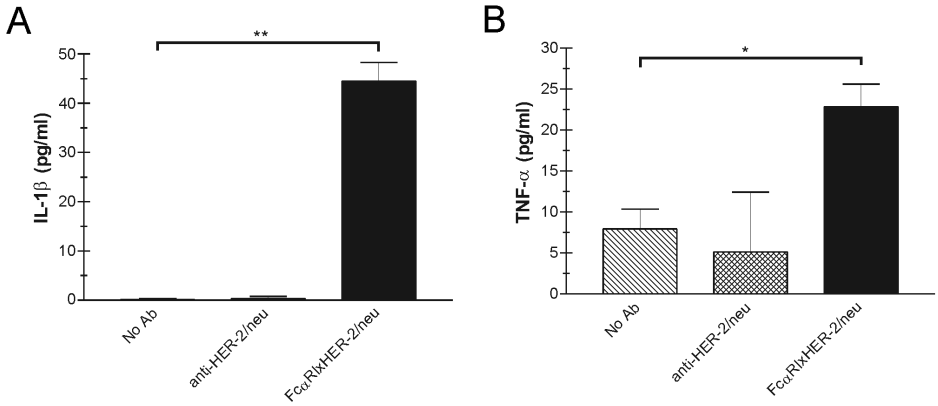

Figure 4. IL-1β and TNF-α levels in supernatants. (A) IL-1β and (B) TNF-α levels in supernatants of SK-BR-3 collagen gels, which were incubated for 24 hours with neutrophils in the absence (hatched bars), or presence of anti-Her-2/neu IgG Ab (cross-hatched bars), or FcαRIxHer-2/neu BsAb (black bars). Data represent mean ± SD of triplicates. Data in panel A and B are representative for one experiment out-of-three. *: $p<0.05$, **: $p<0.01$.

FcαRI-induced release of IL-1β and TNF-α by neutrophils.

Pro-inflammatory cytokines, can induce IL-8 secretion by neutrophils and endothelial cells[24,25]. IL-1β and TNF-α levels were therefore determined in supernatants of SK-BR-3 collagen gels. Neither IL-1β, nor TNF-α levels were altered in supernatants of collagen gels incubated with anti-Her-2/neu IgG, compared to supernatants of control gels (Fig. 4A, and 4B). By contrast, addition of FcαRIxHer-2/neu to collagen gels led to increased IL-1β and TNF-α levels in supernatants. Presence of HUVEC did not affect cytokine levels (data not shown), which indicated that neutrophils were the main source of secreted IL-1β and TNF-α.

Role of IL-8 in tumor-specific neutrophil migration.

To examine the relationship between chemotaxis and IL-8 levels, supernatants were collected from FcαRIxHer-2/neu stimulated SK-BR-3 collagen gels at several time-points. Surprisingly, the chemotactic activity of supernatants of FcαRIxHer-2/neu stimulated collagen gels, either in the presence or absence of HUVEC, peaked around 4 hours (Fig. 5A), whereas IL-8 levels

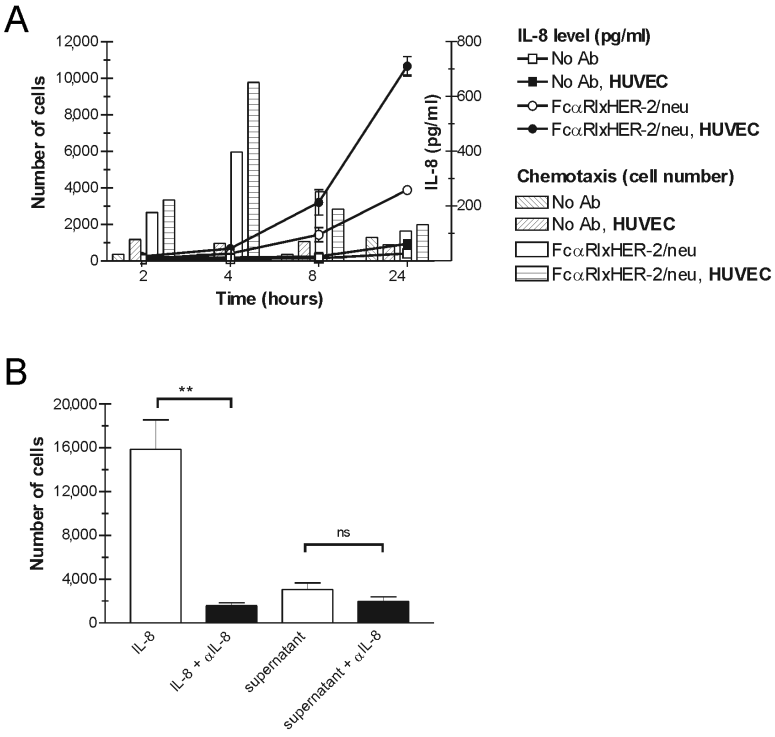

Figure 5. Kinetics of chemotaxis and IL-8 production. (A) Neutrophils were added without, or with FcαRIxHer-2/neu BsAb to collagen gels containing SK-BR-3 colonies, in the absence or presence of a HUVEC layer. Supernatants were collected at the indicated time-points, and chemotactic activities and IL- 8 levels were determined. One representative experiment out-of-three is shown. (B) The chemotactic potential of IL-8 (as a control), or of supernatants of SK-BR-3 collagen gels that were incubated for 4 hours with FcαRIxHer-2/neu BsAb, was measured in the absence (white bars) or presence (black bars) of a blocking anti-IL-8 mAb (αIL-8). Data represent mean ± SD from triplicate samples. Blocking experiments were repeated twice, yielding similar results. **: $p<0.01$, ns: non significant.

started to increase only after 8 hours. After 24 hours, at which time chemotactic activity of supernatants was decreased, significantly enhanced IL-8 levels were observed, most notably in supernatants of FcαRIxHer-2/neu stimulated collagen gels containing HUVEC.

Furthermore, addition of an anti-IL-8 mAb blocked migration of neutrophils into IL-8 containing lower compartments of Boyden chambers (Fig. 5B). Anti-IL-8 mAb, however, did not affect the chemotactic potential of supernatants collected from collagen gels stimulated for 4 hours with FcαRIxHer-2/neu (the time at which maximal chemotactic activity was observed). Moreover, incubation of FcαRIxHer-2/neu stimulated collagen gels with an anti-IL-8 mAb did not affect neutrophil migration into tumor colonies either (data not shown).

DISCUSSION

The therapeutic use of Ab for cancer has received increasing attention in the last decades[26]. We here studied Ab-induced tumor-directed migration of neutrophils and observed that FcαRI targeting on neutrophils consistently induced release of chemokines, pro-inflammatory cytokines, as well as strong neutrophil migration towards tumor colonies, which resulted in tumor cell killing. In contrast, targeting FcγRI was ineffective, as neither release of chemoattractants and cytokines, nor neutrophil migration was observed in the presence of FcγRI BsAb. Interestingly, surface expression, signaling, and effector functions of both FcαRI and FcγRI are mediated via the Fc receptor γ chain (FcR γ chain)[27-30]. FcαRI, however, has a stronger association with the FcR γ chain, due to an additional electrostatic interaction within its transmembrane region[31], which may result in more efficient neutrophil activation via FcαRI. Furthermore, a subpopulation of FcαRI is expressed on neutrophils without associating FcR γ chain[32], suggesting that FcαRI might interact with an - up till now - unidentified molecule. These observations might explain the functional differences between FcαRI and FcγRI. In addition, responses in the presence of anti-tumor IgG were minimal, and tumor colony destruction was not observed. Moreover, neutrophils from several donors did not respond at all to anti-tumor IgG. This is presumably attributable to a polymorphism in FcγRIIa, as neutrophils of FcγRIIa-H/H131 donors have previously been shown less effective in triggering ADCC compared to FcγRIIa-R/R131 neutrophils[9,33].

Targeting of neutrophil FcαRI resulted in elevated IL-8 levels in the supernatants of collagen gels. No correlation, however, was found between IL-8 levels and the chemotactic potential of supernatants. Furthermore, an anti-IL-8 blocking mAb did not affect neutrophil migration towards tumor colonies, supporting that IL-8 was not the key chemoattractant in our assays. This does, however, not necessarily exclude a role for IL-8 in FcαRI-induced neutrophil migration, *in vivo*. In our *in vitro* model, 30 ng/ml IL-8 was required to induce neutrophil migration, whereas only picograms of IL-8 were detected in supernatants. These low IL-8 levels may be due to the limited numbers of neutrophils that can be added to collagen gels, and

a short half-life of isolated neutrophils[23]. FcαRI-mediated neutrophil migration in collagen gels was therefore likely induced by other granulocyte chemoattractants, like complement component C5a, platelet activating factor (PAF; mostly an eosinophil chemoattractant), or leukotriene B4 (LTB4)[6]. However, C5a was not present in our cultures as heat-inactivated sera were used. Furthermore, in chemotaxis assays, PAF induced only minor neutrophil influxes, whereas LTB4 initiated massive neutrophil migration (data not shown). Because neutrophil activation leads to upregulation of LTB4 receptor expression, peaking at 4 hours[34], and as this

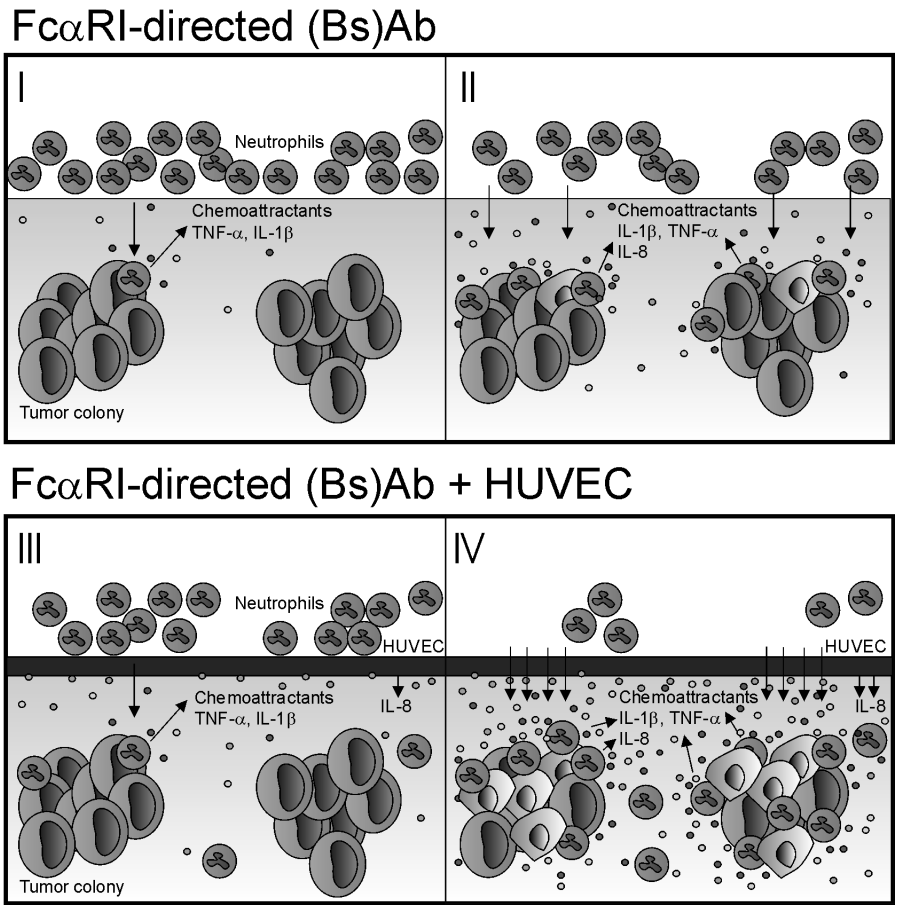

Figure 6. Model of FcαRI-induced neutrophil migration. (I) Some neutrophils migrate randomly into collagen gels, thereby interacting with tumor colonies. In the presence of FcαRI-directed BsAb, these contacts lead to neutrophil activation followed by release of chemotactic stimuli and inflammatory mediators. (II) Consequently, more neutrophils are attracted and activated, leading to tumor cell lysis and secretion of cytokines IL-1β and TNF-α, which enhance neutrophil IL-8 production. (III) In the presence of HUVEC, neutrophils randomly cross endothelial cells, which become activated and start to secrete IL-8, thereby attracting additional neutrophils. In the presence of FcαRI-directed BsAb, interaction of neutrophils with tumor cells triggers release of chemotactic stimuli and inflammatory mediators, (IV) thereby attracting even more neutrophils. IL-1β and TNF-α enhance IL-8 production by neutrophils and endothelial cells, resulting in a profound neutrophil accumulation and tumor colony destruction.

coincides with the observed kinetics of chemotaxis in supernatants, LTB4 may well contribute to the chemoattractant activity in collagen assays.

Thus, we hypothesize that in our model several neutrophils migrate randomly into collagen gels, thereby interacting with tumor colonies. If FcαRI is triggered (via mAb or BsAb), this contact results in neutrophil activation and release (or synthesis) of chemotactic stimuli, hereby attracting other neutrophils. In addition, activated neutrophils, lyse tumor cells and secrete inflammatory mediators, like IL-1β and TNF-α[6]. As IL-1β and TNF-α enhance IL-8 production by neutrophils[35], and IL-8 and TNF-α were shown to upregulate FcαRI expression levels[14,15], an autocrine loop might be initiated (Fig. 6, panels I, and II).

FcαRI-induced chemokine release (amongst which IL-8), neutrophil migration and tumor colony destruction were furthermore enhanced in the presence of a HUVEC layer. This might be explained by the initiation of an additional paracrine loop, since IL-1β and TNF-α were also reported to induce production of IL-8 by endothelial cells[24,36], which results in more massive neutrophil migration and concomitant tumor destruction (Fig. 6, panels III, and IV). As FcγR neither induced release of chemoattractants, nor inflammatory cytokines, the failure to initiate such a cascade might well underlie the absence of neutrophil migration.

In conclusion, antibody-mediated neutrophil migration towards tumor colonies was only observed via FcαRI engagement, which induced a cascade of events, including cytokine and chemokine release. Moreover, FcαRI-mediated effector functions were further increased in the presence of an endothelial barrier, suggesting a paracrine amplification loop between neutrophils and endothelial cells. Taken together, our data suggest that targeting of FcαRI leads to a more efficient neutrophil recruitment towards tumors, which might result in better tumor cell killing.

ACKNOWLEDGEMENTS

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Chapter 6

Inefficient antigen presentation via the IgA Fc receptor (FcαRI) on Dendritic Cells

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ABSTRACT

Dendritic cells (DC) are professional antigen presenting cells that can induce and regulate adaptive immune responses. For that reason, DC are attractive candidates for vaccination strategies. Recently, expression of the IgA Fc receptor (FcαRI, CD89) was observed on DC, which activation led to DC maturation. We now investigated the potential of DC FcαRI as a target molecule for vaccination against cancer. FcαRI expression was observed on human blood myeloid DC. Furthermore, expression of FcαRI was low on immature DC, cultured from either human monocytes or FcαRI transgenic (Tg) mouse bone marrow cells. Addition of TNF-α to culture regimes of both human and mouse DC, led to more semi-mature DC, on which FcαRI expression was slightly upregulated. FcαRI on both human and FcαRI Tg mouse DC was internalized after receptor crosslinking. Antigen presentation, measured in FcαRI Tg mouse DC, was however minimal. As antigen presentation is crucial to elicit effective T cell responses, these data suggest that targeting of DC FcαRI is not optimal for DC vaccination strategies.

INTRODUCTION

Dendritic cells (DC) are potent antigen presenting cells, and have a unique ability to induce and regulate activation of T cells, B cells, natural killer cells and natural killer T cells[1]. In peripheral tissues, immature DC sample their environment, take-up and process antigens and migrate to draining lymph nodes, where peptides are presented on MHC molecules to T cells. In the absence of co-stimulation, this presentation leads to immunological tolerance to self antigens[2]. Only in the presence of a "secondary signal", like the presence of microbial components or inflammatory cytokines, DC mature, after which they migrate to lymph nodes. Mature DC have a decreased endocytotic functionality, but upregulation of MHC and costimulatory molecules allows priming of T cells and initiation of adaptive immune responses[3,4].

DC which are loaded with tumor-antigens - either via direct binding of peptides to MHC molecules or via processing of endocytosed tumor lysates - can evoke tumor-specific immune responses[1,5]. DC are therefore attractive candidates for cancer vaccination strategies. DC can take-up tumor lysates via micropinocytosis, phagocytosis, or receptor-mediated endocytosis[6], via C-type lectin receptors, like the mannose receptor and DEC-205[7], or Fc receptors[8]. As IgG antibodies (Ab) could enhance the efficiency of DC antigen presentation[9], the potential of leukocyte IgG receptors (FcγR) as target for vaccination therapies has been investigated. Targeting of IgG immune-complexed tumor-antigens to DC FcγR, was shown to elicit DC maturation, and to evoke potent anti-tumor T cell responses[8,10]. However, DC express all three classes of leukocyte FcγR, including the inhibitory FcγRIIb[11], which can downregulate immune responses. FcγR mediated anti-tumor responses were greatly enhanced in FcγRII knockout mice, compared to wildtype mice[12], indicating that responses via IgG immune-complexes can be strongly downregulated by the inhibitory receptor. Therefore, the IgA Fc receptor (FcαRI, CD89), which was recently identified on DC[13-15], might represent an alternative target for vaccination therapies.

FcαRI is expressed on cells of the myeloid lineage[16,17], and plays a role in the clearance of bacteria[18]. Several DC-types express FcαRI, including interstitial-type DC, $CD34^+$ progenitor-derived DC and monocyte-derived DC (MoDC), but not Langerhans-type DC[13-15]. After DC activation, FcαRI expression is upregulated, which is in contrast to other Fc receptors[15]. Furthermore, FcαRI crosslinking induces receptor internalization and DC activation[13]. However, in addition to efficient uptake of tumor antigens, effective antigen processing and presentation to T cells are prerequisites for the induction of successful immune responses[1]. It was previously demonstrated that FcαRI can take-up and present IgA-Ovalbumin (OVA) immune-complexes to OVA-specific T cells, using FcαRI-transfected IIA1.6 cells (FcαRI-IIA1.6)[19]. In the current study, we therefore investigated the antigen presenting capacity of FcαRI on DC.

MATERIALS AND METHODS

Cell lines.

OVA-specific mouse $CD4^+$ T cells (DO-11-10 cell line), and IIA1.6 cells (murine B cell line) were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK), supplemented with 10% heat-inactivated FCS and antibiotics (RPMI/10%). FcαRI/FcR γ chain-transfected IIA1.6 cells (FcαRI-IIA1.6 cells)[20], were cultured in the presence of methotrexate (10 μM).

Antibodies and flow cytometry.

FcαRI expression on cultured DC was determined by incubation with PE-labeled A59 (Becton Dickinson, Franklin Lakes, NJ). Human DC were stained with FITC-labeled anti-CD14 Ab (clone TUK4, DAKO, Glostrup, Denmark), PE-labeled anti-CD11c Ab (clone B-ly6, BD), PE-labeled CD1a Ab (clone HI149, BD), FITC-labeled anti-HLA-DR Ab (clone L243, BD), FITC-labeled anti-CD80 Ab (clone DAL-1, Serotec, Oxford, UK), or PE-labeled anti-CD86 Ab (clone IT2.2, BD).

Mouse DC were incubated with FITC-labeled anti-CD11b Ab (clone M1/70, BD), biotin-labeled anti-CD11c Ab (clone HL3, BD) followed by PE-labeled streptavidin (BD), PE-labeled anti-CD40 Ab (clone 3.23, Immunotech, Marseille, France), or unconjugated anti-MHC class II Ab (clone M5/114.15.2, BD), anti-CD80 Ab (clone 16-10A1, BD), or anti-CD86 Ab (clone GL1, BD), followed by FITC labeled anti-rat IgG Ab (Jackson Sanbio, West Grove, PA). FcαRI expression on myeloid DC in whole blood was determined by incubation with anti-FcαRI mAb (clone A77, mIgG1, produced from hybridoma obtained from Medarex, Bloomsbury, NJ) followed by biotin-labeled horse anti-mouse IgG1 (Vector Laboratories, Burlingame, CA) and APC-labeled streptavidine (BD). Blood was stained with Cy-5-labeled anti-CD19 Ab (clone HIB19, BD), FITC-labeled anti-HLA-DR Ab (clone L243, BD), and PE-labeled anti-CD1c (BDCA-1) Ab (clone AD5-8E7, Miltenyi Biotec, Auburn, CA).

For analysis, aspecific binding to DC was blocked by incubation with 5% mouse serum (30 minutes, room temperature). Cells were washed with FACS buffer (1%BSA, 0.1% sodium azide diluted in PBS), after which cells were incubated with different monoclonal Ab (mAb). Unconjugated Ab were detected with a fluorescently labeled secondary Ab, or a secondary biotin-conjugated Ab followed by APC-labeled streptavidin. All cells were incubated for 30 minutes at 4°C with Ab, after which cells were washed with FACS buffer. Unstained cells, isotype controls and secondary Ab were used as negative controls. Cells were analyzed on a FACScan (BD).

Generation of human DC.

