Functional specialization and cooperation between the neonatal- and classical IgG receptors, FcRn and FcγR

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IMMUNOGLOBULIN LIGHT CHAIN AFFECTS THE FUNCTION OF IgG2 THROUGH FcγRIIA

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ABSTRACT

The effector functions of IgG are encoded by their heavy chain Fc-region, providing binding epitopes for Fcγ-Receptors (FcγR) and the neonatal Fc receptor (FcRn). The four IgG subclasses bind with distinct affinity to these effector molecules due to differences in the structural composition of the Fc domains. No function has been assigned to the light chain isotypes despite their lack of sequence homology (~33% identity between constant κ and λ domains). In this study, we compared the phagocytic properties of human neutrophils against targets opsonized with human IgG1 and IgG2 with either κ or λ light chains but identical variable regions. The light chain isotype had little or no effect on IgG1 effector function. However, a clear difference between IgG2κ- and IgG2λ-mediated neutrophil phagocytosis was observed, with IgG2κ being more efficient. This difference could not be explained by the A/B isomerization found in IgG2κ. Although we observed a differential binding of IgG2κ and IgG2λ to FcRn, the receptor was not responsible for this effect. In contrary, preventing binding to FcRn by using IgG2-Fc mutants unable to bind FcRn, exaggerated the difference between IgG2κ- and IgG2λ-mediated phagocytosis. FcγRIII-blocking reduced IgG2-mediated phagocytosis, but without affecting the κ/λ-difference. However, IgG2κ displayed stronger binding affinity than IgG2λ to recombinant FcγRlla and to FcγRlla-transfectants, explaining how phagocytes discriminate between either κ- or λ- IgG2 opsonized particles. Thus, it is not only the Ig-isotype and subclass that can encode for different effector functions, but also the light chain.
INTRODUCTION

Antibodies are the backbone of the adaptive humoral response, each consisting of two identical heavy chains covalently linked to each other and each to one of the two identical smaller light chains, forming a Y-shaped molecule.

Five separate isotypes of antibodies exist, defined by their heavy chain; IgG, IgA, IgM, IgE and IgD. IgG is furthermore divided into four subclasses named IgG1-4, in order of decreasing abundance in serum. Each IgG possesses unique characteristics due to differential affinities for the complement factor C1q and the different types of FcγRs. Two different constant light chain isotypes also exist, κ and λ, with a κ to λ ratio for IgG produced being approximately 2:1 in humans, usually only deviating in case of a monoclonal gammopathy or other B cell pathologies. While the heavy chain type dictates antibody effector function, the two light-chain isotypes are considered interchangeable with respect to antibody function despite their low homology of only 33% amino-acid sequence identity of the constant regions. However, they do share the same structural immunoglobulin-domain characteristics.

The nature of each immune challenge dictates which IgG subclass is evoked in response. The four IgG subclasses differ greatly in their abilities to initiate complement activation and FcγR-mediated phagocytosis, with IgG3 and IgG1 being more efficient than IgG2 and IgG4 (IgG3>IgG1>>IgG2>IgG4). While IgG1 and IgG3 are most important for T-cell dependent protein antigens such as viruses, class switching to IgG2 occurs typically in response against T-cell independent antigens such as bacterial capsular polysaccharide antigens.

Polymorphonuclear cells (PMNs) are the immune system’s first line of defense against invading bacteria, and express three different FcγRs; the classical FcγRIIA and FcRIIB as well as FcRn. While the GPI-linked FcγRIIB does not bind IgG2, FcγRIIA is the only classical FcγR on PMNs with significant affinity to IgG2, which binds stronger to the H131- than the R131-allotypic variant of the receptor. However, it cannot be excluded that FcγRIIB also contributes to phagocytosis mediated by IgG2 as FcγRIIB resides in lipid microdomains rich in signaling molecules required for signaling and function of other FcγRs, and can be recruited to the signaling complex through interaction through molecules such as CD11b/CD18.