Monocyte derived DC (MoDC) were obtained as described by Chen *et al.*[21]. Briefly, mononuclear cells were isolated from heparanized peripheral blood of healthy volunteers by standard Ficoll-Paque (Sigma Aldrich, Steinheim, Germany) density gradient centrifugation. Monocytes were purified by discontinuous percoll gradient centrifugation (successively 60%,

47.5% and 30% of percoll). Cells (0.5 x 10^6) were incubated for 30 minutes in RPMI/10% at 37°C, after which non-adherent cells were removed. Monocytes were cultured for 6 days in RPMI/10%, supplemented with 500 U/ml granulocyte/macrophage-colony stimulating factor (GM-CSF; kindly provided by Amgen Inc., Thousand Oaks, CA) and 50 ng/ml interleukin-4 (IL-4; Strahtmann, Hanover, Germany), or 500 U/ml GM-CSF, 50 ng/ml IL-4 and 100 ng/ml tumor necrosis factor alpha (TNF-α; HyCult Biotechnology, Uden, The Netherlands), after which DC were harvested. Purity of MoDC was determined by staining cells for CD1a, CD11c and CD14.

Mice.

FcαRI (CD89) transgenic (Tg) mice[22], were bred and maintained at the Central Animal Facility of the Utrecht University, The Netherlands. For all experiments, Tg mice were matched with non-transgenic (NTg) littermates. Experiments were approved by the Utrecht University animal ethics committee and performed according to institutional and national guidelines.

Generation of murine DC.

Bone marrow-derived DC (BMDC), were obtained as described by Inaba *et al*.[23]. In short, bone marrow cells were flushed from mouse femurs and incubated on ice with a lysis solution of pH 7.4 (0.16M ammonium-chloride, 0.01M potassium bicarbonate and 0.1mM sodium-edetate, pH7.4) for 2 minutes to remove erythrocytes. Cells (1x10^6/ml) were grown in filtered RPMI/10% supplemented with 15 ng/ml GM-CSF (Cell Sciences Inc., Norwood, MA), 15 ng/ml GM-CSF and 50 ng/ml IL-4 (Strahtmann, Hanover, Germany), or 15 ng/ml GM-CSF and 50 ng/ml TNF-α (Biosource International, Camarillo, CA). Non-adherent cells were replated on day 1. At day 2 and 4, culture medium was refreshed. On day 7, non-adherent and loosely adherent DC were harvested. DC purity was determined by staining cells for CD11b and CD11c.

Internalization assay.

DC were incubated for 30 min at 4°C with 10 μg/ml anti-FcαRI (A77) mAb, diluted in 5% BSA (in PBS). Cells were washed and incubated with $F(ab)'_2$ fragments of goat anti-mouse IgG1 mAb (SBA, Birmingham, AL) (30 min at 4°C), after which samples were split. One sample was kept at 4°C for analysis of total FcαRI surface expression, whereas the other samples were put at 37°C to allow internalization for the indicated time-points. Internalization was stopped by adding ice-cold FACS-buffer. Remaining surface expression of FcαRI was visualized by staining for 30 min at 4°C with $F(ab)'_2$ fragments of FITC-labeled rabbit anti-goat IgG (H+L) Ab (Jackson Sanbio). Cells were analyzed on a FACScan (BD).

Antigen presentation.

FcαRI-mediated antigen presentation was performed as described by Shen *et al.*[19]. In short, IgA_2 anti-NIP (200 μg/ml) was complexed with OVA-NIP (1mg/ml) for 20 minutes at 37°C to

form IgA anti-NIP/NIP-OVA (IgA-OVA) immune complexes. BMDC or FcαRI-IIA1.6 cells (1 x 10^5) were incubated with DO-11-10 cells (2 x 10^4) and various concentrations of IgA-OVA complex for 20 hours at 37°C. Excess OVA (400 μg/ml) was used as positive control. IL-2 production by DO-11-10 cells was measured in supernatants by ELISA; 96-wells maxisorp plates (NUNC, Roskilde, Denmark) were coated overnight at 4°C with 1 μg/ml anti-IL-2 capture Ab (BD). After blocking with 1% BSA (1 hour, room temperature), samples were added for 2 hours. Plates were washed and further incubated at room temperature with 2 μg/ml anti-IL-2 detection Ab (BD), followed by incubation for 1 hour horseradish peroxidase-labeled streptavidine (Beckman Coulter, Fullerton, CA). ABTS (Roche Diagnostics, Mannheim, Germany) was used as a substrate and optical density was measured at a wavelength of 405 nm.

RESULTS AND DISCUSSION

FcαRI expression on human DC.

To determine FcαRI expression on blood DC populations, blood was collected from healthy volunteers. Similar to FcγR[24], FcαRI expression was not detected on BDCA-4$^+$ plasmacytoid DC (data not shown). HLA-DR$^+$, BDCA-1$^+$, CD19$^-$ myeloid DC, consisted of both a FcαRI-positive (28% ± 13.5% SEM) and a FcαRI-negative population (Fig. 1). As FcαRI expression was increased on activated DC compared to immature DC[15], a mixture of immature and activated myeloid DC might explain the heterogeneous expression pattern on BDCA-1$^+$ DC in blood.

As the low percentage of FcαRI-positive blood myeloid DC precluded functional studies, we cultured CD11c$^+$, CD14$^-$ immature MoDC in the presence of GM-CSF and IL-4. These DC expressed low levels of MHC, CD80 and CD86 (data not shown). Using this culture regime, MoDC of only ~40% of donors expressed low FcαRI levels (Fig. 2A). Because TNF-α has been shown to upregulate FcαRI expression on myeloid cells[17,25], and effector functions of MoDC

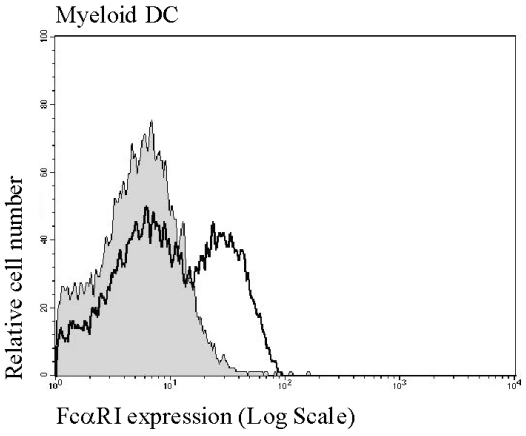

Figure 1. FcαRI expression on myeloid dendritic cells from blood. Surface expression of FcαRI (bold line) determined by flow cytometry on HLA-DR$^+$, BDCA-1$^+$, CD19$^-$ human myeloid DC in whole blood. Cells were stained with A77 (anti-FcαRI) mAb, followed by incubation with biotin-labeled horse anti-mouse IgG1, and APC-labeled streptavidine, and gated for HLA-DR$^+$, BDCA-1$^+$, CD19$^-$ cells. The filled area represents secondary and third Ab alone. One representative experiment out-of-two is shown.

like induction of allegeneic T cell proliferation and antigen presentation, have been documented to be enhanced by this cytokine[21,26], TNF-α was added to the cultures. In the presence of TNF-α, MoDC differentiated towards more semi-mature DC, with increased expression of HLA-DR, CD80 and CD86 (data not shown, and ref 21). This culture regime resulted in FcαRI expression in 75-80% of donors (Fig. 2B), which was similar to observations by Pasquier *et al.*, who showed that activated MoDC expressed higher FcαRI levels[15].

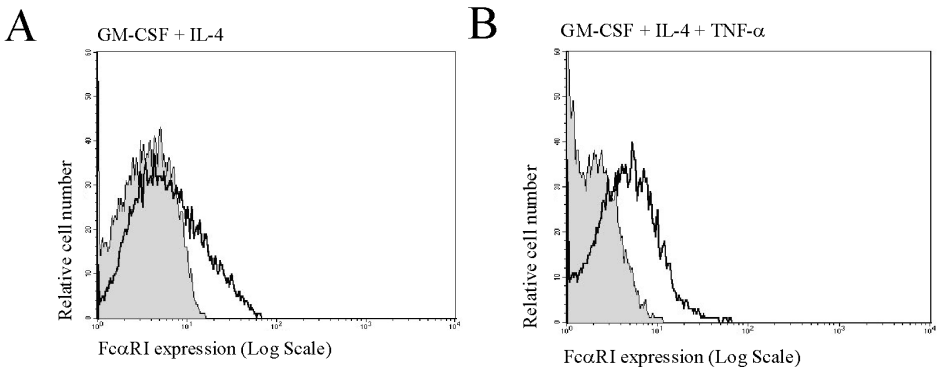

Figure 2. FcαRI expression on human monocyte-derived dendritic cells. FcαRI surface expression levels (bold lines) determined by flow cytometry on human CD11c$^+$, CD14$^-$ MoDC, cultured with (A) GM-CSF and IL-4, or (B) GM-CSF, IL-4 and TNF-α. Cells were stained with PE-labeled A59 (anti-FcαRI) mAb, or as a control PE-labeled irrelevant mAb (filled areas) and gated for MoDC. Data in panels A and B are representative of 8 independent experiments.

FcαRI expression on transgenic mouse DC.

A well-documented model to study antigen presentation by DC, is the murine model in which OVA peptides are internalized, processed, and presented on MHC class II molecules to CD4$^+$ OVA-specific murine T cells[27]. As MHC molecules of human MoDC are not recognized by these OVA-specific T cells, murine DC need to be used to investigate antigen presentation. However, mice do not express FcαRI. As such, transgenic (Tg) mice were made, which express human FcαRI. The cell distribution of FcαRI in these mice mirrors the cell distribution in humans[22].

Expression and FcαRI-internalization by transgenic mouse DC was determined first, to assess whether murine DC express FcαRI. As only limited numbers of monocytes can be isolated from murine blood, bone marrow cells were used as a source for DC progenitors (BMDC)[23]. Either culturing with GM-CSF and IL-4, or GM-CSF alone induced differentiation of mouse bone marrow cells towards bonafide CD11b$^+$, CD11c$^+$ immature DC[28,29], which expressed low levels of MHC class II, CD80 and CD86 (data not shown, n=8). Furthermore, both culture conditions led to phenotypically and functionally similar DC, compared to human immature MoDC[28,29]. The levels of FcαRI expression were slightly increased on BMDC cultured with GM-CSF alone (MFI ± SEM of 16.1 ± 5.2, n=8), compared to BMDC cultured with GM-CSF and IL-4 (10.9 ± 5.2,

n=2) (Fig. 3A, and B). Similar to human MoDC, addition of TNF-α to GM-CSF culture regimes led to more activated CD11b$^+$, CD11c$^+$ BMDC[30,31], which was shown by upregulated expression of MHC molecules, CD80 and CD86 (data not shown, n=8). Similar to activated MoDC[15], FcαRI expression was upregulated on BMDC by TNF-α (MFI ± SEM of 25.5 ± 7.1, n=8, and Fig. 3C). Background MFI of BMDC in all three culture regimes was 3.13 ± 0.33 (SEM). Expression patterns of all tested DC markers were similar on BMDC of FcαRI Tg and NTg mice, indicating that FcαRI transgeneity did not affect DC maturation.

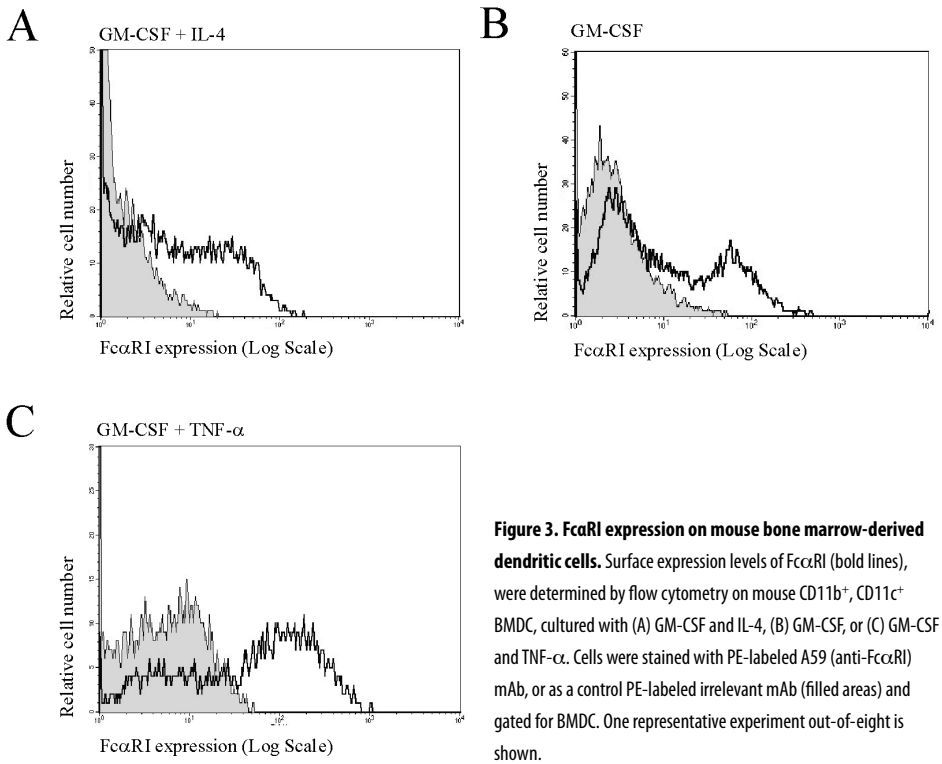

Figure 3. FcαRI expression on mouse bone marrow-derived dendritic cells. Surface expression levels of FcαRI (bold lines), were determined by flow cytometry on mouse CD11b$^+$, CD11c$^+$ BMDC, cultured with (A) GM-CSF and IL-4, (B) GM-CSF, or (C) GM-CSF and TNF-α. Cells were stained with PE-labeled A59 (anti-FcαRI) mAb, or as a control PE-labeled irrelevant mAb (filled areas) and gated for BMDC. One representative experiment out-of-eight is shown.

FcαRI mediated internalization and antigen presentation by dendritic cells.

As receptor internalization precedes antigen processing and presentation, FcαRI-internalization was addressed next. Human MoDC internalized crosslinked FcαRI within 5 minutes (Fig. 4A). Furthermore, FcαRI Tg mouse BMDC, cultured with either GM-CSF, or GM-CSF and TNF-α, were equally effective, and also displayed FcαRI receptor internalization within 5 minutes (Fig. 4B).

Next, we investigated whether FcαRI-internalization led to FcαRI-mediated antigen presentation. FcαRI-IIA1.6 cells were used as a positive control, as these cells efficiently take-up IgA-immune complexed OVA (IgA-OVA) and present antigens to OVA-specific T cells, which activation leads to IL-2 secretion[19]. FcαRI-Tg BMDC cultured with GM-CSF alone, were unable

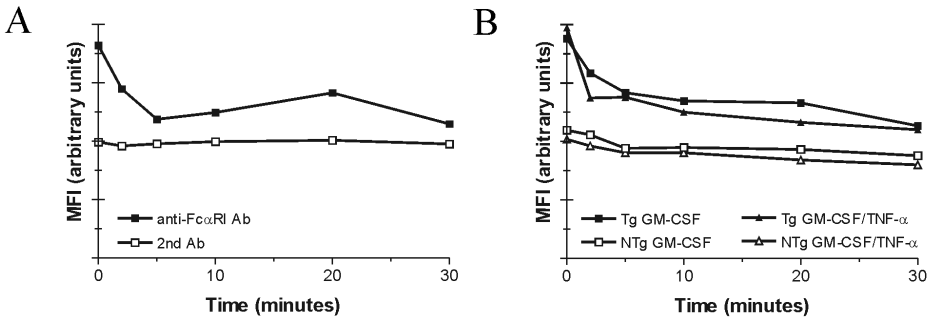

Figure 4. FcαRI internalization on dendritic cells. FcαRI internalization by (A) human MoDC, cultured with GM-CSF, IL-4 and TNF-α, or (B) FcαRI Tg mouse BMDC cultured with GM-CSF (■), or GM-CSF and TNF-α (▲) was measured by crosslinking the Fc receptor (anti-FcαRI mAb, ■) for the indicated time periods. In (A) receptor internalization with secondary Ab only (□) was used as a control. In (B) internalization by NTg mouse BMDC (□ for GM-CSF cultured BMDC, Δ for GM-CSF and TNF-α cultured BMDC) served as controls. Experiments were repeated 3 times, yielding similar results.

to mediate antigen presentation (Fig. 5A). GM-CSF/TNF-α cultured FcαRI-Tg BMDC, however, did induce FcαRI-mediated antigen presentation, although responses were minimal (Fig. 5B). Antigen presentation to OVA-specific T cells was only induced at IgA-OVA concentrations between 1,000 and 5,000 ng/ml. IgA-OVA concentrations higher than 5,000 ng/ml induced non-specific uptake by NTg BMDC, as well (data not shown). No differences were observed between FcαRI-Tg BMDC and NTg BMDC in fluid phase uptake and processing of excess Ova, which was used as a positive control for antigen-presenting capability of DC. Earlier work showed that the capability of DC to cross-present antigens was increased after LPS or TNF-α DC activation[21,32]. Therefore, as culturing of BMDC in presence of TNF-α led to more activated DC, shown by increased MHC class II, CD80 and CD86 expression levels, differences in DC activation status might explain the discrepancy in FcαRI-induced antigen presentation between GM-CSF cultured BMDC and GM-CSF/TNF-α cultured BMDC. Alternatively, BMDC, which were cultured with GM-CSF and TNF-α, expressed enhanced levels of FcαRI compared to BMDC cultured with GM-CSF alone, which might lead to increased uptake of antigens, and thus better presentation to T cells. Furthermore, FcαRI-IIA1.6 cells, which have higher FcαRI expression levels compared to DC, induced more effective FcαRI-mediated antigen presentation, even though DC represent the most potent antigen presenting cells (Fig. 5). The observed antigen presentation was FcαRI-specific, as My43 mAb (an anti-FcαRI IgM blocking Ab) effectively blocked IgA-OVA induced antigen presentation (data not shown). We therefore hypothesize that although efficient FcαRI internalization by DC was observed, only small amounts of immune complexes can be internalized / processed via this receptor. As a consequence, this might lead to a suboptimal presentation of peptides on MHC molecules, and inefficient activation of T cells. A threshold level of FcαRI might thus be needed for proper antigen presentation. As FcαRI expression on human MoDC was even lower compared to FcαRI Tg BMDC (Fig. 2, and 3), we postulate that human MoDC will be unable to present antigens via FcαRI.

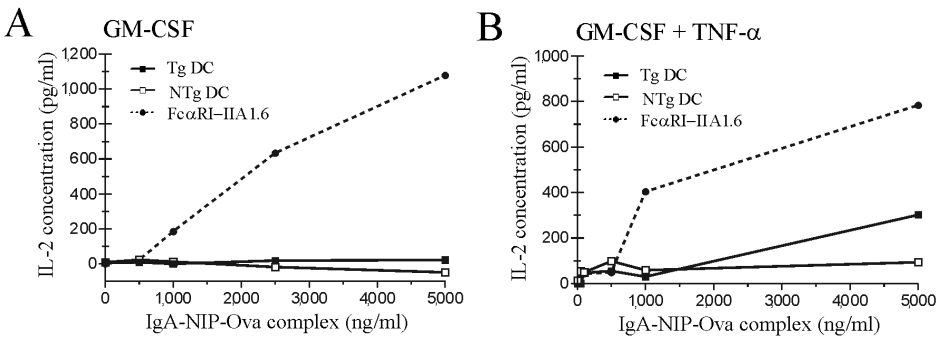

Figure 5. FcαRI-mediated antigen presentation by dendritic cells. Presentation of IgA-OVA immune complex by FcαRI-IIA1.6 cells (●, dotted line), FcαRI Tg mouse BMDC (Tg DC, ■), or as a control, NTg mouse BMDC (NTg DC, □). BMDC were cultured with (A) GM-CSF, or (B) GM-CSF and TNF-α. IL-2 production by T cells served as readout. Data are representative of 9 individual experiments.

Taken together, we documented that blood myeloid dendritic cells, as well as cultured human and FcαRI Tg murine dendritic cells express FcαRI. In both species, DC FcαRI crosslinking induces receptor internalization. However, FcαRI-induced antigen presentation by FcαRI Tg murine DC was minimal. Therefore, our data suggest that targeting of tumor antigens to FcαRI on DC does not represent an optimal strategy for DC vaccination.

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Chapter 7

Potential pitfalls of murine cancer models for antibody therapy

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ABSTRACT

Murine cell lines expressing human tumor associated antigens (TAA) are commonly used for evaluation of immunotherapeutic approaches. Because these cells are not rejected in immunocompetent mice it has been assumed that anti-human immune responses do not develop. However, in our experiments, in which murine CMS7 cells - engineered to express human HER-2 (CMS7HE) - were used, we observed treatment-independent regression of tumors in several mice. This was associated with occurrence of ulceration of the skin covering the tumor, whereas less tumor regression was observed in the absence of ulceration. Re-challenge of cured mice with CMS7HE, and control empty vector transduced CMS7neo cells, led to regression of CMS7HE tumors, indicating that specific anti-human HER-2 immune responses had developed. Additionally, vaccination with lysed CMS7HE-, but not CMS7neo (control) cells partially protected mice from a challenge with live CMS7HE cells.

These observations indicate that human antigens may not trigger immune responses in mice per se, but specific anti-human HER-2 immunity can be induced during inflammatory conditions, or so-called 'danger signals'. As many approaches aiming to stimulate the immune system inherently represent 'danger signals', development of murine anti-human responses in anti-tumor treatment experiments might skew results. Consequently, the present data question the accuracy of mouse models expressing human antigens for evaluation of novel immunotherapeutic approaches.

INTRODUCTION

In spite of significantly improved therapeutic options, cancer still represents one of the main causes of death in Western society. Conventional treatments, such as chemo- and radiotherapy are effective to a point, but dose-limitations - due to severe side effects - and the induction of resistance confine their application. Need for new therapies are, therefore, eminent. Tumors are genetically unstable and alterations such as mutations, chromosomal translocations or gene amplifications can lead to expression of aberrant antigens, or over-expression of normal antigens on tumor cells[1]. These so-called tumor associated antigens (TAA) are potentially recognized by the immune system, which makes them attractive candidates for immunotherapy, and a large number of attempts have been made to enhance immune responses to TAA[2]. To study effectiveness and safety of such efforts, pre-clinical studies are necessary and numerous animal models have therefore been developed.

After the observation that human tumors can be engrafted in athymic or severe combined immunodeficient (SCID) mice, a plethora of human cancer cells and tissues have successfully been grown in these animals[3]. Nonetheless, several restrictions with respect to immunotherapy need to be considered. Athymic or SCID mice possess an intact innate immune system, hereby enabling the study of anti-tumor responses initiated by monocytes / macrophages, granulocytes, and soluble factors. Specific acquired immune responses can, however, not be mounted due to deficiencies in T, or T and B cells.

To overcome these limitations, syngeneic animal models have been developed. These models serve very well in testing the efficacy of cytokines or vaccination strategies using tumor lysates, but general application of tumor-specific cytotoxic T lymphocytes (CTL) or antibodies (Ab) is hampered by the limited number of identified murine tumors antigens[4-7]. Hence, an increasing number of laboratories has moved to use syngeneic murine tumor cell lines that have been engineered to express human TAA (hTAA-syngeneic). This approach has the additional advantage that Ab developed for clinical applications in man can be evaluated in immunocompetent mice. Currently, several murine cell lines engineered to express hTAA like carcinoembryonic antigen[8], prostate-specific antigen[9], Ep-CAM[10], and HER-2[11] have been described, and proposed as suitable models for evaluation of experimental immunotherapies.

Hence, we inoculated mice subcutaneously (s.c.) with syngeneic CMS7 cells that were stably transduced to express human HER-2 (CMS7HE[12]) to investigate the efficacy of anti-HER-2 bispecific antibodies (BsAb). However, during the course of these experiments it was observed that - depending on the mode of injection - several mice were able to clear the developing s.c. tumor, regardless of treatment. Concerned by this outcome we investigated the suitability of this hTAA-syngeneic murine mouse model for testing immunotherapies of cancer. We show that CMS7HE cells, which grow in immunocompetent mice, are rejected under "inflammatory" conditions, due to development of immune responses against human HER-2. These

results suggest that murine cell lines expressing human HER-2 – or possibly other human TAA – may be inapt for the evaluation of novel immunotherapeutic approaches.

MATERIAL AND METHODS

Antibodies.

A panel of rat anti-mouse monoclonal antibodies (mAb) was used to detect different murine effector cell populations; anti-F4/80 mAb was purchased from Serotec (Oxford, UK). mAbs RB6-8C5 (anti-Ly-6G; GR-1), RA3-6B2 (anti-CD45R/B220), RM4-5 (anti-CD4) and 53-6.7 (anti-CD8) were all obtained from Becton Dickinson (San Diego, CA). Biotinylated rabbit anti-rat IgG antibodies were purchased from Vector Laboratories Inc. (Burlingame, CA). mAb TA-1 (murine IgG1; Calbiochem, San Diego, CA) and FITC-labeled F(ab')2 fragments of goat anti-mouse IgG antibody (Jackson Laboratories, West Grove, PA) were used to analyze HER-2 expression.

Cell lines.

Transduction of the 3-methylcholantrene-induced murine sarcoma cell line CMS7 with the human HER-2 construct (CMS7HE) or control vector (CMS7neo) was described previously[12], and cell lines were kindly provided by Prof. Dr. Shiku (Mie University School of Medicine, Mie, Japan). Cells were cultured in RPMI 1640 medium (GIBCO, Paisley, Scotland), supplemented with 10% fetal calf serum (FCS), and antibiotics (50 μg/ml streptomycin and 50 IU/ml penicillin; GIBCO BRL). 1 mg/ml Geneticin (G418 sulfate; GIBCO) was added to select for transduced cells. Cells were harvested by incubation with trypsin-EDTA (GIBCO). HER-2 expression of CMS7HE was confirmed by FACS analysis prior to each experiment.

Quantification of tumor-specific murine serum IgG.

Serum of mice was diluted 1:30 in PBS. CMS7neo and CMS7HE cells ($2x10^5$) were incubated with diluted serum (30', 4°C), washed twice with PBS/1%BSA, and further incubated (30' 4°C) with FITC-conjugated F(ab')2 fragments of goat anti-mouse IgG (1:100). Cells were washed twice and analyzed on a FACScan (Becton Dickinson).

Immuno-histochemistry.

Tumor sections were fixed in either 10% buffered formalin or zinc salts-based fixative (0.5 g/L calcium acetate, 5.0 g/L zinc acetate, 5.0 g/L zinc chloride in 0.1 M Tris buffer)[13], and embedded in paraffin. Alternatively, tumor samples were snap-frozen in liquid nitrogen to enable anti-CD4 and anti-CD8 immuno-stainings. After deparaffinization, endogenous peroxidase and non-specific binding were blocked by incubation with 0.3% H_2O_2 in methanol (30 min), and 10 % rabbit serum (15 min), respectively. Sections were incubated with mAbs directed against macrophages (F4/80; 1:50), granulocytes (GR-1; 1:300), B cells (CD45R/B220; 1:500), T

helper cells (CD4; 1:100) or cytotoxic T cells (CD8; 1:100) for 1 hour. After washing in 0.05 % Tween/ PBS (3 x 10 min) slides were incubated with biotinylated rabbit anti-rat IgG antibodies (1:300). Slides were washed in 0.05 % Tween/ PBS (3 x 10 min), and further incubated with streptavidin-HRP (Vector Laboratories Inc.; 1:500) or avidin-biotin complex-HRP (DAKO, Glostrup, Denmark; 1:100) for 30 min. Immunoreactivity was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB). Slides were counter stained with Mayer's hematoxilin, dehydrated and mounted in Entellan (Merck, Darmstad, Germany). The presence of plasma cells was evaluated in slides stained with Hematoxylin-Eosin (H&E) without additional immunostaining. All stainings were performed at room temperature.

Tumor models.

8-12 week-old Balb/c mice were obtained from Harlan (Horst, The Netherlands), and maintained at the Mouse Facility of the Central Animal Laboratory, Utrecht University, The Netherlands. Mice were housed and handled under animal welfare regulations, and all experiments were approved by the Utrecht University ethical committee, and performed according the institutional and national guidelines.

Mice were injected subcutaneously (s.c.) in the right flank with $1.5x10^6$ CMS7HE cells to establish solid s.c. tumors ('deeply' s.c.; n=10). Alternatively, tumor cells were injected as closely beneath the skin surface as possible ('superficial' s.c.; n=10). Tumor sizes were measured three times a week with calipers and are expressed as length x wide x height (mm^3). Mice, in which tumors went into regression were re-challenged after one month with $1.5x10^6$ CMS7HE cells and $1.5x10^6$ CMS7neo cells s.c. in right and left flanks, respectively. Previous unchallenged mice served as control for outgrowth of tumors (challenge group). Tumor sizes were measured over time. After termination of experiments, mice were sacrificed and serum and tumors samples were collected. Several mice were sacrificed just prior to complete tumor eradication to enable microscopical analyses of regressing tumors.

Lysis CMS7neo or CMS7HE cells - required for vaccination experiments - was achieved by freezing and thawing three times in PBS. Mice were injected s.c. with PBS, lysed CMS7neo or lysed CMS7HE cells ($1.5x10^6$ lysed cells/mouse; 5 mice/ group). One month later, mice were inoculated s.c. with $1.5x10^6$ vital CMS7HE and CMS7neo cells in right and left flanks, respectively. Tumor sizes were measured over time.

Statistical analyses.

Results were analyzed by means of unpaired two-tailed Welch T-, Chi-square- or ANOVA tests. Results are expressed as mean ± standard error of the mean (SEM), and significance was accepted at $p < 0.05$.

RESULTS

Treatment-independent regression of CMS7HE tumors.

In order to evaluate the efficacy of BsAb - targeting the human TAA HER-2 - in vivo, a subcutaneous tumor model was established by s.c. injection of CMS7HE cells in syngeneic mice. During the course of the experiments, we noted that, depending on the mode of tumor cell injection, tumors went into regression in several mice, irrespectively of treatment. After 'deep' s.c. tumor cell injection, tumors did not always grow uniformly, which hindered exact measurements with calipers (Fig. 1A, left panel). To increase accuracy, it was aimed to inject tumor cells as closely beneath the skin surface as possible (referred to as 'superficial' injection), which however resulted in extensive ulceration of the skin covering the tumor (Fig. 1A, right panel). Unexpectedly, ulceration was found associated with treatment-independent regression of tumors. This observation prompted us to further investigate the mechanism underlying the 'spontaneous' regressions.

Syngeneic Balb/c mice were s.c. injected either 'deeply', or 'superficially' with CMS7HE tumor cells and tumor growth was followed in time. After approximately 18 days the majority of 'superficially' injected tumors went into regression, and after 26 days ~70 % of tumors had disappeared, compared to ~30 % of tumors that developed after 'deeply' s.c. injection (Fig. 1B). Cured mice were re-challenged one month later with CMS7HE and CMS7neo cells in their right, and left flanks, respectively. Except for one mouse, CMS7neo tumors did not go into regression in the re-challenge group, but 8 out-of-9 CMS7HE tumors were eradicated (Fig. 1C, upper and lower panel, respectively). In the control challenge group, only 1-out-of 7 CMS7HE tumors, and no CMS7neo tumors went into regression.

Vaccination with lysed CMS7neo or CMS7HE cells.

The observation that ulceration of the skin correlated with spontaneous regression of emerging CMS7HE tumors led us to hypothesize that concomitant inflammatory responses, as a result of ulceration, mounted anti-human HER-2 immune responses. It was, therefore, investigated whether lysed cells could induce anti-human HER-2 immunity. Balb/c mice were vaccinated with either lysed CMS7neo or CMS7HE cells. Control mice were injected with PBS. After one month, mice were challenged with vital CMS7neo and CMS7HE cells in their left and right flanks, respectively (Fig. 1D). No differences in outgrowth of CMS7neo tumors were observed between groups (Fig. 1D, upper panel). However, vaccination with lysed CMS7HE cells led to diminished outgrowth of CMS7HE tumors (Fig. 1D, lower panel). Vaccination with lysed CMS7neo cells did not yield protection.

Both cellular and humoral anti-tumor immunity participate in tumor rejection.

To investigate which immune cells were involved in tumor clearance, several mice were sacrificed prior to complete tumor eradication in order to enable microscopical analyses of tumor

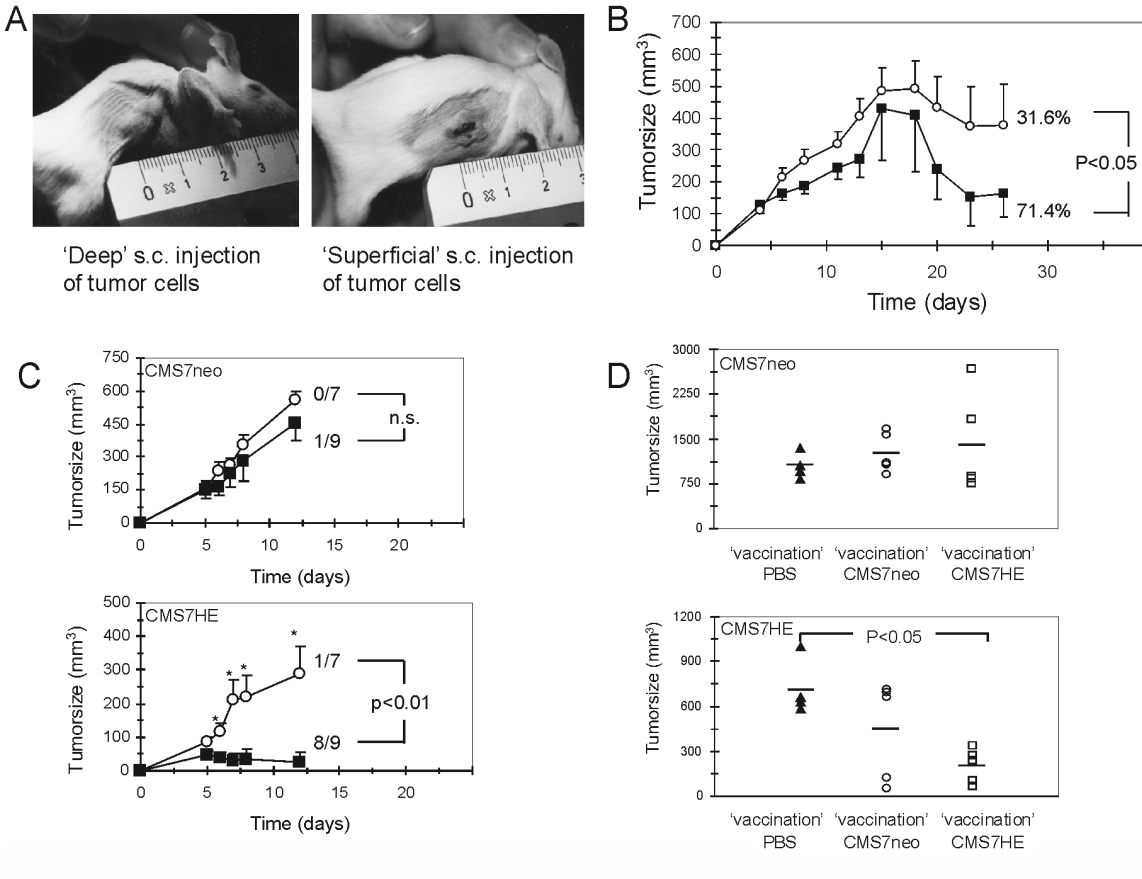

Figure 1. Subcutaneous tumor growth of CMS7HE or CMS7neo in syngeneic balb/c mice in the absence or presence of inflammation. (A) Macroscopical aspects of 10 day-old s.c. CMS7HE tumors that were injected either 'deeply' s.c. (left panel) or 'superficially' (right panel). (B) Mean size ± SEM of s.c. CMS7HE tumors injected 'superficially' (■) or 'deeply' (○) followed over time. Percentages reflect numbers of tumors that disappeared at day 26 in two separate experiments. (C) Mice in which CMS7HE tumor disappeared (■) were re-challenged a month later with CMS7neo (upper panel), and CMS7HE (lower panel) cells in their left, and right flanks, respectively. Naïve mice served as controls for tumor outgrowth (○). Tumor growth was measured over time. Numbers indicate number of mice that cleared the tumor / total number of mice per group. (D) Mice were 'vaccinated' with PBS (▲), lysed CMS7neo- (○), or lysed CMS7HE (□) cells. After a month, mice were challenged with CMS7neo (upper panel), and CMS7HE (lower panel) cells in their left, and right flanks, respectively. A representative experiment out-of-two is shown. Differences in tumor size, or numbers of tumors that were eradicated were tested with unpaired two-tailed Welch T-tests, or Chi-square- tests, respectively. Differences between groups were analyzed with ANOVA. *p< 0.05.

sections. Microscopical examination of progressing CMS7HE tumors showed solid tumors that exhibited an expansive growth pattern, which was clearly demarcated from the adjacent muscular tissue (Fig. 2A; panel 1). Only low numbers of immune cells were observed (Fig. 2A; panels 2-5). In contrast, demarcation between regressing tumors and muscular tissue was blurred by infiltration of large numbers of inflammatory cells (Fig. 2A; panel 6). Closer examination of immune cell infiltrates in regressing tumors demonstrated the presence of conglomerates of CD45R/B220-positive cells and plasma cells (Fig. 2A; panels 7 and 8). Additionally, large numbers of CD4-positive cells and CD8-positive cells were observed, indicating that regression of CMS7HE tumors was associated with the presence of various types of immune effector cells (Fig. 2A; panels 9 and 10). Microscopical analyses of regressing or progressing CMS7HE tumors showed no significant differences in the numbers of F4/80-positive macrophages, or GR1-positive granulocytes (data not shown).

Because of the presence of large numbers of both B and plasma cells in regressing tumors, Ab titers against CMS7neo and CMS7HE cells were determined. Ab titers against CMS7HE cells were increased in mice that were able to eradicate CMS7HE tumors after re-challenge, suggesting involvement of humoral responses (Fig. 2B, right panel). Mice that were challenged with CMS7HE cells for the first time did not show Ab titers. Notably, mice in the re-challenge group expressed higher Ab titers against CMS7neo cells, as well (albeit lower than Ab titers against CMS7HE), suggesting development of additional immune responses against endogenous murine tumor antigens (Fig. 2B, left panel). Moreover, one mouse with the highest serum Ab titer against CMS7neo cells (Fig. 2B, left panel; arrow) was able to eliminate the CMS7neo tumor (see also Fig. 1C, upper panel).

DISCUSSION

The need to develop clinically relevant murine tumor models in immunocompetent mice has led to the establishment of a number of syngeneic murine cell lines, engineered to express known human TAA[8-12]. Because these cell lines are not rejected in immunocompetent mice, it has been suggested that anti-human TAA immune responses do not develop. In this paper, we, however, observed that under inflammatory conditions mice did develop specific anti-human HER-2 immunity, as both ulceration of the skin covering the tumor, and vaccination with lysed cells led to the accumulation of extensive immune cell infiltrates in tumors, leading to tumor regression. Increased T cell numbers, as well as elevated specific anti-HER-2 Ab titers, were demonstrated in mice that were able to eliminate CMS7HE tumors. This is in accordance with earlier reports demonstrating that development of both cellular and humoral anti-HER-2 responses were required for effective protection against tumor cell challenge[14,15], and supports that strategies including both cellular and humoral immune responses against TAA may lead to more potent anti-tumor immunity than induction of specific CTL alone.

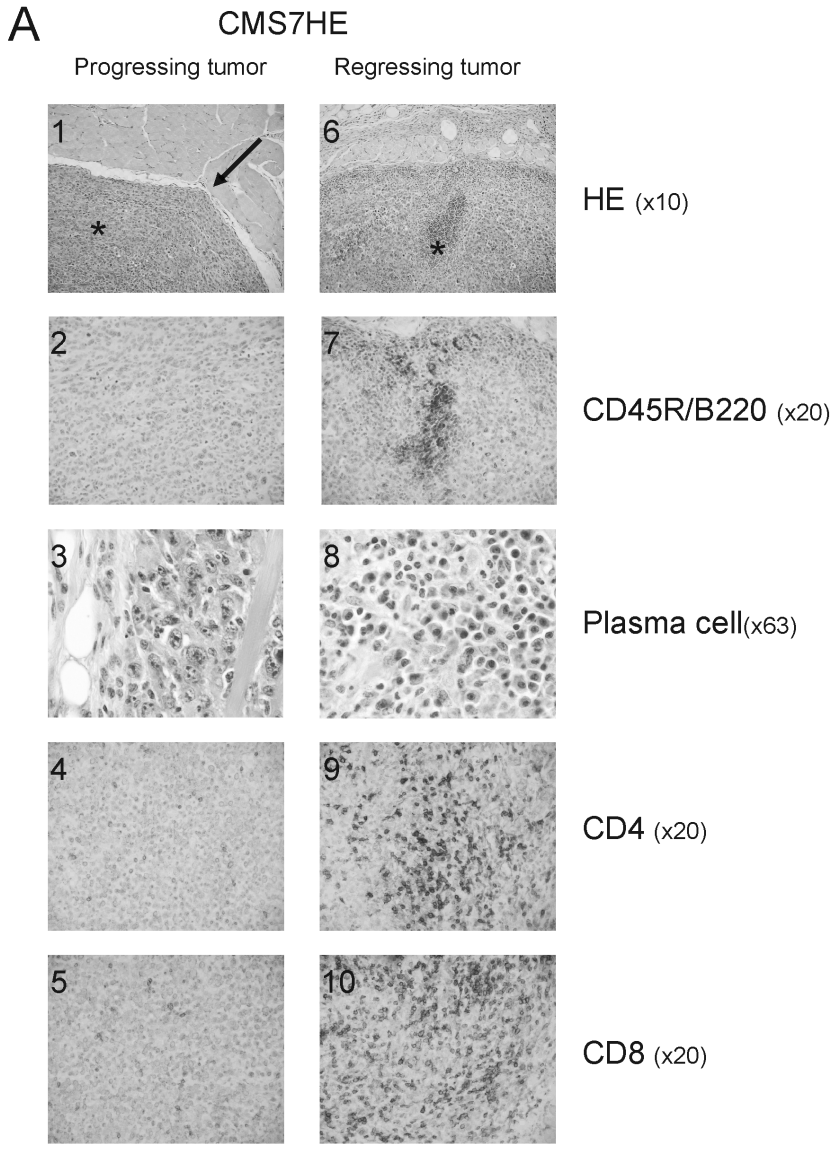

Figure 2. Development of cellular and humoral anti-tumor responses. For a full-color picture, see Appendix page 172. (A) Analysis of cellular immune cell infiltrates in progressing (left panels) and regressing (right panels) CMS7HE tumors. Left Panels: (1) Low power magnification (x10). Tumor (marked by asterisk) showed solid, expansive growth and was clearly demarcated from muscular tissue (arrow) (HE staining). (2) Immuno-staining for the presence of B cells; no B cells were detected (x20). (3) Examination of the peripheral rim of the tumor revealed only presence of tumor cells, which demonstrated cellular pleomorphism, a high nucleus:cytoplasma ratio and multiple prominent nucleoli per nucleus. No plasma cells were found. (HE staining; x63) (4) Immuno-staining for CD4 or (5) CD8 showed the presence of few, disseminated positive cells (x20). Right Panels: (6) Low power magnification (x10). The demarcation between the solid CMS7HE tumor and muscular tissue was blurred by infiltration of inflammatory cells. A condense nodule, indicative of clustered B cells, was present at the margin of the tumor (indicated by asterisk) (HE staining). (7) Immunostaining for the presence of B cells on identical area depicted in (6). Note the CD45R/B220 immunoreactivity of the clustered cellular infiltrate, supporting the presence of B cells. (8) At the rim of the tumor, conglomerates of plasma cells were found. Note the typical cellular characteristics of plasma cells; Elongated, oval cells with peripheral round nuclei, fairly condensed nuclear chromatin patterns, a juxta-nuclear luscent area (reflecting the Golgi system) and a basophilic cytoplasm. (9) High numbers of CD4-positive cells, as well as high numbers of (10) CD8-positive cells, were present within the tumor.