All the IgG subclasses have N-linked Fc glycosylation at Asn297, which is attached to the IgG by glycosyltransferases during antibody synthesis, folding and secretion in the endoplasmic reticulum and Golgi. These sugars are biantennary, usually core-fucosylated and partially truncated oligosaccharides which may or may not carry sialic acid, galactose and bisecting N-acetylglucosamine residues. The presence of these oligosaccharides is a prerequisite for FcγR binding and variation in the sugar moieties influences the affinity of IgG to different subtypes of the receptors. For
instance, the lack of fucosylation improves binding to FcγRIIa and FcγRIIib while Fc sialylation has been reported to reduce binding affinity to Fcγ receptors.10-12

The non-classical FcRn, a structural homologue of major histocompatibility complex class I (MHCI) proteins, is less selective for IgG-subclass binding, and is not affected by IgG-Fc glycosylation.13-15 Whereas the classical FcγRs are expressed as surface membrane proteins, FcRn is expressed in intracellular vacuoles of many cell types, in particular in phagocytes such as monocytes and neutrophils. We and others have previously shown that this receptor, which is best known for feto-maternal IgG transport as well as for half-life extension of IgG in serum, works in tandem with FcγR to mediate phagocytosis of opsonized particles by neutrophils and monocytes.9;16-18 While FcRn engages IgG only at the acidic pH found in phago-lysosomes, it displays negligible affinity to IgG at neutral extracellular pH. Two histidines at positions 435 and 310 in the IgG Fc region are key players mediating pH dependent binding due to their protonation at pH < 6.5, with mutations of these amino acids effectively abrogating binding to the receptor.19;20 FcRn not only has a different optimal pH range for IgG binding than the classical FcγRs, it also has a distinct binding site on the Fc region which lies between the CH2 and CH3 domains, in contrast to FcγRs which bind to the lower hinge and the upper CH2 domains. This means that binding of FcRn and FcγR to IgG can potentially take place simultaneously, although the possibility exists that binding to FcRn is dominant at acidic pH when FcγR binding affinity seems reduced.16;21

While no functional difference has yet been attributed to the two IgG light-chain isotypes, Montano and Morrison found that for human IgG2, but none of the other IgG subclasses, the κ isotype had longer half-life than the λ in mice.22;23 Recently, it has become increasingly clear that IgG2κ, to a greater extent than IgG2λ, exists as different isoforms, distinguished by the covalent linkage between the two heavy chains at the hinge.24;25 This mixture consists of, in addition to the classical A form, a novel B form, which has only two disulfide bonds interconnecting the heavy chains, as well as a hybrid A/B structure. The intermediate asymmetrical A/B form exhibits characteristics of both isoforms, where one side of the molecule has the A formation while the other has the B. While IgG2λ predominantly displays the traditionally known A-form, IgG2κ exists as mixture of all three isoforms. It would seem that while most IgG2κ is originally synthesized as an IgG2κA, it quickly converts to the A/B isoform during maturation in serum and then slowly folds into the B formation.26;27

In this study we constructed and produced a panel of IgG1 and IgG2 antibodies in combination with κ and λ light chains, with identical V-genes encoding for specificity to pneumococcal polysaccharide of serotype 6A/B, and investigated their capacity to stimulate phagocytosis by neutrophils. We found that IgG2κ mediated more effective phagocytosis than IgG2λ, a process which was not affected by the structural isomerization of IgG2κ and was not due to altered interaction with FcRn but was rather a consequence of a distinct difference in binding affinity to FcγRIIa.
MATERIAL AND METHODS

Antibodies
The cloning and expression of a human anti-pneumococcal serotype 6A/B IgG2\(\kappa\) GDob1 antibody as IgG1\(\kappa\) and IgG2\(\kappa\) was previously described.\(^2\) The original \(\lambda\)-light chain cDNA was also cloned and expressed in pcDNA3.1, enabling the expression of IgG1 and IgG2 with either \(\kappa\) or \(\lambda\) light chains.\(^1\)^\(^4\)^\(^2\)\(^8\) Mutations abrogating binding of IgG2 to FcRn (H310A and H435A) were introduced by ligations of synthesized DNA-fragments (Geneart, Invitrogen). After co-transfecting the heavy and light chain plasmids into HEK 293 Freestyle cells, using 293-Fectin transfection reagent (Invitrogen) according to the manual instructions, secreted antibodies were isolated from the supernatant on a protein G HiTrap HP column (GE Healthcare Life Sciences, Little Chalfont, UK) using the Acta Prime Plus system (GE Healthcare Life Sciences), followed by dialysis against PBS overnight.