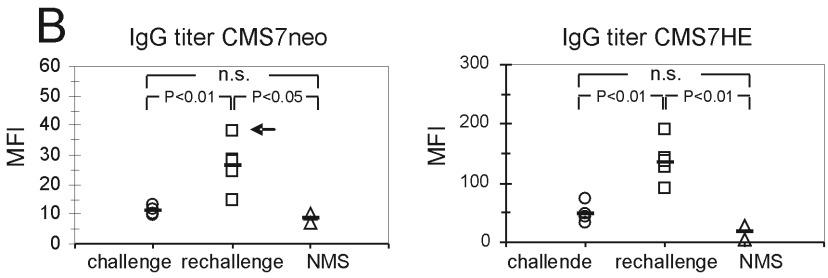

Figure 2. Development of cellular and humoral anti-tumor responses. (B) Serum IgG titers against CMS7neo (left panel) and CMS7HE (right panel) are shown of challenged (○), re-challenged (□) and control (Δ) mice (NMS; normal mouse serum). An arrow indicates the anti-CMS7neo titer of the mouse that was able to clear the CMS7neo tumor (see also Fig. 1C, upper panel). Differences were analyzed with ANOVA tests. MFI = mean fluorescence intensity.

In retrospect, the observation that human TAA may provoke immune responses under inflammatory conditions is not surprising. Already in the early nineties Matzinger proposed her 'danger signal' theory, which postulates that the main goal of the immune system is not to discriminate between self and non-self[16]. Instead, immune responses may also be triggered by endogenous danger signals, originated from damaged tissues, in order to protect the organism from further harm. In view of this hypothesis, it might be anticipated that human antigens with homology to their murine counterparts do not provoke immune responses per se, but can evoke anti-human immunity in the presence of danger signals, released by inflamed or necrotic tissues.

Many strategies designed to induce anti-tumor immunity, and as such inherently represent "danger signals", have been tested in immunocompetent mice with the use of hTAA-syngeneic cell lines. These include vaccination strategies as well as the use of TAA-specific Ab that are coupled to either cytokines or toxins (Table 1, refs. 10,11,17-30). Although it is not likely that all anti-tumor responses observed in such *in vivo* models are the result of development of anti-human responses, it cannot be excluded either.

For instance, in our experiments we aimed to study the *in vivo* efficacy of BsAb targeting HER-2 and the human Fc receptor for IgA (FcαRI), a potent trigger molecule for tumor cell killing[31]. Two weeks after BsAb treatment, CMS7HE tumors went into complete regression because of the development of anti-human HER-2 responses (data not shown). FcαRI is highly expressed on macrophages, and to a lesser extent on dendritic cells[32,33]. Although DC do not efficiently present antigens via FcαRI (own unpublished data), FcαRI-mediated antigen presentation can be induced by macrophages[34]. It is thus possible that anti-FcαRI x anti-HER-2 BsAb induce specific antigen presentation of HER-2 expressing tumor cells, resulting in anti-tumor responses (Fig. 3A). However, histological examinations of tumor sections showed that anti-FcαRI BsAb induced necrosis in tumors (data not shown). Consequently, it cannot be excluded that anti-FcαRI BsAb merely induced danger signals necessary (and sufficient) for the murine immune system to develop anti-human antigen responses (Fig. 3B). If the latter

explanation holds true, results with FcαRI-directed BsAb may have been positively skewed, and the relevance of observations in this mouse model for human cancer patients remains to be seen. The use of mice that are transgenic for the human TAA of interest may serve as a suitable alternative, as such mice will be tolerant for this molecule.

Table 1

Strategy	Therapy	Human TAA	References
Vaccination	Antigen + HSP70 DNA	MAGE	17
	Antigen + CD80 + CD86 DNA	MUC1	18
	DNA + dendritic cells	MUC1	19
	Antigen + HA epitope in liposome	HER-2	20
	Irradiated cells	MUC1	18
	HER-2 expressing plasmids	HER-2	21
	CEA-GM-CSF plasmids	CEA	22
(Bispecific) antibody	Anti-FcγRI x anti-HER-2	HER-2	23
	Anti-CD20	CD20	24
Adjuvants	Anti-MUC1 + β-glucan	MUC1	25
Ab-cytokine fusion proteins	Anti-Ep-CAM Ab x IL-2	Ep-CAM	10,26
	Anti-Ep-CAM Ab x IL-2 + chemokine MIG	Ep-CAM	27
	Anti-HER-2 Ab x IL-2	HER-2	28
	Anti-HER-2 Ab x IL-12	HER-2	29
	Anti-HER-2 Ab x GM-CSF	HER-2	28
Ab-toxin fusion proteins	Anti-HER-2 x Exotoxin	HER-2	11
	Anti-EGFR x Exotoxin	EGFR	30

Table 1. Examples of *in vivo* immunotherapeutic approaches involving the use of murine cell lines expressing human TAA.
HSP70; heat shock protein 70, MAGE; Melanoma Antigen-Encoding Genes, MUC1; human epithelial mucin, HA; T-helper epitope derived from influenza haemagglutinin, HER-2; human epidermal growth factor receptor 2, CEA; carcinoembryonic antigen, EpCAM; epithelial cell adhesion molecule, IL; interleukin, MIG; monokine induced by interferon-γ, GM-CSF; granulocyte-macrophage stimulating factor, Exotoxin; Pseudomonas aeruginosa exotoxin A, EGFR; epidermal growth factor receptor.

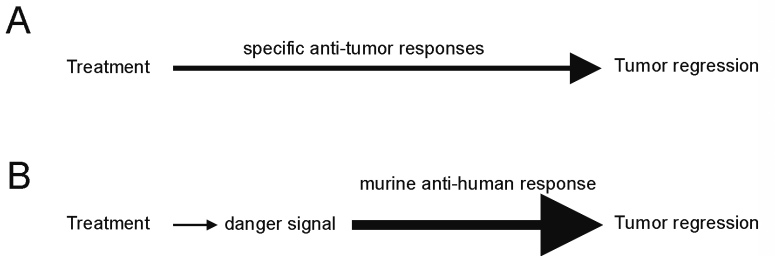

Figure 3: Model illustrating alternative explanations for results obtained with murine cell lines expressing human antigens.
(A) Treatment induces specific **anti-tumor** responses leading to tumor regression. (B) Treatment results in some 'danger signals' necessary and sufficient for the murine immune system to develop an **anti-human** antigen response, leading to tumor regression.

In conclusion, we have shown that a murine cell line - expressing human HER-2 - that is initially not rejected by the murine immune system, triggers a potent murine anti-human response in the presence of danger signals. Because many therapies, aiming to improve immune responses, inherently represent danger signals, engineered murine cell lines may not be suitable to evaluate immunotherapeutic approaches. Therefore, caution should be taken with interpretation of results, since positively skewed data obtained in such models may lead to disappointing outcome in clinical trials.

ACKNOWLEDGEMENTS

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Chapter 8

Role of FcγR in experimental antibody therapy of liver metastases

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ABSTRACT

In colorectal cancer, many patients will ultimately develop liver metastases, even after successful surgical removal of the primary tumor at a time at which no visible metastases are present. Peri-operative adjuvant treatment might therefore help to prevent secondary disease. We studied the potential of antibody therapy to prevent outgrowth of liver metastases in immuno-competent mice. Peri-operative treatment with mouse anti-gp75 monoclonal antibody prevented outgrowth of B16F10 liver metastases in more than 90% of the mice. Therapeutic efficacy was maintained in either C1q-, or complement receptor 3-deficient mice, but was abrogated in FcR γ chain-deficient mice, which lack all activatory IgG receptors (FcγR). These results indicate that interaction with activatory FcγR is necessary for successful therapy. Because antibody treatment was still effective in FcγRI$^{-/-}$, FcγRIII$^{-/-}$ and FcγRI/III$^{-/-}$ mice, an important role for the newly identified FcγRIV is implicated.

INTRODUCTION

Colorectal cancer represents the third most common malignancy in the western world. Worldwide, one million cases are recorded annually, and over half a million patients die from this disease each year[1]. The initial treatment for patients is surgical removal of the primary tumor. Development of incurable liver metastases in these patients is the main cause of death. At the time of resection, 10% - 25% of the patients are already diagnosed with liver metastases[2]. Furthermore, an additional 25% - 50% of patients who do not have visible liver metastases at the time of surgery, develop secondary disease within 3 years[3,4].

Several mechanisms might be involved in the development of liver metastases after removal of the primary tumor. Circulating tumor cells were found prior to surgery in 70% of patients with colorectal cancer[5]. In addition, manipulation of the tumor during resection can lead to dissemination of tumor cells[6]. Wound healing processes, including release of angiogenic and inflammatory factors, may contribute to enhanced outgrowth of tumor cells[7,8]. Moreover, depending on the duration of surgery, the degree of trauma, and the amount of blood loss, surgery induces immuno-suppression[6,9]. This immuno-suppression temporally impedes the arrest and elimination of blood-borne tumor cells by immune cells, which may lead to growth of micro-metastases[10]. Rats, which underwent an operation prior to tumor cell inoculation in the portal circulation, developed increased numbers of liver metastases, hereby strongly supporting the impact of surgery on tumor outgrowth (van der Bij *et al.*, unpublished data). As the liver is the first organ in which disseminated tumor cells are trapped, formation of liver metastases might thus, paradoxically, be initiated during surgical resection of primary colorectal carcinoma. Although surgical procedures have been optimized, and patients have been treated with additional therapies like chemotherapy and radiotherapy, prognosis remains poor[11]. Therefore, preventive treatment of patients during and after surgery that helps to diminish development of secondary disease, may thus improve clinical outcome.

Anti-tumor antibodies (Ab), which provide a link between immune cells and tumor cells, have been shown to represent promising drugs against cancer[12]. Treatment of malignancies with anti-tumor Ab demonstrated encouraging results, which led to the approval of eight therapeutic Ab by the United States FDA[13]. Ab treatment was previously demonstrated to reduce liver metastasis outgrowth in nude mice, which have an incomplete immune system[14-16]. Additionally, in one clinical trial it was reported that 30% of patients with colorectal cancer had benefit of post-operative treatment with anti-EpCAM Ab[17]. Understanding the mode of action of therapeutic Ab in the prevention of liver metastases formation may help to further improve clinical outcome of patients.

The mechanisms of tumor cell killing via mAb are, however, not yet completely understood. mAb might influence signaling of tumor antigens, leading to pro-apoptotic, or anti-proliferative effects of tumor cells[18]. mAb can recruit the classical complement pathway[19]. Furthermore, mAb bind to IgG Fc receptors (FcγR) on immune cells, which can activate im-

mune effector functions including Ab-dependent cellular cytotoxicity (ADCC)[20]. *In vivo*, FcγR have been shown to be crucial for therapeutic activity, as protection against tumor growth was abrogated in FcR γ chain-deficient mice, lacking all activatory FcγR[21]. Furthermore, a role for complement receptor 3 (CR3), which can enhance Fc receptor mediated cell killing, has been documented, as Ab therapy was diminished in CR3 knock-out (KO) mice[22]. In human, 3 classes of FcγR have been identified, which all have their own expression pattern on immune cells[20]. Recently, a fourth class of FcγR, FcγRIV, has been identified in mice[23]. FcγRI, III, and IV are activatory IgG receptors, and depend for cell surface expression and effector functions on interaction with the signaling, ITAM containing, FcR γ chain[24,25]. FcγRII, containing an ITIM motif, represents an inhibitory IgG receptor, which activation results in downregulation of immune responses[20].

In the present study, we investigated the mechanisms of peri-operative Ab therapy in the prevention of outgrowth of liver metastases in immuno-competent mice. We, therefore, established a syngeneic liver metastases model, in which tumor cells were inoculated during surgical procedure.

MATERIALS AND METHODS

Mice

C57Bl/6 wildtype mice were obtained from Janvier (Le Genest Saint Isle, France). FcR γ chain KO mice[26], FcγRI$^{-/-}$ (CD64 KO) mice[27], FcγRIII$^{-/-}$ (CD16 KO) mice[28], and CR3$^{-/-}$ (MAC-1 KO) mice[29], were bred and maintained at the Central Animal Facility of the Utrecht University, The Netherlands. FcγRI/III$^{-/-}$ (CD64/CD16 double KO) mice and C1q$^{-/-}$ (C1q KO) mice[30] were bred at the LUMC, Leiden, The Netherlands. FcγRI$^{-/-}$ mice were backcrossed for 6 generations to the C57bl/6 background. All other KO mice were backcrossed for more than 10 generations to the C57Bl/6 background. For each experiment, mice between 8 and 16 weeks old were used. All experiments were approved by the Utrecht University animal ethics committee, and performed according to institutional and national guidelines.

Cell culture

The mouse melanoma cell line B16F10, which expresses gp75, was obtained from the Netherlands Cancer Institute (Frederick, MD). Cells were cultured in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated FCS and antibiotics. For experiments, B16F10 cells were harvested using trypsin-EDTA (Gibco BRL), washed three times with PBS, and collected in HBSS medium (Gibco BRL). In all experiments, cell viability exceeded 95%, as determined by trypan blue exclusion.

Antibodies and flow cytometry

Monoclonal antibody (mAb) TA99 (mIgG2a, anti-gp75) was produced from hybridoma HB-8704 (ATCC, Manassas, VA) under serum-free conditions with HyQ ADCF-mAb medium (Hyclone, Logan, UT), and purified by protein A Sepharose chromatography (Amersham Biosciences, Uppsala, Sweden).

B16F10 gp75 surface expression was determined by staining cells with mAb TA99 (10 μg/ml, 30 min at 4°C), followed by incubation with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG Ab (Protos, San Francisco, CA, 30 min at 4°C). To measure total gp75 expression, B16F10 cells were permeabilised with ice-cold methanol/acetone (1:1, 15 min at 4°C) before cells were stained. Cells were analyzed on a FACScan (BD Biosciences).

B16F10 liver metastasis model

Mice were anesthetized, and a small incision was made in the left flank to reveal the spleen. 2 x 10^5 B16F10 tumor cells (100 μl) were injected intrasplenically at a constant rate to allow flow of tumor cells towards the liver. After one minute, the spleen was removed to prevent early death due to a high tumor load in the spleen, and the incision was sutured (for photographs, see Fig. 1). All steps within this surgical procedure were standardized to minimize differences between mice. Mice were injected intraperitoneally on day 0, 2, and 4, with 200 μg mAb TA99, or PBS as a control (250 μl). At day 21, mice were sacrificed, and the number of liver metastases was scored in each mouse.

Statistical analysis

Statistical differences were determined using the two–tailed unpaired Student's *t*-test or ANOVA. Significance was accepted when $p < 0.05$. All experiments were repeated at least two times, yielding similar results.

RESULTS AND DISCUSSION

To address the potential of anti-tumor Ab as a peri-operative treatment modality to prevent outgrowth of liver metastases, we established a liver metastasis model in immuno-competent C57bl/6 mice. For induction of liver metastases, tumor cells can be inoculated into the portal circulation[31]. Due to the small size of both mesenteric and portal veins, direct injection in either vein would lead to a high risk of internal bleeding and unacceptable losses of mice. We therefore established an alternative model, in which tumor cells were injected into the spleen, from which tumor cells directly flow towards the liver (see Fig. 1)[32]. Splenectomy was performed after injection of tumor cells to prevent early death due to high spleen tumor load. To study Ab therapy, C57bl/6 mice were inoculated with B16F10 tumor cells, expressing gp75 (Fig. 2A), and the therapeutic effect of mAb TA99 (mouse anti-gp75 IgG2a) was assessed. Following tumor

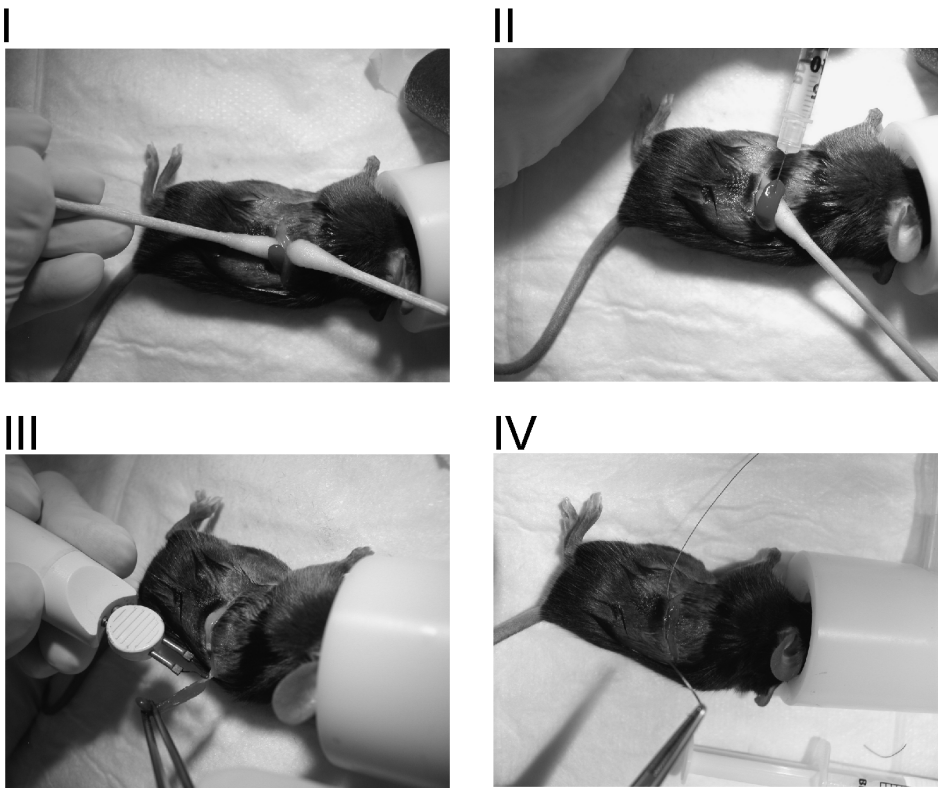

Figure 1. Mouse liver metastasis model. For a full-color picture, see Appendix page 173. (I) In anesthetized C57bl/6 mice, the spleen was exposed through a small incision, (II) after which B16F10 tumor cells were injected intrasplenically. (III) Removal of the spleen occurred with a searing devise to cauterize afferent and efferent blood vessels, (IV) after which the incision was sutured.

inoculation, black and grey metastases developed in untreated mice (overall mean of 25) within 3 weeks. Treatment of mice with mAb TA99 prevented tumor outgrowth in 90% - 95% of mice (n=60) (Fig. 2B, and C). The remaining 5% - 10% of mAb TA99 treated mice developed merely 1 to 4 liver metastases, a considerably lower number compared to untreated animals.

The underlying mechanisms of antibody-mediated prevention of liver metastases was evaluated by performing experiments in C57bl/6 mouse strains, deficient for specific complement components or IgG receptors. No difference was observed in anti-tumor activity of TA99 in $C1q^{-/-}$ mice, compared to wildtype mice (Fig. 3A, and B). As binding of C1q to IgG antibodies is crucial for activation of the classical pathway of complement[19], these results excluded a major role for complement in TA99 therapy against liver metastasis formation. TA99 mAb treatment was still effective in $CR3^{-/-}$ mice, as well (Fig. 3A, and C). Because CR3 is important for efficient immunological synapse formation by neutrophils[33], and additional treatment with G-CSF - which increases the number of neutrophils in the blood[34] - did not enhance therapy (data not shown), our data support that neutrophils are not involved in therapy against liver

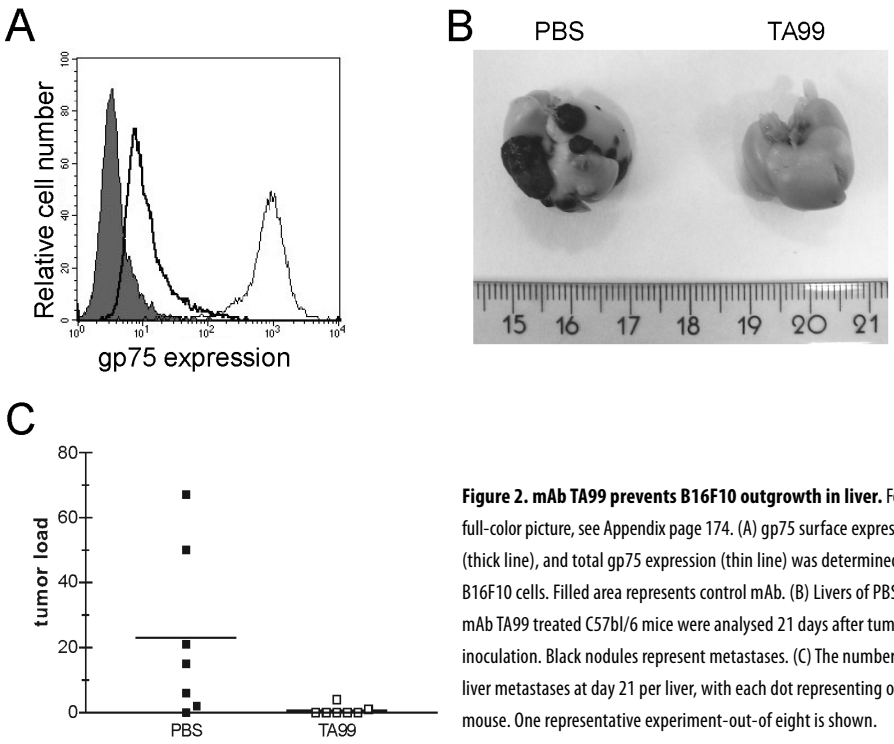

Figure 2. mAb TA99 prevents B16F10 outgrowth in liver. For a full-color picture, see Appendix page 174. (A) gp75 surface expression (thick line), and total gp75 expression (thin line) was determined on B16F10 cells. Filled area represents control mAb. (B) Livers of PBS or mAb TA99 treated C57bl/6 mice were analysed 21 days after tumor inoculation. Black nodules represent metastases. (C) The number of liver metastases at day 21 per liver, with each dot representing one mouse. One representative experiment-out-of eight is shown.

metastasis development. In a B16F10 lung metastases model, a major role for complement was excluded, as well, by the use of cobra venom factor[35]. Since TA99-therapy was however dependent on CR3[22], and treatment with G-CSF augmented therapy, neutrophils most likely play a role in the lung metastases model[22].

TA99 therapy against liver metastases was dependent on the presence of activatory FcγR, as therapy was abrogated in FcR γ chain$^{-/-}$ mice (Fig. 4. A and B), which is in accordance with earlier reports demonstrating a critical role for FcγR in Ab therapy[36-38]. Next, the role of individual activatory FcγR was evaluated. TA99 treatment effectively prevented tumor outgrowth in FcγRI$^{-/-}$ mice (Fig. 4C). In addition, therapy was not affected in FcγRIII$^{-/-}$ mice, either (Fig. 4D). To address the role of redundancy between FcγRI and FcγRIII, studies were performed in FcγRI/III$^{-/-}$ mice. Because TA99 treatment still prevented tumor outgrowth in these double KO mice (Fig. 4E), a specific role for either one of these receptors was excluded, which supports a putative role for FcγRIV in TA99 therapy against liver metastases. In the B16F10 lung metastasis model, a role for both FcγRI[39] and FcγRIV[40] have been implicated. In the lung metastasis model, infiltrates of macrophages were observed after Ab treatment[35]. Furthermore, a role for neutrophils in Ab therapy was supported[22]. Recruitment of these two immune cell subsets might explain the involvement of FcγRI and FcγRIV in Ab therapy against experimental lung metastases.

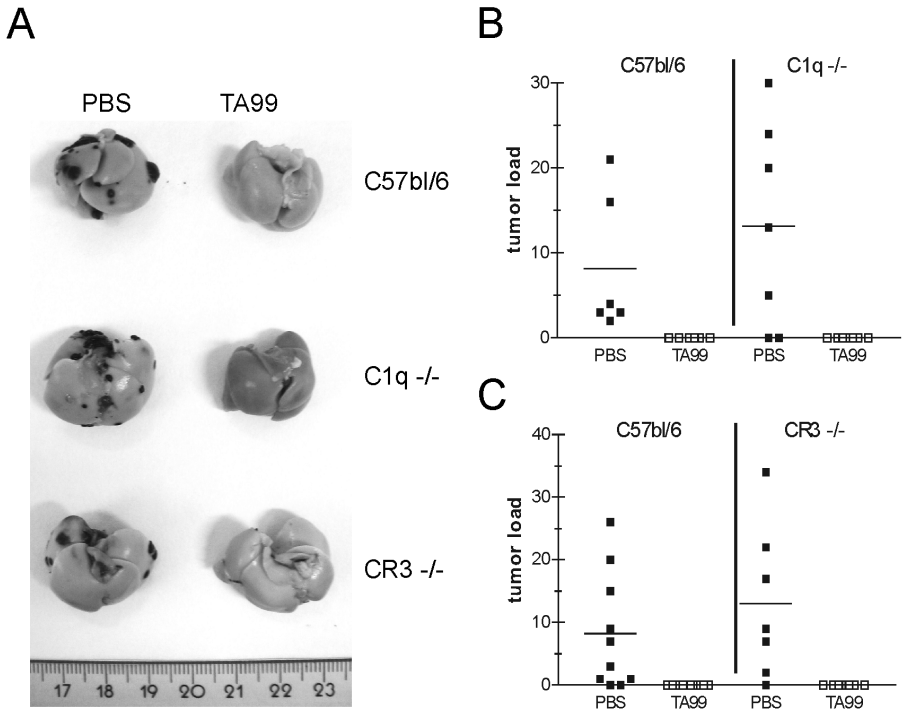

Figure 3. Complement is not essential for mAb TA99 therapy of liver metastases. For a full-color picture, see Appendix page 175. Livers of PBS or mAb TA99 treated C57bl/6 mice (panels A-C), C1q$^{-/-}$ mice (panels A, and B), or CR3$^{-/-}$ mice (panels A, and C) were excised 21 days after B16F10 tumor inoculation, and numbers of liver metastases quantified. One representative experiment out-of-two is shown.

FcγRIV is selectively expressed on myeloid cells, including dendritic cells, monocytes, macrophages, and neutrophils[23]. Natural killer cells, which are important for the natural defense against liver metastases[41], were excluded as effector cells for TA99 therapy against liver metastasis, as these cells specifically express FcγRIII[20], which was not required for TA99-mediated therapy (Fig. 4D). Furthermore, our data in CR3$^{-/-}$ mice (Fig. 3C) suggest that neutrophils were not involved either. We, therefore, postulate that macrophages are the main effector cell population for TA99-induced anti-tumor effects in the liver. Kupffer cells, which are resident liver macrophages, are essential for clearance of bacteria that enter the blood from the gastro-intestinal tract during inflammation[42]. Kupffer cells, however, can also trap and phagocytose tumor cells, which enter the liver[10,43]. Moreover, freshly isolated Kupffer cells mediate effective tumor cell killing[44], and significant outgrowth of tumor cells in the liver has been observed after depletion of these cells in rats[45] and mice (data not shown, n=3). These data, thus, support an important role for Kupffer cells in the defense against development of liver metastases[46]. Furthermore, Ab can recruit macrophages as effector cells; Ab-mediated tumor cell lysis has been well-documented, *in vitro*[47], and Ab-induced depletion of B-cells was shown to be mediated by macrophages, *in vivo*[48]. The fact that we predict that arrest of

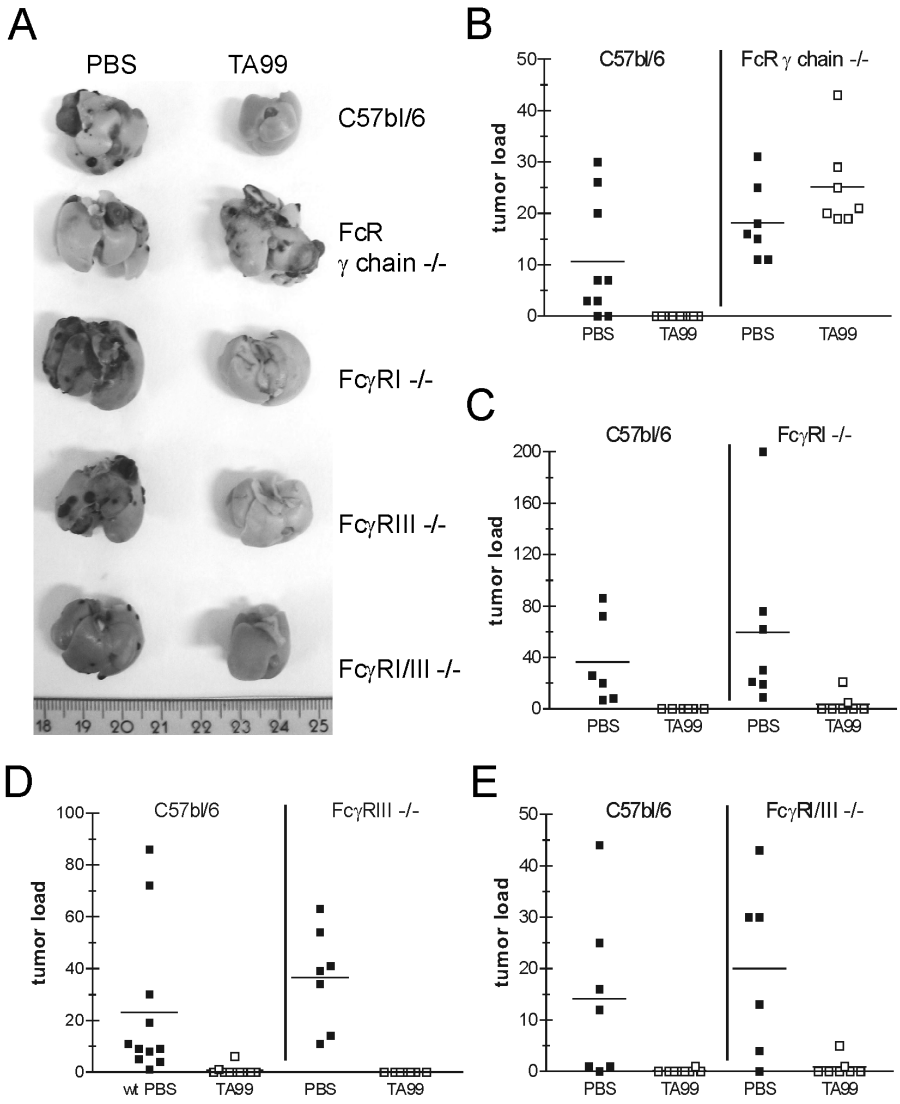

Figure 4. Activatory FcγR are essential for mAb TA99 therapy of liver metastases. For a full-color picture, see Appendix page 176. Mice inoculated with B16F10 cells were treated with either PBS or mAb TA99. After 21 days, livers were excised, and tumor load was determined in C57bl/6 mice (panels A-E), FcR γ chain$^{-/-}$ mice (panels A, and B), FcγRI$^{-/-}$ mice (panels A, and C), FcγRIII$^{-/-}$ mice (panels A, and D), or FcγRI/III$^{-/-}$ mice (panels A, and E). Experiments were repeated at least 2 times, yielding similar results.

circulating tumor cells by Kupffer cells, plays a role in Ab therapy against formation of liver metastases, may also explain our observations that neither effective treatment of mice, 1 or 2 weeks after tumor inoculation, nor induction of memory responses was found (data not shown, n=3).

In conclusion, TA99 antibody therapy against development of liver metastases was not dependent on activation of the classical complement pathway, but was completely abrogated in FcR γ chain$^{-/-}$ mice, indicating a role for activatory FcγR. As prevention of liver metastases was independent of FcγRI and FcγRIII, a role for FcγRIV is proposed. Importantly, because Ab therapy completely prevented formation of liver metastases in 90% - 95% of mice, antibody therapy in patients during surgical removal of colorectal cancer might prevent outgrowth of liver metastases, as well.

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Chapter 9

Summarizing discussion

RATIONALE FOR FcαRI AS TARGET FOR ANTIBODY THERAPY

In the 20th century, surgical resection, chemotherapy, and radiotherapy represented the main treatment options for cancer. To improve cancer therapy, scientists are now evaluating the use of more tumor-specific strategies. Because tumors express tumor-associated antigens (TAA)[1] to which specific antibodies (Ab) can be obtained, the role of anti-tumor Ab in tumor therapy is currently investigated[2]. This work led to the approval of eight anti-tumor monoclonal Ab (mAb) by the United States FDA within the last decade. Therapeutic Ab are nowadays broadly used[3], and a large number of Ab are under investigation. A number of potential drawbacks of IgG Ab have, however, been observed. IgG Ab can bind to all types of leukocyte IgG receptors (FcγR) and can, thus, also interact with the inhibitory FcγRIIb, which might lead to down-modulation of immune responses[4,5]. FcγR are furthermore widely distributed and are also expressed on non-cytotoxic cells, like platelets, B cells, and endothelial cells. Binding of IgG to such cells may act as an antibody "sink". In addition, polymorphisms have been described for FcγRIIa (CD32) and FcγRIIIa (CD16), which influence clinical responses to therapeutic Ab[6-9]. A number of new strategies are under investigation to tackle these drawbacks.

One strategy involves engagement of the IgA receptor (FcαRI, CD89) as an alternative target for Ab therapy. FcαRI is selectively expressed on myeloid cells, and can potently trigger immune effector functions, like phagocytosis, cytokine release, and oxidative burst (**Chapter 2**, and refs. 10,11). However, as large quantities of anti-tumor IgA Ab were not available, and since mice do not express a murine homologue for FcαRI, it has been difficult to study the role of FcαRI in Ab therapy. In the last few years, new molecular techniques enabled the construction of anti-tumor IgA Ab[12]. Additionally, human FcαRI-transgenic (Tg) mice became available, in which the cell distribution of FcαRI mirrors the cell distribution in humans[13]. Due to the availability of these tools, the role of FcαRI in tumor therapy could be addressed. Early studies showed that triggering of neutrophil FcαRI effectively induced tumor cell lysis *in vitro*[14,15]. Treatment of FcαRI Tg mice with FcαRIxIdiotype bispecific Ab (BsAb) against BCL1 lymphoma, resulted in prolonged survival of FcαRI Tg mice (M. van Egmond and M.J. Glennie, unpublished results). This work identified FcαRI as a candidate target for Ab therapy, and its role was investigated in this thesis.

NEUTROPHIL FcαRI AS TARGET FOR ANTIBODY THERAPY

Neutrophils exert well-documented anti-tumor properties, and have been shown to play a role in tumor rejection *in vivo*, as depletion of these cells resulted in enhanced tumor outgrowth in rats and mice[16-18]. Their numbers can be increased in blood by treatment of patients with granulocyte-colony stimulating factor (G-CSF)[19]. Furthermore, G-CSF treatment induces expression of neutrophil FcγRI (CD64)[20]. As FcγRI is the most potent leukocyte FcγR on neu-

trophils for induction of Ab-mediated cellular cytotoxicity (ADCC)[20,21], clinical studies were performed in which patients with breast cancer were treated with G-CSF and BsAb, targeting FcγRI and the TAA Her-2/neu. However, only limited therapeutic effects were shown[22-26]. To investigate whether targeting of neutrophils via FcαRI would improve immune cell responses, we studied FcαRI and FcγRI-mediated neutrophil functions in detail in **Chapters 3 to 6**.

Targeting neutrophil FcαRI with IgA (or BsAb), recognizing HLA class II, EpCAM or Her-2/neu positive tumors, resulted in more effective lysis of tumor cells, compared to targeting neutrophil FcγRI. Furthermore, signaling initiated in mature neutrophils was also more efficient after FcαRI triggering (**Chapter 3**). As treatment of patients with G-CSF induces mobilization of immature neutrophils from bone marrow[19], we investigated Fc receptor functions in bone marrow neutrophils, as well. Notably, although immature neutrophils mediated signaling and effector functions by targeting FcαRI, most FcγRI-mediated functions were hampered, which might explain the disappointing results in patients treated with G-CSF and FcγRI-directed BsAb[22-26] (**Chapters 3, and 4**).

To study tumor-directed migration of neutrophils in response to anti-tumor Ab, collagen cultures were established in which tumor colonies were grown. Targeting of neutrophil FcαRI induced massive neutrophil migration, followed by tumor colony destruction (**Chapter 3**). As these responses were not observed by targeting of FcγR, we further evaluated the underlying mechanisms of FcαRI-induced migration in **Chapter 5**. We observed that triggering of FcαRI, either via IgA or BsAb, induces release of chemoattractants and pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α. We hypothesize that this leads to enhanced neutrophil recruitment towards tumor colonies, resulting in colony destruction. FcαRI-mediated responses were enhanced in the presence of an endothelial layer. We postulate that cytokines released by activated neutrophils, stimulate IL-8 production by endothelial cells[27,28], which may trigger increased neutrophil migration and tumor cell killing. In contrast, FcγRI-triggering did not induce cytokine/chemoattractant-release, which may underlie the observed lack of neutrophil recruitment and tumor destruction.

Remarkably, although neutrophil FcαRI not only triggered ADCC more efficiently than FcγRI (even more obvious in immature neutrophils), but also induced tumor-specific migration, both receptors depend on association with the FcR γ chain for induction of downstream effector functions[29,30]. Fc receptor internalization was the only receptor activity in immature neutrophils which could readily be triggered via FcγRI (**Chapter 4**). As receptor internalization represents an FcR γ chain independent function[31,32], our results suggest that the discrepancy between FcαRI and FcγRI-mediated functions may be related to differences in association with the FcR γ chain. The interaction with the FcR γ chain was, therefore, investigated in co-immuno-precipitation studies, and showed a less stable association of FcγRI with the FcR γ chain, compared to FcαRI (**Chapter 4**). Earlier work showed that FcαRI bears a positively charged amino acid in its transmembrane region, that may form an electrostatic interaction with a negatively charged amino acid in the FcR γ chain[30]. Because FcγRI does not bear such a

charged residue, this might underlie the less stable association of the receptor with the FcR γ chain. As neutrophils express relatively low FcR γ chain levels, compared to monocytes[33], and FcγRI-FcR γ chain interactions are crucial for stable cell surface expression[34], we hypothesize that an inability of FcγRI to trigger effector functions, as well as downregulation of FcγRI expression during neutrophil maturation (**Chapter 4**), may be the result of competition between FcαRI and FcγRI for available FcR γ chain. Additionally, a less stable association of FcγRI with the FcR γ chain might affect downstream signaling in mature neutrophils. In contrast to FcγRI-targeting, triggering of neutrophil FcαRI induced release of chemoattractants and cytokines (**Chapter 5**). Although it has been shown that FcαRI surface expression depends on association with the FcR γ chain[13], select neutrophil populations have been identified to express some FcαRI not associated with this signaling chain[32]. FcαRI expression on these cells suggests that FcαRI may associate with other (signaling) molecules as well.

Taken together, we postulate that FcαRI-directed Ab therapy might trigger neutrophil recruitment, leading to tumor lysis. Because activated neutrophils can secrete chemoattractants and inflammatory cytokines, other immune cells, like monocytes, dendritic cells and T cells, may be attracted, as well, resulting in more generalized anti-tumor immune responses[35,36].

To investigate the role of neutrophils in FcαRI-mediated immunotherapy *in vivo*, we established an "*in vivo* ADCC", in which Her-2/neu-transfected mouse tumor cells (CMS7-HE) were used as tumor targets. On days 4 and 6 after tumor inoculation, FcαRIxHer-2/neu BsAb-opsonised neutrophils were injected in FcαRI Tg mice. Tumors went into regression when opsonised neutrophils were injected directly into tumors, which suggested that targeting of neutrophil FcαRI induced lysis of tumor cells. Tumor growth was, however, unaffected when opsonised neutrophils were injected intravenously (M.A. Otten and M. van Egmond, unpublished data). In **Chapter 5**, we showed that triggering of neutrophil FcαRI *in vitro* efficiently induced tumor-directed migration, which was in contrast with our *in vivo* observations. *In vitro*, several neutrophils may migrate randomly in collagen gels as a result of gravity, leading to "spontaneous" interactions between neutrophils and tumor cells. In the presence of FcαRI-directed BsAb, these neutrophil-tumor cell interactions may trigger the cascade of events (like cytokine/chemokine release) leading to neutrophil accumulation and tumor cell killing. Additional chemotactic signal(s) might, in our opinion, be needed *in vivo*, to enforce the "first" type of interaction between neutrophils and tumor cells.

DENDRITIC CELL FcαRI AS TARGET FOR ANTIBODY THERAPY

Dendritic cells (DC) are potent antigen presenting cells, with a unique role in regulation of adaptive immunity[37]. To acquire optimal T cell activation, DC take-up and process antigens to peptides, which are then presented on DC MHC molecules to T cells[37]. In addition, inflammatory signal(s) are necessary, as these lead to upregulation of co-stimulatory molecules,

and DC maturation[38]. DC can, thus, be used to achieve tumor-specific T cell and memory responses by targeting of tumor antigens to DC[37].