Synthesis of IgG2\(\kappa\)A and \(\kappa\)B isoforms
Enrichment of IgG2\(\kappa\)\(A\) isoforms by redox treatment was performed as described in Dillon et al 2008.\(^2\)\(^5\) Shortly, for the synthesis of the B isoform, IgG2\(\kappa\) was incubated at a concentration of 3 mg/ml in 200mM Tris buffer at pH 8 with 6 and 1 mM of cysteine and cystamine, respectively. For IgG2\(\kappa\)A enrichment, 0.9 M guanidine hydrochloride (GuHCl) was also added. The samples were protected from light and placed at 2–8°C for 48–72 h. Afterwards the antibodies were run through a Zeba spin desalting column (Pierce) for buffer exchange into PBS, and stored at -20°C. To verify the enrichment, samples were run on a Dionex ProPac WCX-10 (4.0 x 50 mm) HPLC column.\(^2\)\(^4\)

Bacteria
Streptococcus pneumoniae was kept at -80°C in Thodd-Hewitt broth with 10% glycerol, were spread on blood agar plates and cultured overnight. Picked colonies were expanded at 37°C shaking for approximately 8 hours in Todd-Hewitt broth with 10% FCS. Bacteria were then heat-killed for 30 min at 56°C before staining with 7 µl 10 mg/ml Dy488 in 1 ml 100 mM NaHCO\(_3\) for 60 minutes at RT in the dark. After extensive washing, bacteria were suspended in PBS 1% BSA and aliquoted for storage at 10\(^9\) CFU/nl at -20°C.

Isolating neutrophils from peripheral blood
After diluting 1:1 with PBS, a Ficoll Hypaq density gradient was performed and the serum and white fraction was carefully removed. Lysis was then performed on the red fraction by diluting it with an ammonium chloride buffer and incubating for 15 minutes at 4°C. A second incubation was performed with a washing step in between. After extensive washing with cold PBS, the neutrophils were dissolved in RPMI phagocytosis medium with 10% FCS (without the addition of antibiotics or HEPES).
Phagocytosis assay

Uptake was performed in Li Hep coated microvette tubes (Biotang Inc., Lexington, Massachusetts, USA) for 30 min at 37°C shaking, with 3 x 10^5 PMN and 15 x 10^4 bacteria in a final volume of 450 µl phagocytosis medium. When applicable, PMNs were incubated with FcγR blockers (or PBS) for 15 min at RT. Blockers used were AT10 (whole mouse IgG1 anti-CD32, GTX74628 from Genetex) and 3G8 (Sanquin). Phagocytosis was stopped by washing twice with cold PBS, 7 min, 1200 RPM, 4°C.

PMNs were stained for 30 min at 4°C with 8 µl of Pacific blue conjugated anti-CD45 (PB986, Dako, Glostrup Denmark), diluted 1:1 with PBS 0.5% BSA. After washing, cells were fixed with 50-100 µl 2% PFA for 30 minutes at 4°C and stored ON cold and dark before measurement on the Imagestream X (Amnis, Seattle, WA) on a 40x collection filter. The data was analyzed with the Amnis Ideas software, using the Internalization Wizard application. The internalization score (IS) reflects the relative amount of bacteria that has been successfully internalized, rather than just attached to the surface.

\[ IS = \frac{\log(\frac{(a)}{1-a})}{m_{L} + m_{B}} \]

(m_L = Mean intensity of upper quartile pixels in I, m_B = Mean intensity of upper quartile pixels in B, p_L = Peak intensity of upper quartile pixels in I, p_B = Peak intensity of upper quartile pixels in B).

Binding assays

Human myeloma-IgG2 kappa or-IgG2 lambda antibodies (Sigma-Aldrich) were coupled to M-270 Epoxy Dynabeads (Dynal®, Oslo, Norway) according to manufacturer’s descriptions. After incubation on ice for 30 minutes with human PMNs ( homozygous H131- or R-131 FcγRIIa donors as indicated) or IIA1.6 cells (FcγRIIa-H131-transfectants), and washing, the binding was assessed by FACS after labeling with RPE-conjugated goat F(ab')2 anti-human IgG Fc (Southern biotechnology associates, Birmingham).