Because IgG immune complexes trigger DC maturation and may induce anti-tumor T cell responses[39-41], FcγR are considered as candidate targets for DC-based vaccines. In FcγRII-deficient mice, however, anti-tumor responses were enhanced[42], which showed that the inhibitory FcγRIIb on DC was capable of down-regulating immune responses. As FcαRI has been described on DC as well, and engagement of this receptor triggered DC activation[43-45], we studied the suitability of DC FcαRI as an alternate DC target molecule (**Chapter 6**).

Both blood and cultured DC expressed low levels of FcαRI, and FcαRI-triggering induced receptor internalization. FcαRI-mediated antigen presentation, however, was minimal. Targeting of this receptor on FcαRI-transfected IIA1.6 cells leads to presentation of ovalbumin peptides (**Chapter 6,** and ref. 46), indicating that FcαRI is capable of inducing efficient antigen presentation. We, therefore, hypothesize that DC FcαRI expression is simply too low to induce internalization and processing of significant amounts of antigens, leading to minimal presentation on MHC molecules and low levels of T cell activation. As such, we do not regard FcαRI a suitable target for DC based vaccines.

However, *in vivo*, we observed that targeting of neutrophils via FcαRI triggered tumor necrosis. Furthermore, chemokines and pro-inflammatory cytokines can be secreted as well by FcαRI-triggered neutrophils (**Chapter 5**). This may lead to chemokine-induced attraction of other immune cells, like DC and macrophages[35,36]. DC may consequently take-up tumor lysates, and become activated by cytokines released by neutrophils, leading to antigen presentation[47]. Neutrophil FcαRI-targeting might, therefore, indirectly induce anti-tumor T cell responses via DC.

MACROPHAGE FcαRI AS TARGET FOR ANTIBODY THERAPY

Macrophages can trigger tumor cell lysis in the presence of anti-tumor Ab[48]. Furthermore, macrophage-mediated depletion of B-cells has been observed after *in vivo* administration of anti-CD20 Ab[49]. In the liver, Kupffer cells (resident liver macrophages) express FcαRI under inflammatory conditions[50]. These cells can trap and phagocytose IgA-coated bacteria to prevent septicemia[50]. In addition, Kupffer cells exhibit prominent cytotoxicity against tumor cells, and are involved in the first line defense against development of liver metastases[51,52]. Many patients with colorectal cancer, the third most common malignancy in the western world[53], ultimately develop incurable liver metastases, even after successful surgical removal of the primary tumor[54,55]. Paradoxically, formation of liver metastases might be initiated during surgical resection, as surgery induces immuno-suppression[56,57]. We therefore evaluated whether FcαRI-directed Ab therapy could be used as a peri-operative treatment modality to prevent liver metastases formation.

Because most anti-tumor IgA Ab or FcαRI-BsAb are directed against human TAA, such as Her-2neu, we initially used Her-2/neu transfected mouse tumors (CMS7-HE). During the course of our studies, however, we observed that subcutaneously grown CMS7-HE, which was injected superficially, often regressed spontaneously, in contrast to more deeply injected tumors (**Chapter 7**). Ulceration usually preceded regression, as tumors grew through the skin. Furthermore, increased levels of mouse anti-Her-2/neu Ab, and elevated numbers of T and B cells were observed in tumors of these mice. We, therefore, postulate that the observed spontaneous tumor regression was attributable to an activated immune system, giving rise to mouse anti-human responses. A similar phenomenon has been described for GFP-transfected tumor cells. Inoculation of such cells in immuno-competent mice elicited T and B cell responses against GFP[58]. As anti-tumor Ab direct the immune system towards the tumor, human TAA-transfected tumors might inevitably induce mouse anti-human responses, which may lead to tumor regressions, independent of therapeutic effects elicited by the Ab itself.

To circumvent this problem, we switched to a syngeneic immuno-competent mouse model, in which B16F10 melanoma cells, expressing the TAA gp75, were used. Treatment of FcαRI Tg mice with FcαRIxgp75 BsAb, however, did not lead to prevention of B16F10 liver metastases development (data not shown). Treatment of mice with the parental anti-gp75 IgG Ab resulted in complete prevention of tumor outgrowth in livers (**Chapter 8**). *In vitro*, macrophage FcαRI can trigger efficient tumor cell lysis[59]. Macrophage recruitment, however seems more efficient via FcγR-targeting[60]. Although we can not exclude that the dose / treatment schedule of FcαRIxgp75 BsAb in our *in vivo* experiment was suboptimal, we focused our experiments on the mechanism of anti-tumor IgG in prevention of liver metastases.

Therapy with anti-gp75 IgG against liver metastases formation was dependent on the presence of FcγR, most probably the newly-identified FcγRIV[61] (**Chapter 8**). Mouse FcγRIV is expressed on dendritic cells, neutrophils, monocytes and macrophages[61]. In neutrophils, CR3 is essential for immunological synapse formation[62]. Because Ab therapy against liver metastasis development was not dependent on CR3, our data suggest that neutrophils were most likely not involved. We hypothesize that macrophages (Kupffer cells) were recruited as effector cells (**Chapter 8**).

The therapeutic mechanism underlying Ab therapy against liver metastases formation differed from the mechanism observed in the lung metastasis model; in lungs, therapy could be enhanced by G-CSF treatment, and therapy was dependent on CR3, supporting a role for neutrophils[63]. Furthermore, in addition to FcγRIV, a role for FcγRI has been implicated in the lung metastasis model, as well[64,65]. We hypothesize that the (immunological) environment of organs has an impact on the therapeutic mechanism and efficacy of Ab therapy. As Ab treatment after surgery prevented tumor outgrowth in mouse livers (**Chapter 8**), peri-operative Ab treatment during surgical removal of primary tumors, might provide a therapeutic modality to increase clinical outcome in patients.

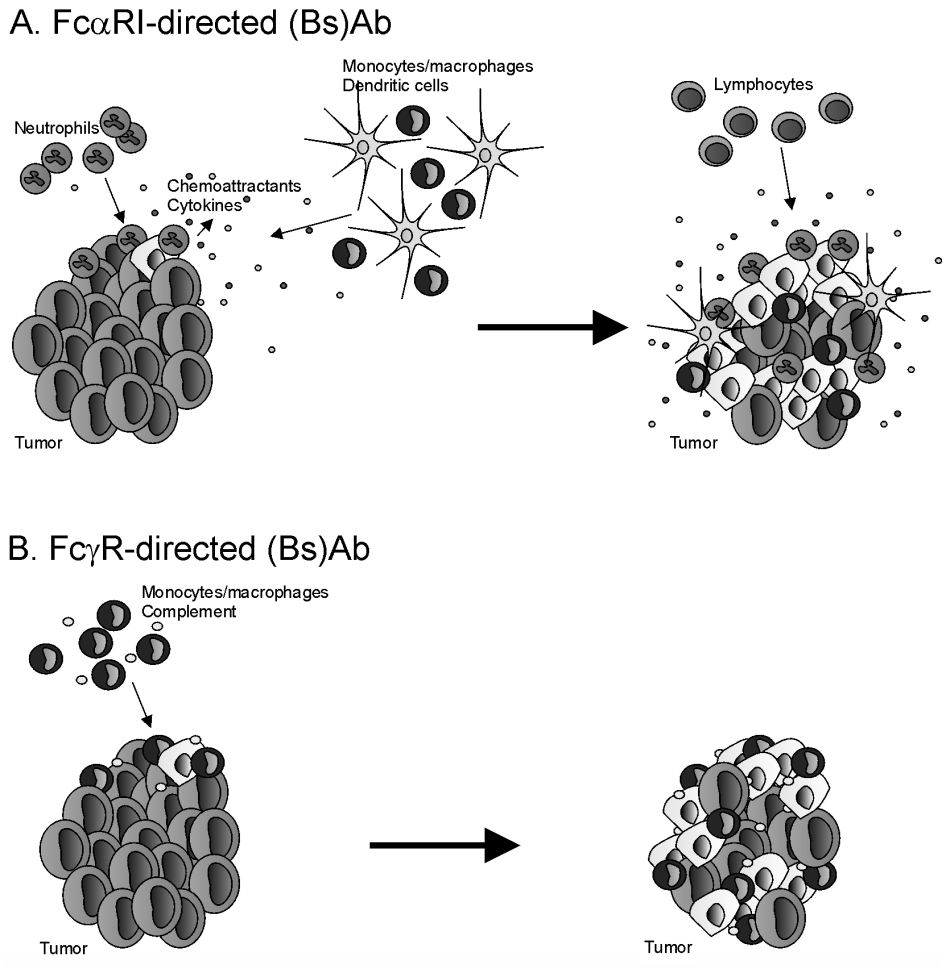

Figure 1. Model for FcαRI- and FcγR-directed antibody therapy. (A) FcαRI-directed (Bs)Ab trigger recruitment of neutrophils. Activated neutrophils lyse tumor cells and secrete cytokines/chemokines, thereby attracting monocytes/macrophages and dendritic cells. Lysed tumor cells are taken-up by DC. In addition, DC are activated due to cytokines released by neutrophils (such as TNF-α), leading to generation of lymphocyte responses. (B) FcγR-directed Bs(Ab) recruit macrophages. In addition, the complement pathway can be activated by IgG Ab, (further) enhancing tumor cell lysis.

CONCLUDING REMARKS

Immunotherapy represents a growing field, in which clinical challenges consistently trigger new biological questions. Unraveling these questions will hopefully lead to improved therapies. In this thesis, we aimed to further clarify the role of FcαRI as target for Ab therapy. We observed that FcαRI-triggering efficiently recruits neutrophils. Because triggering of neutrophil FcαRI induced secretion of chemokines and cytokines, additional immune cells, such as monocytes / macrophages and DC, may be recruited / activated, leading to more generalized anti-tumor responses (Fig. 1A). We, furthermore, showed that IgG therapy resulted in prevention of tumor outgrowth in livers, which was possibly mediated by Kupffer cells. FcγR-targeting may, thus, attract macrophages for induction of anti-tumor responses (Fig. 1B). As both FcαRI and FcγR seem to recruit different immune cell subsets, the use of an antibody "cocktail" consisting of FcαRI-directed (Bs)Ab and FcγR-directed Bs(Ab), both recognizing diverse tumor antigens, may lead to a broad recruitment of immune cell populations, hereby inducing maximal anti-tumor responses. We hope that the results presented in this thesis may trigger the development of better / more effective (immuno)-therapeutic approaches for cancer.

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Nederlandse samenvatting

Methodes van antilichaam therapie voor kanker

INTRODUCTIE TOT HET IMMUUNSYSTEEM

Dagelijks wordt de mens blootgesteld aan een uitgebreid scala van bacteriën, virussen en parasieten. De huid speelt een belangrijke rol bij de bescherming tegen deze ziekteverwekkers. Wanneer ziekteverwekkers toch de huid passeren zijn er ook nog andere afweermechanismen om de indringers te herkennen en te vernietigen: het immuunsysteem. Het is belangrijk dat het immuunsysteem snel tot actie komt om blijvende schade aan het lichaam te voorkomen. Deze snelle reactie wordt in gang gezet door het aangeboren immuunsysteem dat algemene patronen van lichaamsvreemde binnendringers kan herkennen. Het aangeboren immuunsysteem bestaat uit witte bloedcellen en uit complement-eiwitten. Na herkenning van een indringer kunnen complement-eiwitten in het bloed worden geactiveerd, wat leidt tot rechtstreekse doding van de indringer. Verder worden de witte bloedcellen aangezet om de indringer "op te eten" (fagocyteren).

Om een meer specifieke afweerreactie te verkrijgen, kunnen fagocyterende cellen (zoals dendritische cellen) stukjes van de ziekteverwekker presenteren aan zogenoemde T-cellen en B-cellen. De T- en/of B-cellen die deze ziekteverwekker herkennen worden geactiveerd en gaan vermenigvuldigen. Geactiveerde T-cellen kunnen geïnfecteerde cellen rechtstreeks vernietigen. B-cellen kunnen dit niet rechtstreeks, maar produceren eiwitten (antistoffen / antilichamen) die specifiek de ziekteverwekker binden. Soms kan deze binding al voldoende zijn om de ziekteverwekker te neutraliseren en af te voeren. In andere gevallen fungeren de gebonden antilichamen als "vlaggetjes", om zo het aangeboren immuunsysteem specifiek de ziekteverwekker aan te laten vallen. Aangezien de mens deze specifieke reactie verwerft terwijl de ziekteverwekker al in het lichaam aanwezig is, komt deze een stuk langzamer op gang vergeleken met het aangeboren immuunsysteem. Dit verworven systeem "onthoudt" een eenmaal opgetreden reactie echter voor lange tijd (jaren/decennia), waardoor deze zeer snel weer geactiveerd kan worden op het moment dat dezelfde ziekteverwekker nog een keer binnendringt.

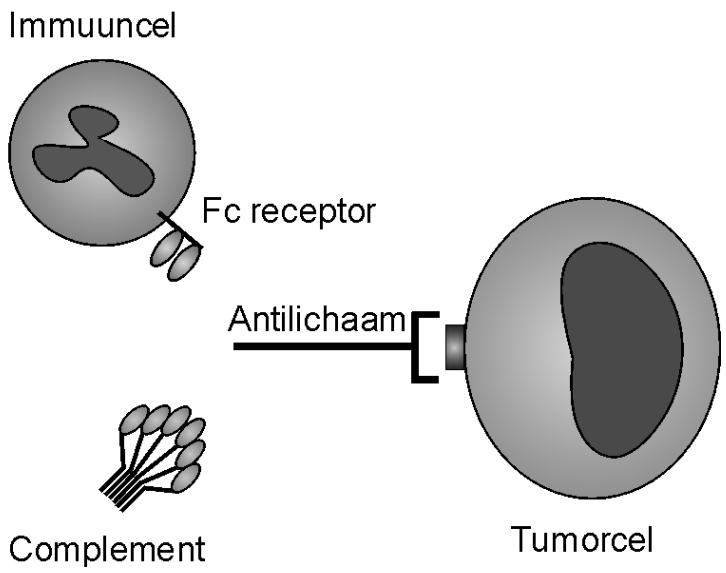
INTRODUCTIE TOT ANTILICHAAMTHERAPIE

De geneeskunde maakt gebruik van de wetenschap over het immuunsysteem. Door mensen te vaccineren tegen ziektes als hepatitis, polio en tetanus, wordt het immuunsysteem aangezet een immuunreactie tegen deze ziekteverwekkers op te bouwen zonder dat de persoon op dat moment daadwerkelijk geïnfecteerd is. Aangezien het verworven immuunsysteem deze reactie kan blijven "onthouden", is de mens voor jaren/decennia beschermd tegen deze ziekteverwekkers.

In de laatste jaren is duidelijk geworden dat het immuunsysteem ook gebruikt kan worden tegen kanker. Tot op heden zijn operatieve verwijdering van de tumor, bestraling en chemo-

therapie de meest gebruikte methodes voor het bestrijden van kanker. Bestraling en chemotherapie zijn echter niet specifiek tegen de tumor gericht en veroorzaken vaak ongewenste bijwerkingen. In vergelijking met gezonde cellen hebben tumorcellen veel hogere hoeveelheden van bepaalde eiwitten (tumorantigenen) op hun celmembraan zitten. Antilichamen die deze tumorantigenen herkennen (anti-tumor antilichamen) binden daardoor specifiek aan de tumorcel. Omdat deze gebonden antilichamen herkend kunnen worden door de andere componenten van het immuunsysteem, wordt op deze manier het immuunsysteem in gang gezet om de tumor op te ruimen. Dit is het basisprincipe van antilichaamtherapie (figuur 1).

Op dit moment zijn er al acht verschillende anti-tumor antilichamen goedgekeurd door de FDA (Food and Drug Administration in de Verenigde Staten). Ieder tumorantigen is specifiek voor een bepaald type kanker. Zo kunnen anti-CD20 antilichamen (rituximab) gebruikt worden voor een groot aantal patiënten met non-Hodgkin Lymfoom (tumorantigen is CD20), terwijl een groot aantal borstkankerpatiënten (tumorantigen is Her-2/neu) behandeld kunnen worden met anti-Her-2/neu antilichamen (trastuzumab). Hoe antilichaamtherapie precies werkt is tot op heden nog niet volledig bekend. Verschillende mechanismen, die ook nog kunnen variëren tussen de verschillende type antilichamen, zijn beschreven (zie ook figuur 1): Binding van een antilichaam aan een tumorantigen kan veroorzaken dat het antigen niet



Figuur 1. Schematische voorstelling van de werking van antilichaam therapie. Anti-tumor antilichamen herkennen een eiwit (tumorantigen) wat op de tumorcel tot expressie komt. Antilichamen kunnen de functie van dit eiwit verstoren, wat leidt tot geprogrammeerde celdood (apoptose) van de tumorcel. Verder kan de Fc staart van het antilichaam herkend worden door receptoren op immuuncellen (Fc receptoren), wat leidt tot activatie van de immuuncel. Tenslotte kunnen eiwitten in het serum van het bloed (complement-eiwitten) ook aan het antilichaam binden. Deze geactiveerde complement-eiwitten vormen een complex, dat in staat is de tumorcel te doden.

meer goed kan functioneren, wat kan leiden tot geprogrammeerde celdood (apoptose). Een anti-tumor antilichaam kan ook het complement-systeem activeren, om via deze weg de tumorcel te doden. Verder kan de "staart" (het Fc deel) van het anti-tumor antilichaam binden aan zogenoemde receptoren (Fc receptoren) die op het oppervlak van immuuncellen zitten. Deze geactiveerde Fc receptoren zetten vervolgens de immuuncel aan tot het doden van de tumorcel. De mens heeft verschillende klassen van antilichamen (IgG, IgA, IgD, IgE, en IgM) die ieder een specifiek type Fc receptor bindt. Tot op heden zijn alle FDA goedgekeurde antilichamen van het IgG type. Deze kunnen herkend worden door IgG-specifieke Fc receptoren, die ook wel FcγR genoemd worden.

PROMOTIEONDERZOEK

Dit proefschrift beschrijft onderzoek naar de manier hoe anti-tumor antilichamen gebruikt kunnen worden om leveruitzaaiingen te voorkomen. Verder hebben we gekeken of antilichaamtherapie ook gemedieerd kan worden via IgA-specifieke Fc receptoren (ook wel FcαRI genoemd).

Antilichaamtherapie tegen leveruitzaaiingen

Darmkanker is, na longkanker en borstkanker, het meest voorkomende type kanker in de westerse wereld. Deze tumor kan operatief worden verwijderd. Echter, zelfs als patiënten tijdens deze operatie geen zichtbare uitzaaiingen hebben, krijgt 25 tot 50% van deze personen uiteindelijk toch leveruitzaaiingen. Een van de gedachtes hierover is, dat tijdens de operatie je afweer tijdelijk vermindert. Hierdoor kunnen losgeraakte tumorcellen, die normaal gesproken opgeruimd zouden worden door het immuunsysteem, ongestoord gaan uitgroeien in de lever (het eerste orgaan na de darm). Het geven van antilichaamtherapie tijdens en vlak na de operatie zou bij deze patiënten uitgroei van levertumoren mogelijk kunnen voorkomen.

Om dit uit te testen hebben we een systeem opgezet in muizen, waarbij bestudeerd werd of het geven van antilichaamtherapie rondom de operatie leidde tot preventie van tumor-uitgroei in de lever. Veel anti-tumor antilichamen zijn gericht tegen humane tumorantigenen. Omdat humane tumoren echter niet groeien in muizen met een volledig functioneel immuunsysteem, werd veelal gebruik gemaakt van muizen tumorcellen die humane tumorantigenen op hun celmembraan tot expressie brengen (getransfecteerde cellen). In **hoofdstuk 7** hebben we echter gezien dat het humane tumorantigen op deze getransfecteerde cellen "spontaan" herkend kan worden door het immuunsysteem in de muis. Hierdoor verdween de tumor in deze muizen ook al zonder antilichaam-behandeling. Het bestuderen van antilichaamtherapie tegen leveruitzaaiingen (**hoofdstuk 8**) hebben we om deze reden uitgevoerd met een muizen tumorcellijn B16F10 (tumorantigen is gp75). Hierbij zagen we dat onbehandelde muizen na 3 weken gemiddeld 25 tumoren in de lever hadden, terwijl 92%

van de muizen die vlak na operatie behandeld waren met anti-gp75 IgG (TA99) antilichamen volledig tumorvrij waren. De overige 8% van de TA99-antilichaam behandelde muizen had slechts enkele (1 tot 4) tumoren. Deze data tonen aan dat het geven van antilichaamtherapie tijdens operatieve verwijdering van darmtumoren zou kunnen helpen bij het verminderen van het aantal leveruitzaaiingen.

Om de werking van antilichaamtherapie in de lever beter te begrijpen, hebben we ook gekeken naar het mechanisme van deze therapie. Kennis over het mechanisme kan namelijk leiden tot optimalisatie van antilichaamtherapie in de mens. Hiervoor hebben we gebruik gemaakt van verschillende muizenstammen die een bepaald eiwit missen (knock-out muizen). In muizen die een bepaald gedeelte van het complement-systeem missen (C1q, klassieke route) werkte de therapie nog volledig, wat aantoont dat complement waarschijnlijk geen belangrijke rol speelt. De therapie was echter niet meer werkzaam in muizen die activerende IgG Fc receptoren (FcγR) missen. Dit toont aan dat Fc receptoren cruciaal zijn. FcγRIV, die tot expressie komt op immuuncellen zoals monocyten, macrofagen, dendritische cellen en neutrofielen, lijkt hierbij de belangrijkste FcγR te zijn. Aangezien er veel residentiële macrofagen in de lever zitten (ook wel Kupfferse stercellen genoemd), hypothetiseren wij dat deze immuuncellen het belangrijkste zijn voor TA99-gemedieerde antilichaamtherapie tegen leveruitzaaiingen.