Surface plasmon resonance

Surface plasmon resonance was conducted using a Biacore 3000 instrument (GE Healthcare) with CM5 sensor chips. The coupling was performed by injecting 5 µg/ml of the each protein into 10 mM sodium acetate pH 4.5 using the amine coupling kit (GE Healthcare). Titrated amounts (8,000.0-62.50 nM) of monomeric human FcRn were injected over immobilized IgG variants (500 RU) using phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 6.0 as running buffer whereas HBS-P buffer at pH 7.4 was used for regeneration of the flow cells. Titrated amounts (2,000.0-30.0 nM) of IgG2 variants were injected over site-specific biotinylated human FcγRIIa-H131 (Sino Biological Inc; 200 RU) captured
on immobilized neutravidin (Pierce). HBS-P buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% surfactant P20) at pH 7.4 was as running buffer, and the flow surfaces were regenerated using 10 mM NaOH. Experiments were conducted at 25°C with a flow rate of 40 μl/min. All sensorgrams were zero adjusted and reference cell values subtracted before analyzed using the BIAevaluation 4.1 software.

IgG Glycosylation

IgG glycosylation analysis was performed as described previously, with minor modifications. Briefly, 4-10 μg of IgG were cleaved with trypsin (500 ng, sequencing grade; Promega, Leiden, The Netherlands) in 40 μl 25 mM ammonium bicarbonate by over-night incubation at 37°C and stored at -20°C until usage. Resulting glycopeptides were analyzed by nano-reverse phase-LC-ESI-MS using a Ultimate 3000 HPLC system interfaced to a quadrupole-TOF-MS mass spectrometer (maXis impact; Bruker Daltonics, Bremen, Germany) with a standard ESI source (Bruker Daltonics) and a sheath-flow ESI sprayer. A sheath-flow of 50% IPA, 20% propionic acid and 30% water was applied at 2 μl/min to support ESI spray formation and reduce TFA ion suppression. The total analysis time per sample was 16 min. Data processing was performed as described previously. Briefly, LC-MS datasets were calibrated internally using a list of known glycopeptides and were exported to the open mzXML format. Glycopeptide species were pre-defined as peak maxima in specific mass and retention time windows and were extracted from each dataset using the in-house developed software “Xtractor2D”. On the basis of the normalized intensities of IgG Fc glycopeptides the level of galactosylation, sialylation, bisecting N-acetylglucosaminylation, and fucosylation were calculated as described previously.

RESULTS

IgG1 and 2 antibodies produced as κ and λ have similar characteristics

The variable light and heavy chains from GDob1, a human IgG2λ hybridoma recognizing pneumococcal polysaccharides of serotype 6, were cloned and produced as recombinant IgG1κ or IgG2κ as described previously, but were now also expressed with the original lambda constant region for comparison. By SDS-PAGE, all the purified IgGs displayed expected molecular weights and assembly (Fig. 1A) and bound Streptococcus pneumoniae similarly (Fig. 1B-C), suggesting that the affinity of the antibodies to their cognate antigen was not affected by the light chain isotype.

IgG2κ mediates enhanced phagocytosis when compared to IgG2λ

In order to study the influence of light chain isotype on function, we compared the efficiency of these matching κ and λ IgG1 and IgG2 antibodies to promote phagocytosis using freshly isolated human PMNs (Fig. 2A). GDob1 IgG1 antibodies mediated similar phagocytosis of pneumococci (Fig. 2B-E). Using titrated amounts of
IgG1 and IgG2 κ and λ antibodies, clear differences between the light-chain isotypes were observed, with higher phagocytosis for the κ variants at lower antibody concentrations (Fig. 2B-D). While IgG1 was more efficient in promoting phagocytosis