Antilichaamtherapie via IgA Fc receptoren (FcαRI)

Zoals gezegd zijn tot op heden alle door de FDA goedgekeurde antilichamen van het IgG type. Er zitten echter een aantal nadelen aan IgG antilichamen. FcγR zitten op immuuncellen die tumorcellen kunnen aanvallen (cytotoxische cellen), maar komen ook tot expressie op niet-cytotoxische cellen. Deze laatste cellen kunnen de IgG antilichamen "wegvangen" van de FcγR op cytotoxische cellen. Verder bestaat er ook een type FcγR (FcγRIIb) die immuuncel functies remt. Binding van IgG antilichamen aan deze receptor zorgt voor vermindering van de immuunreactie. Tenslotte zijn er bij de mens polymorfismen (interindividuele variaties) gezien in FcγRIIa en FcγRIIIa, die de antilichaam-gemedieerde anti-tumor responsen in patiënten flink kunnen beïnvloeden. De IgA Fc receptor (FcαRI) zit specifiek op cytotoxische cellen, zoals monocyten, macrofagen, dendritische cellen, eosinofielen en neutrofielen (zie ook **hoofdstuk 2**). Activatie van immuuncellen via FcαRI leidt tot vergelijkbare functies als activatie via FcγR (doding via uitscheiden van toxische stoffen en/of opname en afbraak). Daarom hebben wij *in vitro* (in de reageerbuis) bestudeerd of antilichaamtherapie via deze receptor een goed alternatief zou kunnen zijn.

Dendritische cellen zijn goed in het opnemen en presenteren van stukjes tumorantigen aan T- en B-cellen, hetgeen kan leiden tot een specifieke anti-tumor immuunrespons. Aangezien FcαRI op deze cellen tot expressie komt, zou deze opname ook via FcαRI kunnen geschieden. Daarom hebben we in **hoofdstuk 6** gekeken of het specifiek opnemen van tumorcellen via anti-tumor IgA antilichamen (en dus FcαRI) leidt tot goede T-cel activatie. Wij zagen echter

dat T-cel activatie minimaal was. Dit wordt waarschijnlijk veroorzaakt door de erg lage expressie van FcαRI op deze dendritische cellen.

Neutrofielen zijn zeer goed in staat ziekteverwekkers, zoals bacteriën, maar ook tumorcellen te vernietigen. Verder is het percentage neutrofielen in het bloed goed te verhogen door patiënten te behandelen met granulocyte-colony stimulating factor (G-CSF). Daarbovenop kunnen geactiveerde neutrofielen eiwitten/vetten (chemoattractanten) uitscheiden, die andere immuuncellen naar de "plaats delict" toe trekken om zo een nog bredere immuunreactie te verkrijgen. Om deze redenen hebben we gekeken in welke mate activatie van FcαRI zou leiden tot anti-tumor responsen door neutrofielen. Aangezien anderen hadden aangetoond dat FcγRI de meest efficiënte FcγR is voor neutrofiel activatie hebben we FcαRI-gemedieerde therapie vergeleken met FcγRI-gemedieerde therapie. We zagen dat G-CSF-gestimuleerde neutrofielen beter in staat waren een borstkankercellijn (SK-BR-3, tumorantigen = Her-2/neu) te doden via FcαRI, dan via FcγRI (**hoofdstuk 3**). Dit hebben we vervolgens ook aangetoond voor andere tumorantigenen, zoals EpCAM en HLA klasse II. Omdat G-CSF behandeling in patiënten leidt tot een mobilisatie van onvolwassen neutrofielen uit het beenmerg (waar de neutrofielen worden aangemaakt) naar het bloed, hebben we ook gekeken naar de capaciteiten van deze onvolwassen beenmerg-neutrofielen. Beenmerg-neutrofielen die geactiveerd werden via FcγRI bleken echter niet in staat te zijn om tumorcellen te doden, terwijl activatie van beenmerg-neutrofielen via FcαRI wel effectief was. De resultaten met deze beenmerg-neutrofielen zou kunnen verklaren waarom de anti-tumor responsen van borstkankerpatiënten, die behandeld waren met G-CSF en een FcγR-gericht antilichaam, minder goed bleken dan verwacht.

In **hoofdstuk 4** hebben we beter uitgezocht wat het onderliggende mechanisme is van het uitblijven van de anti-tumor activiteit van beenmerg-neutrofielen via FcγRI vergeleken met de goede anti-tumor activiteit die opgewekt was via FcαRI. Eerdere data hebben namelijk aangetoond dat beide receptoren associëren met hetzelfde signalerende eiwit (FcRγ-keten). Dit eiwit is belangrijk voor expressie van de receptor op de celmembraan en voor het activeren van Fc receptor-gemedieerde immuuncel functies. Wij zagen dat alle FcRγ-keten afhankelijke immuuncel functies van beenmerg-neutrofielen (signalering, uitscheiding toxische moleculen, tumorcel doding) niet geactiveerd konden worden via FcγRI, maar wel via FcαRI. Een FcRγ-keten onafhankelijke immuuncel functie (naar binnen halen van de receptor, oftewel internalisatie) kon echter geactiveerd worden via zowel FcαRI als FcγRI. Deze data wijzen naar een verschil tussen beide receptoren in de signalerende FcRγ-keten route. Verder bleek dat de associatie van FcγRI met de FcRγ-keten minder stabiel was, vergeleken met de associatie van FcαRI met de FcRγ-keten. Dit zou kunnen komen door een extra (sterke) elektrostatische interactie die FcαRI met de FcRγ-keten heeft, vergeleken met FcγRI. Eerder is al aangetoond dat Fc receptoren met elkaar kunnen competeren voor associatie met signalerende ketens. Aangezien neutrofielen een relatief lage hoeveelheid FcRγ-keten hebben (vergeleken met monocyten) hypothetiseren wij dat dit leidt tot competitie van FcαRI en

FcγRI voor de FcRγ-keten ten gunste voor FcαRI. Dit zou kunnen verklaren waarom FcγRI niet functioneel is en waarom FcγRI expressie verdwijnt op deze beenmerg-neutrofielen (FcγRI komt in bloed-neutrofielen alleen tot expressie na stimulatie met G-CSF).

Voor het opruimen van tumorcellen, moeten neutrofielen vanuit het bloed naar de tumor migreren. Antilichaam-gemedieerde tumor-specifieke neutrofiel-migratie hebben we daarom bestudeerd in **hoofdstuk 5**. Om de situatie van migrerende immuuncellen na te bootsen, lieten wij tumorkolonies groeien in een gel, waarna neutrofielen en anti-tumor antilichamen toegevoegd werden. Neutrofielen migreerden niet of nauwelijks naar de tumorkolonies met behulp van anti-tumor IgG antilichamen. Zeer goede responsen werden echter geïnduceerd via anti-tumor IgA antilichamen: Na FcαRI activatie scheidden neutrofielen chemoattractanten en cytokinen uit (om meerdere neutrofielen aan te trekken en te activeren) en hoge aantallen neutrofielen waren zichtbaar in de tumorkolonies (die na 24 uur zichtbaar vernietigd waren). Een extra endotheellaag - om de natuurlijke barrière tussen het bloed en het weefsel na te bootsen - versterkte deze FcαRI-gemedieerde responsen nog meer, wat leidde tot nog betere destructie van tumorkolonies.

CONCLUSIES

In dit proefschrift hebben we *in vitro* gekeken naar FcαRI als een mogelijke kandidaat voor antilichaamtherapie. Wij zagen dat FcαRI activatie zeer goed in staat was neutrofielen aan te zetten tot tumor-specifieke migratie en het doden van tumorcellen. Ook zagen we dat FcαRI-geactiveerde neutrofielen chemoattractanten en cytokinen uitscheidden, wat zou kunnen leiden tot rekrutering en activering van meer typen immuuncellen, zoals monocyten, macrofagen en dendritische cellen, hetgeen weer kan leiden tot een algemene anti-tumor respons (voor een cartoon, zie figuur 1A van **hoofdstuk 9**: "general discussion").

Verder zagen we in muizen dat het geven van anti-tumor IgG antilichamen rondom operatie zeer efficiënt was om leveruitzaaiingen te voorkomen. Dit was waarschijnlijk gemedieerd via Kupfferse stercellen (macrofagen). Therapie via IgG antilichamen rekruteert dus waarschijnlijk voornamelijk macrofagen (voor een cartoon, zie figuur 1B van **hoofdstuk 9**: "general discussion"). Tenslotte: Aangezien FcαRI-gemedieerde therapie en FcγR-gemedieerde therapie beiden een ander type immuuncel rekruteert, zou een antilichaam-cocktail met daarin anti-tumor IgA en IgG antilichamen kunnen leiden tot een nog grotere immuunrespons tegen tumoren. Wij hopen dat de resultaten beschreven in dit proefschrift zullen leiden tot het ontwikkelen van effectievere methodes van antilichaamtherapie voor het behandelen van kanker.

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Laten we de 15e een feestje gaan vieren!

Marielle

CURRICULUM VITAE

Marielle Otten werd geboren op 30 december 1977 te Helmond. Na het behalen van het VWO diploma op het Dr. Knippenberg College te Helmond in 1996, begon zij in datzelfde jaar met de studie Medische Biologie aan de Universiteit Utrecht. Haar propedeuse werd in augustus 1997 met genoegen behaald. Tijdens haar studie werden stages uitgevoerd bij de afdeling Haemato-oncologie onder begeleiding van Dr. S.B. Ebeling (hoofd: Prof. dr. A. Hagenbeek) en bij de afdeling Experimentele Neurologie onder begeleiding van Drs. J.H. Veldink (hoofd: Prof. dr. P.R. Bär). Een extra stage werd uitgevoerd in Pretoria, Zuid-Afrika, bij de afdeling Klinische Epidemiologie onder begeleiding van Drs. D.G. van Zyl (hoofd: Prof. dr. P. Rheeder). In augustus 2001 werd het doctoraal examen met genoegen behaald. Vanaf oktober 2001 werkte zij als assistent in opleiding bij de afdelingen Immunologie, laboratorium Immunotherapie van het Universitair Medisch Centrum Utrecht onder begeleiding van Prof. dr. J.G.J. van de Winkel en de afdeling Moleculaire Celbiologie en Immunologie van het VU Medisch Centrum onder begeleiding van Prof. dr. R.H.J. Beelen. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

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J.H. Veldink, P.R. Bär, E.A. Joosten, **M. Otten**, J.H. Wokke and L.H. van den Berg. Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscular Disorders 13(9): 737-743, 2003*

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M.A. Otten, I. Groenveld, J.G.J. van de Winkel and M. van Egmond. Inefficient antigen presentation via the IgA Fc receptor (FcαRI) on dendritic cells. *Immunobiology, 2006, in press*

M.A. Otten, C.W. Tuk, M.J. Glennie, A.L. Tutt, R.H.J. Beelen, J.G.J. van de Winkel and M. van Egmond. Antibody targeting to FcαRI triggers neutrophil migration towards tumor colonies. *Submitted for publication*

M. van Egmond, **M.A. Otten**, M.D. Jansen, C.W. Tuk, R.H.J. Beelen, S. Meijer, J.G.J. van de Winkel and E. van Garderen. Potential pitfalls of murine cancer models for antibody therapy. *Submitted for publication*

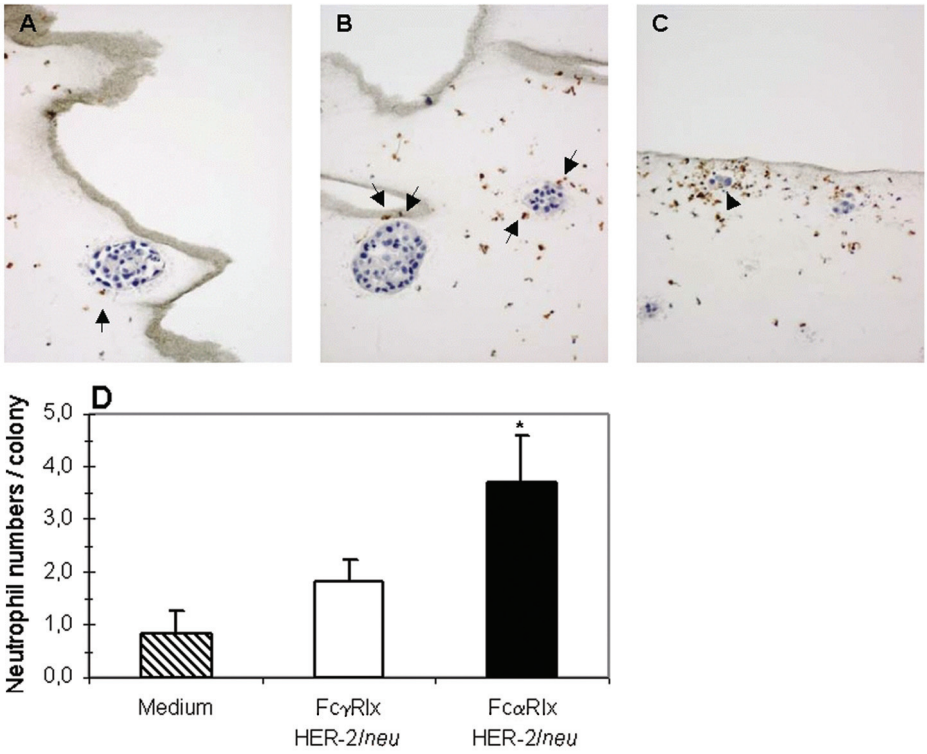
M.A. Otten, J.H.W. Leusen, E. Rudolph, R.H.J. Beelen, J.G.J. van de Winkel and M. van Egmond. FcR γ chain dependent signaling via IgA but not IgG Fc receptors in immature neutrophils. *Manuscript in preparation*

M.A. Otten, G.J. van der Bij, S.J. Verbeek, E. Rudolph, R.H.J. Beelen, J.G.J. van de Winkel and M. van Egmond. Role of FcγR in experimental antibody therapy of liver metastases. *Manuscript in preparation*

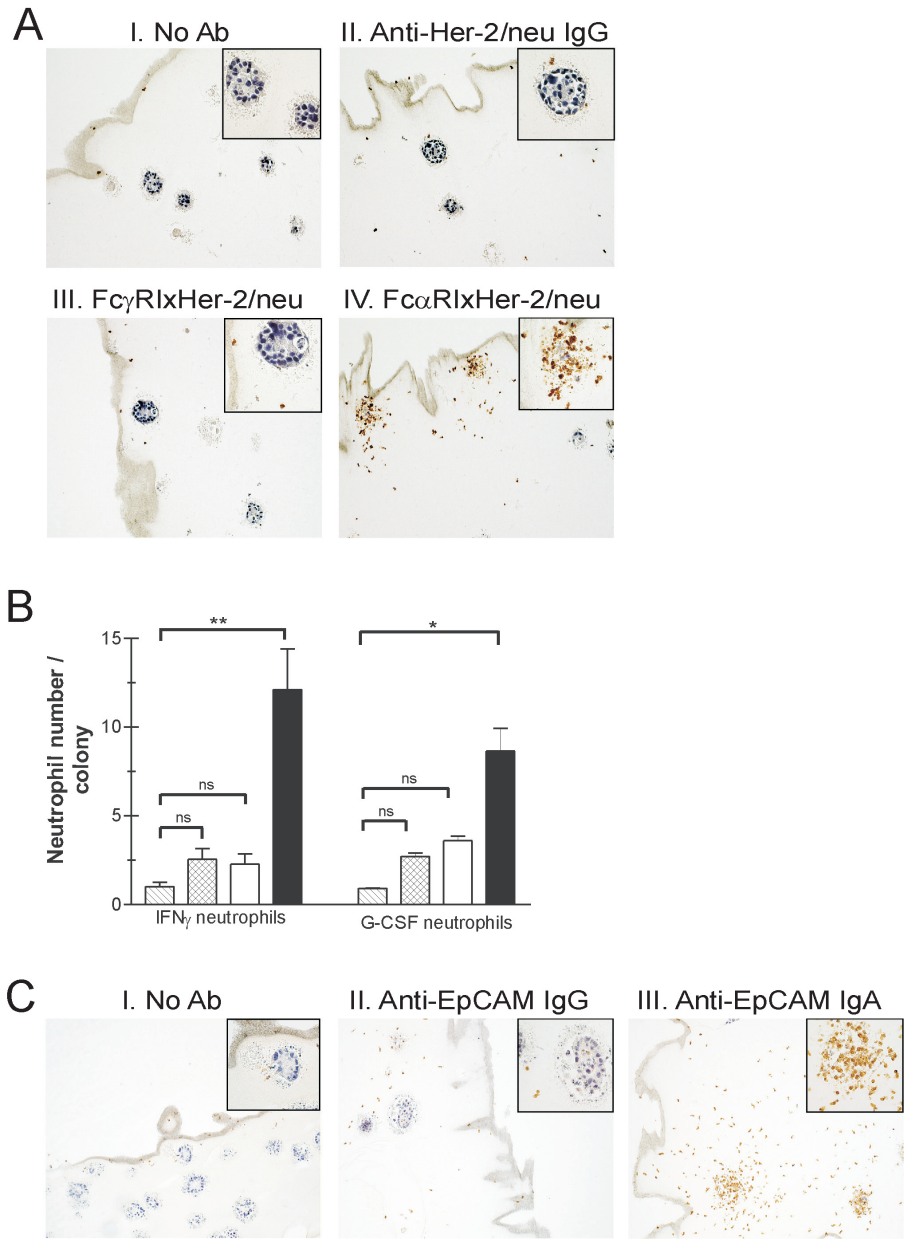
ABBREVIATIONS

-/-	knock-out
3D	3-dimensional
Ab	antibody
ADCC	antibody-dependent cellular cytotoxicity
ASGP-R	asialoglycoprotein-receptor
BMDC	bone marrow-derived dendritic cell
BsAb	bispecific antibody
C1q	complement component 1q
cpm	counts per minute
CR3	complement receptor 3 (CD11b/CD18)
CTL	cytotoxic T lymphocytes
DC	dendritic cell
EpCAM	epithelial cell adhesion molecule
Fab	antigen binding fragment
FACS	fluorescence-activated cell sorter
Fc	constant/crystallisable fragment
FcγR	Fc receptor for IgG
FcαRI	Fc receptor for IgA
FcεRI	Fc receptor for IgE
FcR	Fc receptor
FDA	Food and Drug Administration
fMLP	formyl-methionyl-leucyl-phenylalanine
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GPI	glycosyl-phosphatidylinositol
HER-2	human epidermal growth factor receptor-2
HUVEC	human umbilical vein endothelial cells
IFNγ	interferon-γ
Ig	immunoglobulin
IgAN	IgA nephropathy
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KO	knock-out
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MFI	mean fluorescent intensity

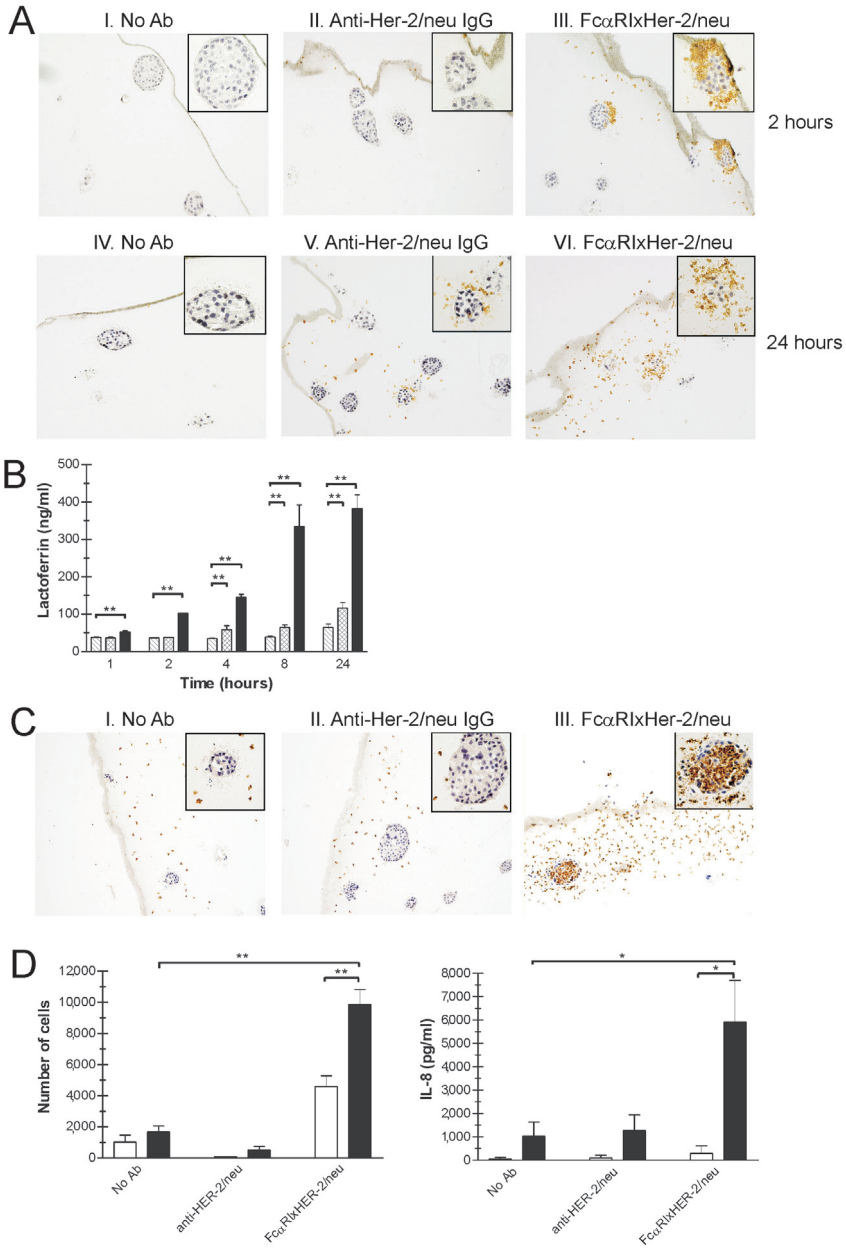
MHC	major histocompatibility complex
MoDC	monocyte-derived dendritic cell
NIP	nitro-iodophenol caproate-o-succinimide
NK cell	natural killer cell
NTg	non-transgenic
OVA	ovalbumin
pIgA	polymeric IgA
pIgR	polymeric Ig receptor
PO	peroxidase
SC	secretory component
SCID	severe combined immunodeficient
SD	standard deviation
SEM	standard error of the mean
SIgA	secretory IgA
TAA	tumor-associated antigen
Tfr	transferrin receptor
Tg	transgenic
TNF-α	tumour necrosis factor-α
wt	wild type