Figure 1. Recombinant IgG assembly and antigen-binding is not affected by either heavy- or light-chain class. A) Purity and assembly of recombinant GDob1 IgG antibodies used in the study. 1 μg of each protein was loaded per well, either non-reduced by the addition of 160 mM iodoacetamide (left) or reduced in 1% β-mercaptoethanol buffer (right). The gel was stained with Coomassie blue. Binding of the GDobl antibodies by B) ELISA to pneumococcal polysaccharide 6B and C) to Dy488 labelled Serotype 6B pneumococci was not affected by light chain. In C) binding of κ-containing IgG are indicated by solid black lines, λ by solid gray lines, conjugate by dotted line, and isotype control by dashed line.
Figure 2. IgG2κ-mediates enhanced phagocytosis when compared to IgG2λ by human neutrophils. A) Recombinant GDob1 IgG antibodies mediated phagocytosis of S.pneumoniae as measured by Imagestream. Image shows four representative events for different Internalization scores (IS) indicated on top in yellow numerals, PMN are stained by anti-CD45 PB (purple) and bacteria are DY488 labelled (green). The IS indicates the relative distribution of internalized vs. external events. An IS>0 indicates that more bacteria phagocytised than adhered to the surface. GDob1-mediated phagocytosis, using either homozygous FcγRIIa-H131 donors (B-C) or FcγRIIa-R131-donors (D-E) of IgG1 and IgG2 of either κ and λ isotypes. B) and D) κ light chain containing antibodies mediated stronger phagocytosis, with IgG1κ and IgG2λ only being significantly different at low concentrations. For IgG2, this difference was more pronounced and increased at high concentrations. As expected, IgG1-mediated phagocytosis was superior to IgG2-mediated phagocytosis for the R131 donor, while IgG2κ performed similarly to IgG1 in H131 donors. Y axis depicts % of PMNs with IS>0 multiplied with the ratio of PMN’s positive for DY488 labeled bacteria. Graphs shows representative data from a single experiment. C) and E) At plateau levels for IgG1 (5 µg/ml), no significant difference was observed for IgG1κ and IgG1λ, while similar comparison for IgG2 was highly significant. Y-axis depicts % phagocytosis of each antibody subclass compared to IgG1κ phagocytosis levels in the same donor. Graphs depicts pooled data from n=6 H131 donors (C) and four R131 donors (E). IgG1 performed better than IgG2 in R131 donors, while IgG2κ performed similarly to IgG1 in H131 donors. Statistical comparisons were performed by one-pair-row multiple t-tests and significance determined by the Holm-Sidak method in B) and D) and a student T-test in C) and E) using Graphpad 6.01. *P ≤ .05; **P ≤ .01. ***P ≤ .001. ns, nonsignificant.
than IgG2 at low concentrations (<1 µg/ml), it did not display the same light chain difference at higher plateau concentrations (Fig. 2B). At plateau levels (5 µg/ml, Fig. 2C), there was a significant difference between κ and λ mediated phagocytosis of *S. pneumoniae* for IgG2, but not for IgG1. This was observed for both H131 (Fig. 2B-C) and R131 donors (Fig. 2D- E). While IgG2κ phagocytosis approached that of IgG1 for H131 donors at plateau concentrations (5 µg/ml), it was intermediate for IgG2λ (Fig. 2B-C). In R131 donors, phagocytosis by IgG2λ was negligible (Fig. 2D-E), while IgG2κ stimulated phagocytosis to a similar level as observed for IgG2λ in H131 donors (see also supplementary Fig. 1).

IgG2 isomers and glycosylation do not explain the difference between IgG2κ- and IgG2λ-mediated phagocytosis

IgG2κ exists as at least three isoforms, differing in the number of covalent bonds between the heavy chains, causing structural heterogeneity in IgG2κ and potentially affecting accessibility to FcγRIIa. We addressed this by subjecting our IgG2κ briefly to mild reducing conditions as described by Dillon et al., which effectively generated IgG2κ fractions, enriched in either A (of which IgG2λ consists entirely) or B form. While recombinant IgG2κ appeared as an isoform mixture, consistent with A/A, A/B and B/B isoforms, the generated IgG2κA or IgG2κB forms were enriched by these procedures (Fig. 3A). When tested for their ability to mediate phagocytosis, no differences were detected between untreated IgG2κ, κA or κB (Fig. 3B), suggesting that the observed difference between IgG2κ and IgG2λ was not caused by the structural isomers exclusive to IgG2κ. Alternatively, the different IgG2κ isomers might affect the accessibility of different glycosyltransferases during synthesis, folding and secretion in the endoplasmic reticulum and Golgi. In turn, this might affect binding to FcγRs as the N-linked glycans attached to Asn-297 in the CH2-domains of the Fc region are critical for binding to FcγRs. However, mapping of the glycosylation profile by mass spectrometry ruled out this possibility, as neither IgG1 nor IgG2-derived glycopeptides encompassing Asn-297 displayed any differences in Fc- fucosylation, galactosylation, sialylation or bisecting N-acetylglucosaminylation (Fig. 3C-D).