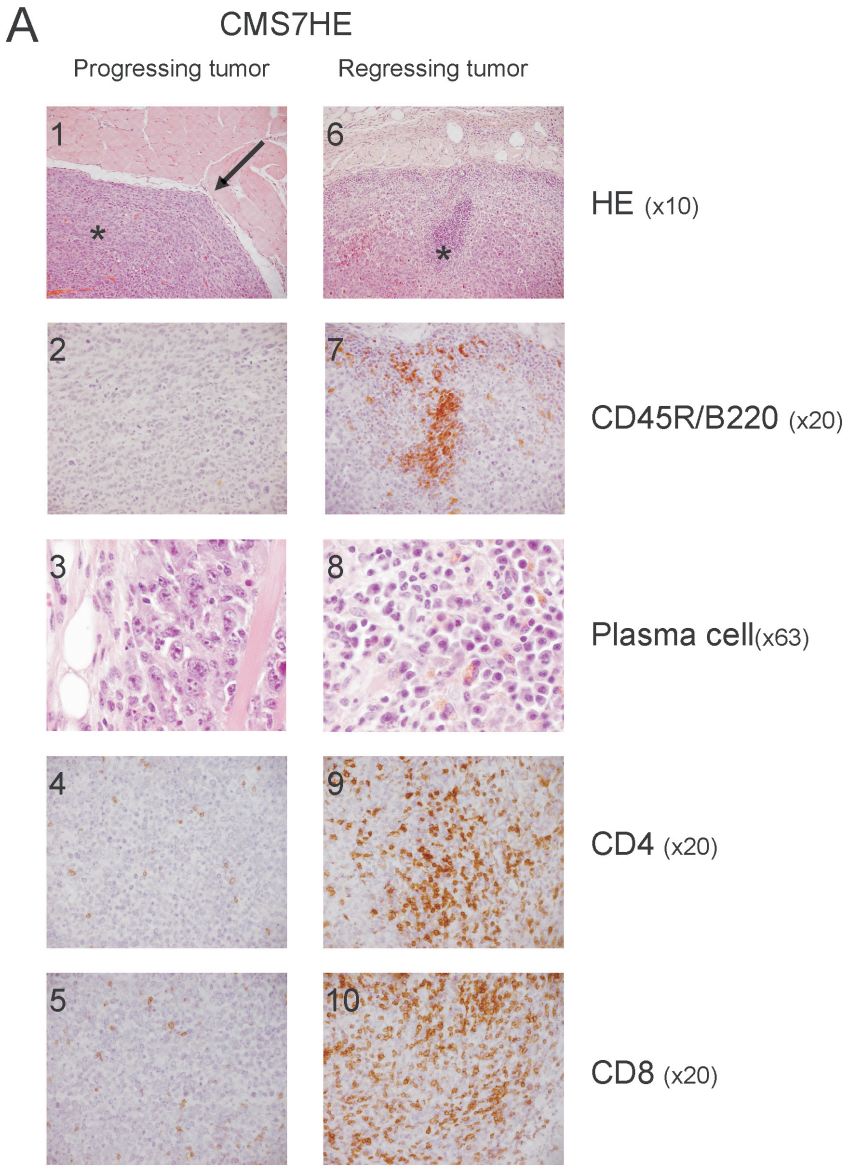
Chapter 3, Figure 3. BsAb-induced neutrophil migration towards tumor colonies. G-CSF neutrophils were added to SK-BR-3 tumor colonies in collagen, either in the absence (A), or presence of 0.5 μg/ml FcγRIxHER-2/*neu* (B) or FcαRIxHER-2/*neu* (C) BsAb. Collagen was fixed and slides were stained for CD66b (neutrophils, brown). Neutrophils attached to tumor colonies are indicated in A and B by arrows. In (C) remnants of a SK-BR-3 tumor colony are marked by an arrowhead. (D) Numbers of neutrophils per colony in the absence (hatched bar), or presence of FcγRIxHER-2/*neu* (white bar) or FcαRIxHER2/*neu* (black bar) BsAb. Results represent mean ± SEM from three individual experiments. * $p < 0.05$, compared to FcγRIxHER-2/*neu* BsAb.



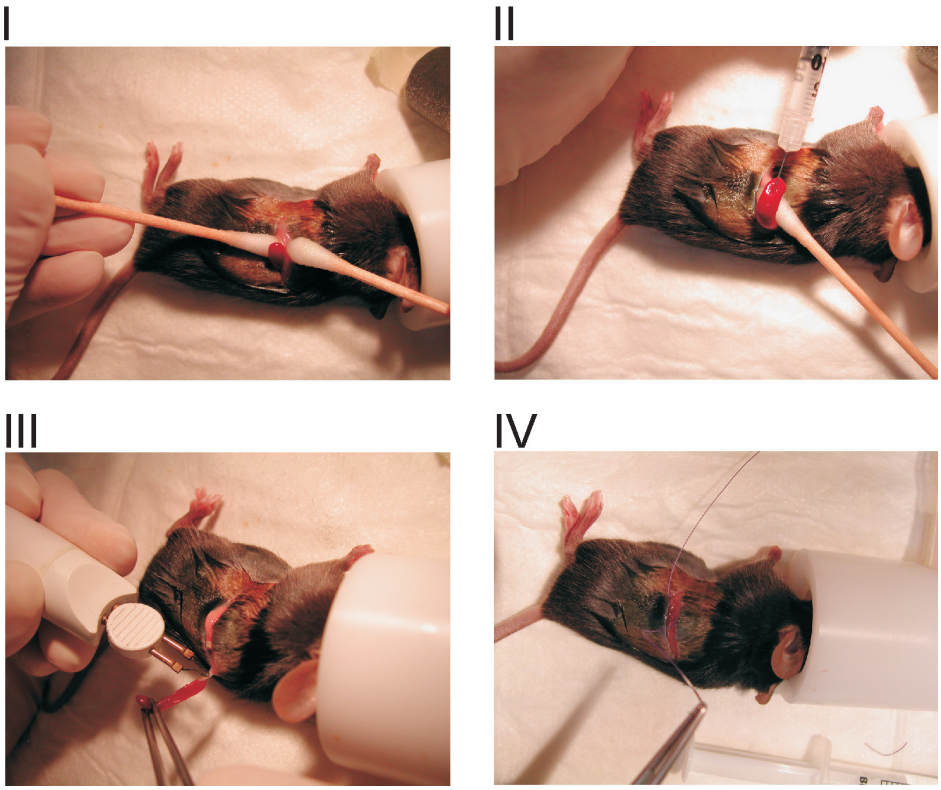
Chapter 5, Figure 1. Antibody-induced neutrophil migration towards tumor colonies. Collagen cultures were fixed 24 hours after addition of neutrophils and slides were stained for CD66b (neutrophils, brown). (A) IFNγ neutrophils were added to SK-BR-3 tumor colonies (blue) in collagen, either in the absence (panel I), or presence of anti-Her-2/neu IgG Ab (panel II), FcγRIxHer-2/neu BsAb (panel III), or FcαRIxHer-2neu BsAb (panel IV). One representative example out-of-eight is shown. (B) Numbers of IFNγ (left) or G-CSF (right) neutrophils per tumor colony in the absence (hatched bars), or presence of anti-Her-2/neu IgG Ab (cross-hatched bars), FcγRIxHer-2/neu BsAb (white bars) or FcαRIxHer2/neu BsAb (black bars). Results represent mean ± SEM from three experiments. *: $p<0.05$, **: $p<0.01$, ns: non significant. (C) IFNγ neutrophils were added to SW-948 tumor colonies (blue), either in the absence (panel I), or presence of anti-EpCAM IgG (panel II) or IgA (panel III) Ab. One representative example out-of-three is shown.



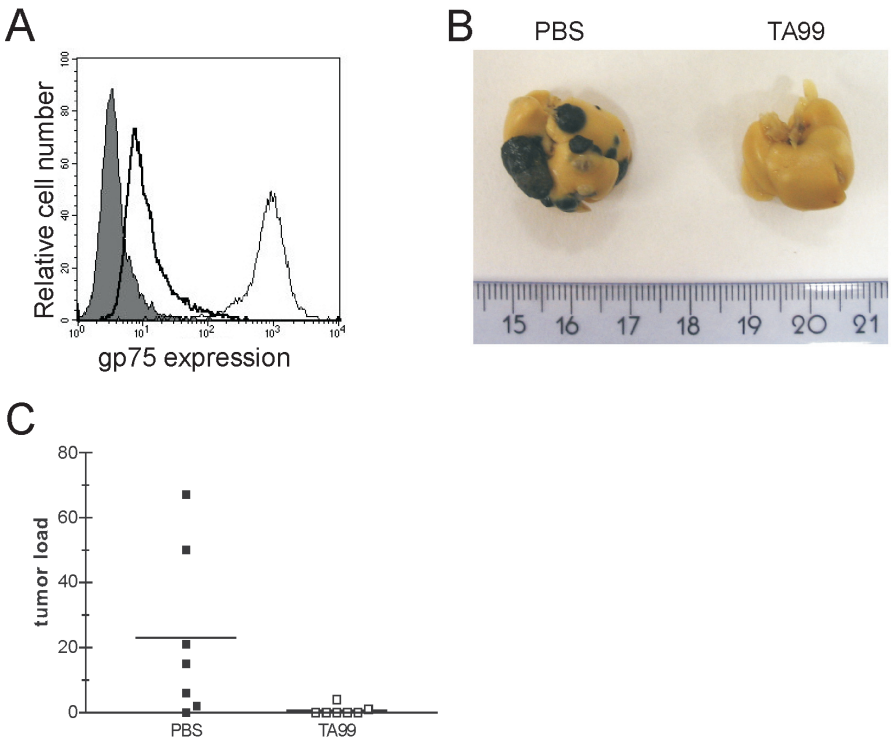
Chapter 5, Figure 3. Neutrophil kinetics and responses to HUVEC layered SK-BR-3 colonies in collagen gels. (A) SK-BR-3 colonies (blue) in collagen gels, incubated without (panels I, IV), or with anti-Her-2/neu IgG Ab (panels II, V), or FcαRIxHer-2/neu BsAb (panels III, VI), were fixed either 2 hours (panels I-III) or 24 hours (panels IV-VI) after addition of IFNγ neutrophils (brown). One representative experiment out-of-five is shown. (B) Lactoferrin levels were determined in supernatants of SK-BR-3 collagen gels incubated without (hatched bars), or with anti-Her-2/neu IgG Ab (cross-hatched bars) or FcαRIxHer-2/neu BsAb (black bars) for the indicated times. Results represent mean levels ± SEM of 5 separate experiments. (C) IFNγ neutrophils (brown) were added for 24 hours to HUVEC-layered collagen gels containing SK-BR-3 colonies (blue), either in the absence (panel I), or presence of anti-Her-2/neu IgG Ab (panel II), or FcαRIxHer-2/neu BsAb (panel III). Data from one representative experiment out-of-four are shown.



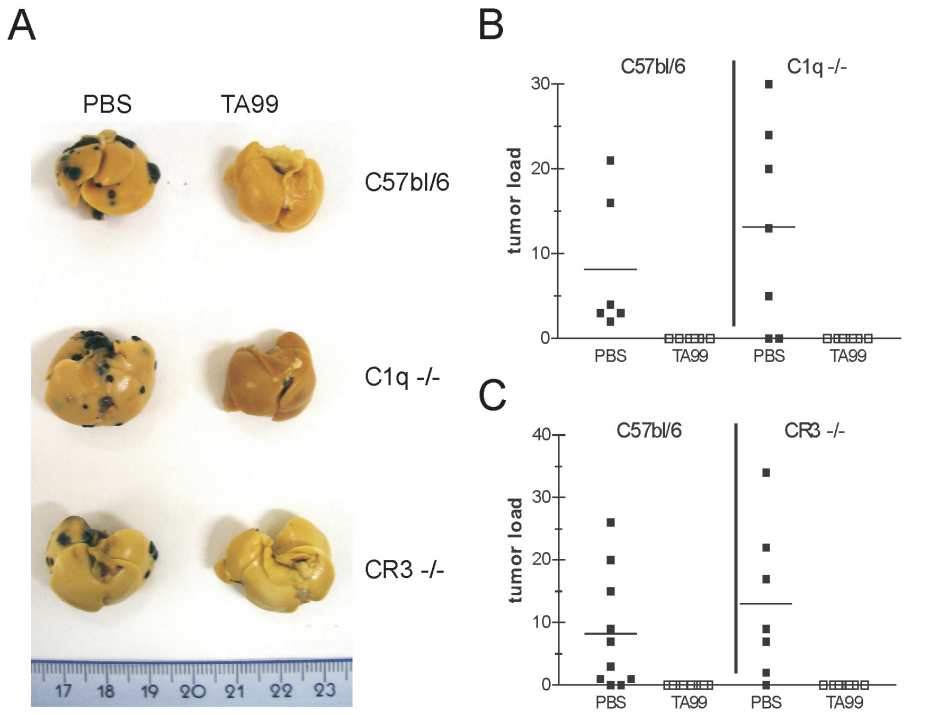
Chapter 7, Figure 2. Development of cellular and humoral anti-tumor responses. (A) Analysis of cellular immune cell infiltrates in progressing (left panels) and regressing (right panels) CMS7HE tumors. Left Panels: (1) Low power magnification (x10). Tumor (marked by asterisk) showed solid, expansive growth and was clearly demarcated from muscular tissue (arrow) (HE staining). (2) Immuno-staining for the presence of B cells; no B cells were detected (x20). (3) Examination of the peripheral rim of the tumor revealed only presence of tumor cells, which demonstrated cellular pleomorphism, a high nucleus:cytoplasma ratio and multiple prominent nucleoli per nucleus. No plasma cells were found. (HE staining; x63) (4) Immuno-staining for CD4 or (5) CD8 showed the presence of few, disseminated positive cells (x20). Right Panels: (6) Low power magnification (x10). The demarcation between the solid CMS7HE tumor and muscular tissue was blurred by infiltration of inflammatory cells. A condense nodule, indicative of clustered B cells, was present at the margin of the tumor (indicated by asterisk) (HE staining). (7) Immunostaining for the presence of B cells on identical area depicted in (6). Note the CD45R/B220 immunoreactivity of the clustered cellular infiltrate, supporting the presence of B cells. (8) At the rim of the tumor, conglomerates of plasma cells were found. Note the typical cellular characteristics of plasma cells; Elongated, oval cells with peripheral round nuclei, fairly condensed nuclear chromatin patterns, a juxta-nuclear luscent area (reflecting the Golgi system) and a basophilic cytoplasm. (9) High numbers of CD4-positive cells, as well as high numbers of (10) CD8-positive cells, were present within the tumor.



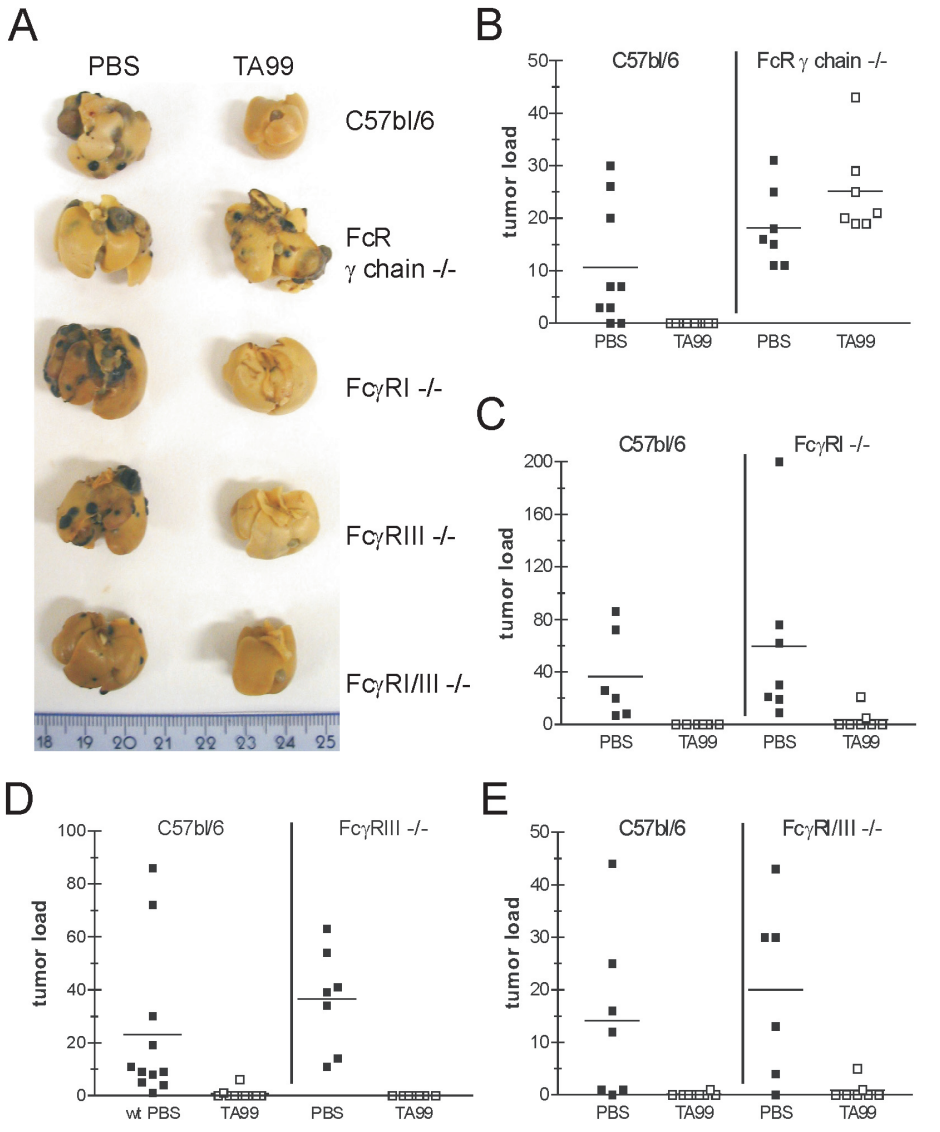
Chapter 8, Figure 1. Mouse liver metastasis model. (I) In anesthetized C57bl/6 mice, the spleen was exposed through a small incision, (II) after which B16F10 tumor cells were injected intrasplenically. (III) Removal of the spleen occurred with a searing devise to cauterize afferent and efferent blood vessels, (IV) after which the incision was sutured.



Chapter 8, Figure 2. mAb TA99 prevents B16F10 outgrowth in liver. (A) gp75 surface expression (thick line), and total gp75 expression (thin line) was determined on B16F10 cells. Filled area represents control mAb. (B) Livers of PBS or mAb TA99 treated C57bl/6 mice were analysed 21 days after tumor inoculation. Black nodules represent metastases. (C) The number of liver metastases at day 21 per liver, with each dot representing one mouse. One representative experiment-out-of eight is shown.



Chapter 8, Figure 3. Complement is not essential for mAb TA99 therapy of liver metastases. Livers of PBS or mAb TA99 treated C57bl/6 mice (panels A-C), $C1q^{-/-}$ mice (panels A, and B), or $CR3^{-/-}$ mice (panels A, and C) were excised 21 days after B16F10 tumor inoculation, and numbers of liver metastases quantified. One representative experiment out-of-two is shown.



Chapter 8, Figure 4. Activatory FcγR are essential for mAb TA99 therapy of liver metastases. Mice inoculated with B16F10 cells were treated with either PBS or mAb TA99. After 21 days, livers were excised, and tumor load was determined in C57bl/6 mice (panels A-E), FcR γ chain$^{-/-}$ mice (panels A, and B), FcγRI$^{-/-}$ mice (panels A, and C), FcγRIII$^{-/-}$ mice (panels A, and D), or FcγRI/III$^{-/-}$ mice (panels A, and E). Experiments were repeated at least 2 times, yielding similar results.