**IgG2λ has higher affinity to FcRn which enhances its phagocytic capacity**

Next, we investigated if the observed differential phagocytosis by IgG2κ or IgG2λ could be explained by different affinities to either FcγRs or FcRn, as they all actively participate in phagocytosis. We therefore measured the affinity of soluble monomeric human FcRn to immobilized IgG variants at pH 6.0 using surface plasmon resonance, and in accordance with a previous study, found FcRn to bind IgG2λ slightly stronger than IgG2κ (Fig. 4A). No difference in FcRn-binding affinity was detected between the IgG1 light chain isotypes.
Differential effector functions of IgG2κ and IgG2λ

Figure 3. Neither IgG2 isomers nor IgG Fc-glycosylation affect IgG-mediated phagocytosis. A) HPLC elution profiles of IgG2κ A and B structural isomers on a Dionex ProPac WCX-10 (4.0_250 mm) column. Enrichment of IgG2λ isoforms by redox treatment was performed as described.25 B) IgG2κ isomers mediate identical phagocytosis of S.pneumoniae. The experiment was carried out as described in Fig.1.C) and D). IgG-Fc glycosylation profiles of the recombinant antibodies used as analyzed by Nano-reverse phase-LC-ESI-MS of IgG-Fc-glycopeptides after trypsin digestion of IgG. No significant difference was detected in N-linked glycosylation between light chain isotypes of IgG1 and IgG2. Fuc= Fucose, Bis= Bisecting N-acetylglucosamine, Gal= Galactose, Sial= Sialic acid.
To investigate if the difference in affinity between IgG2κ and IgG2λ for FcRn could cause the observed differences in phagocytosis of IgG2κ- and IgG2λ-opsonized particles, we first investigated if ablation of FcRn-binding capacity of IgG2 would affect phagocytosis. To this end we used G Dob1 antibodies containing the H310A and H435 substitutions (IgG2κ– or IgG2λ- HH), which dramatically reduce binding to FcRn, with H435A sufficient to significantly reduce FcRn-mediated transcytosis and phagocytosis. Accordingly, aborting the binding capacity to FcRn significantly reduced phagocytosis (Fig. 4B). This reduction was even more noticeable for IgG2λ than for IgG2κ (Fig. 4B). In agreement with the slightly increased affinity of IgG2λ to FcRn compared to IgG2κ and the role of FcRn in promoting phagocytosis, introduction of the H310A and H435 substitutions into IgG2 increased the observed difference between IgG2κ- and IgG2λ-mediated phagocytosis of opsonized bacteria, and increased the significance of the difference (P= 0.0013 for WT IgG2, with P< 0.0001 for IgG2-HH, Fig. 4B). This suggested that the binding characteristics of FcRn counteracted the observed differences between phagocytosis induced by wild-type IgG2κ and IgG2λ.

**Binding to FcγRIIa is sufficient to explain differential phagocytosis by IgG2κ and IgG2λ.**

Neutrophils constitutively express FcγRIIa and FcγRIIib on the cell surface, of which only FcγRIIa is capable of binding to IgG2, while both FcγRs bind IgG1. In agreement with the higher expression of FcγRIIib (100,000-300,000 copies) compared to FcγRIIa (10,000-20,000 copies), IgG1-mediated phagocytosis of opsonized pneumococci occurred predominantly through FcγRIIib, since addition of an anti-FcγRIIib monoclonal blocking antibody (3G8) almost completely blocked uptake while the addition of an anti-FcγRIIa blocking antibody (AT10) only resulted in a partial inhibition (Fig. 5A). Blocking both FcγRIIa and FcγRIIib completely inhibited IgG1-mediated phagocytosis (Fig. 5B). Neither blocking agent had an effect on relative phagocytosis efficiency through IgG1κ or IgG1λ (Fig. 5A).

In contrast, phagocytosis mediated by IgG2 was completely blocked by AT10, in agreement with the inability of IgG2 to bind to FcγRIIib, suggesting FcγRIIa to be indispensable and sufficient for IgG2-mediated phagocytosis (Fig. 5B). Curiously, blocking FcγRIIib also had a significant effect on phagocytosis, suggesting receptor crosstalk as previously reported. Importantly, blocking FcγRIIib did not affect the differential phagocytosis through IgG2κ and IgG2λ. Preventing binding of IgG2 to FcRn with the double mutant (H310A/H435A) again seemed to increase the κ/λ-differences, indicating that FcRn counteracts the enhanced IgG2κ-mediated phagocytosis.

We then measured binding of the IgG2κ and IgG2λ variants to FcγRIIa-H131 by surface plasmon resonance. To mimic a cellular situation, we injected equal amounts of the antibodies over C-terminally site specific biotinylated FcγRIIa-H131 captured on
a neutravidin-coated chip. While FcγRIIa showed no clear preference for either IgG1κ or IgG1λ (Fig. 6A-B, E-F), FcγRIIa-H131 bound IgG2 more avidly than FcγRIIa-R131, with both FcγRIIa-variants displaying preferential binding to IgG2κ (Fig. 6 C-D, G-H, I-J). The obtained sensorgrams show that both IgG2 variants bind FcγRII but IgG2κ

Figure 4. FcRn preferentially binds and enhances phagocytosis of IgG2λ, and masks differential IgG2κ and IgG2λ-mediated phagocytosis. A) Binding of soluble human FcRn (62.5-8000 nM) to human immobilized IgG variants onto CM5 sensor chips. The relative affinity constants derived (KD) are indicated. B) Differential phagocytosis by IgG2κ and IgG2λ becomes more pronounced after preventing FcRn interaction by introduction of the H310A and H435 substitutions. The experiments were performed as described in Fig. 1, and data were pooled from three experiments using H131-FcγRIIa donors. Statistical comparisons were performed with Student’s T-tests. ** P ≤ 0.01; *** P ≤ 0.001.
Figure 5. FcγR promote increased phagocytosis of IgG2κ compared to IgG2λ, while FcRn masks this difference. A) Phagocytosis of *S. pneumoniae* opsonized with 1 µg/ml IgG1 was mostly FcγRIIIb-mediated, as it was efficiently blocked by anti-FcγRIII-blocking antibody (3G8, 10 µg/ml), while the addition of FcγRII-blocking antibody (AT10, 10 µg/ml) had little effect. Experiments were performed as in Fig. 1, using homozygous FcγRIIa-H131 donors, and representative results of one experiment are shown. B) Phagocytosis experiments carried as in A) but using IgG2-Dob1 opsonizing antibodies (5 µg/ml), revealed that FcγRIIa is indispensable for phagocytosis of IgG2-opsonized *S. pneumoniae*, while FcγRIIIb contributes to overall efficiency. Using IgG2-H435A/H310A (HH), inhibiting the involvement of FcRn increased the relative difference between IgG2κ and IgG2λ-mediated phagocytosis. Statistical comparisons were performed with one way Anova with Bonferroni’s multiple comparisons test. Pooled data from from two donors are shown.

binds with distinct kinetics whereas λ-containing IgG2 dissociates faster (Fig. 6I-J). Fitting the data to kinetic binding models revealed that neither of the binding profiles fit to a simple 1:1 Langmuir binding model but instead to a more complex heterogeneous binding model, which assumes the existence of two different binding modes. The high affinity KDs gave rise to a more than 8-fold difference in affinity of IgG2κ and IgG2λ to FcγRIIa- H131 (Fig. 6I-J).

This was further investigated by testing binding of human IgG2κ- and IgG2λ-myeloma antibodies coated beads to FcγRIIa-H131-transfected IIa1.6 cells at 4°C,
Figure 6. 

FcγRIIa bind IgG2κ with enhanced affinity compared to IgG2λ. Site-specific biotynylated (c-terminus) FcγRIIa variants were coupled to neutravidin biosensors, mimicking the natural orientation of the receptor on a cell surface. IgG variants were then injected at identical concentrations. This was done for both FcγRIIa-H131 (A-D and I-J) and FcγRIIa-R131 (E-H), for IgG1κ (A, E), IgG1λ (B, F), IgG2κ (C, G, I), and IgG2λ (D, H, J). K) Binding of myeloma IgG2κ-conjugated beads to FcγRIIa transfectants was increased compared to IgG2λ-beads. Binding index = #beads per 100 (effector) cells. Statistical comparison was performed by Student’s T-test. *P ≤ .05; **P ≤ .01. ***P ≤ .001.
which showed preferential binding of IgG2κ-coated beads (Fig. 6K). This confirmed that the observed light chain differences for IgG2 were not an intrinsic property of our recombinant GDob1 antibodies, but a general feature of IgG2. In conclusion, our study strongly suggests that the discriminatory actions of phagocytes towards IgG2κ- and IgG2λ-opsonized targets are mediated by differential binding to FcγRIIa.

DISCUSSION

Here we studied if light chain isotype-switching of a human hybridoma-derived IgG2λ affected FcγR-dependent phagocytosis. Surprisingly, IgG2κ was more efficient at mediating phagocytosis than IgG2λ. Similar differences were observed for IgG1, but these were smaller and only observed at suboptimal concentrations where IgG1κ outperformed IgG1λ. However, for IgG2, this κ-λ-difference increased with concentration and remained stable at high concentrations. This is not an artifact specific to one clone of antibodies since the same effect was observed with beads opsonized with IgG2 myeloma proteins. The greater phagocytic efficiency of IgG2κ was neither due to any particular IgG2κ structural isomer, since IgG2κA and IgG2κB simulated phagocytosis equally well as native untreated IgG2κ, nor to differential IgG-Fc glycosylation.24-25 The latter result excludes the possibly that glycosyltransferases have differential accessibility to the Fc-glycan at position 297 on different structural light chain isomers, thereby affecting FcγR-binding affinities of either IgG2-light chain isotype.

It has also been suggested that the presence of additional free thiol groups in IgG2κ cysteine compared to IgG1κ encouraged formation of antibody aggregates.37 However, aggregation of IgG2 was only witnessed by Franey et al after weeks of incubation at 45°C, or exposure to harsh circumstances. In this study, we also did not observe aggregation by SDS-PAGE, possibly as care was taken to preserve all antibody aliquots at -20°C, thaw them at 4°C and avoid repeated freeze-thaw cycles, making increased selective aggregation of either of our IgG2κ or IgG2λ preparations unlikely.

Previous publications have hinted at an effect of IgG2 light chain isotypes on FcRn affinity and half-life.22,23 While human IgG2λ was found to have a substantially higher affinity to mouse FcRn than its κ-counterpart as measured by SPR (K_d 4.97 vs 1.78 10^{-4} s^{-1} for κ and λ, respectively), it has also been reported to have shorter serum half-life in WT BALB/c mice.22,23 We now confirm that IgG2λ displays a slightly higher affinity to human FcRn. However, this did not explain the enhanced phagocytosis by IgG2κ, since abrogating binding of IgG to FcRn decreased IgG-mediated phagocytosis, confirming the previous reports on the role of FcRn in this process.9,16-18 More importantly, the relative difference between IgG2κ- and IgG2λ-mediated phagocytosis even increased after preventing participation of FcRn in the
phagocytic response, suggesting the enhanced FcRn-binding to IgG2λ to partly mask the enhanced phagocytosis by IgG2κ.

We then investigated the binding of IgG2 to the neutrophil FcγRs (FcγRIIa and FcγRIIib). Phagocytosis of IgG1-opsonized bacteria was mostly mediated through FcγRIIib, as blocking FcγRIIa had little effect on the process. Conversely, IgG2 phagocytosis was completely dependent on FcγRIIa, but blocking FcγRIIib also had a significant effect on uptake. This was surprising as IgG2 does not have measurable affinity for FcγRIIib. This effect might be explained by the contribution of FcγRIIib through interaction with MAC-1 (CD11b), which together form a coherent complex for phagocytosis. After simultaneously blocking FcγRIIib and eliminating FcRn binding by the H310A and H435A substitutions of IgG2, phagocytosis of IgG2κ became very low but still detectable, while virtually no phagocytosis was observed for IgG2λ, indicating that these two receptors are not responsible for the enhanced phagocytosis induced by IgG2κ. Eliminating phagocytosis all together with an additional FcγRIIa blocker strongly pinpointed FcγRIIa to be sufficient to explain these differences between the IgG2 light chain isotypes. This was further supported by experiments showing that binding to either neutrophils or FcγRIIa-transfected cells also preferred IgG2κ-opsonized beads, while no binding was observed for mock-transfected cells. Preferential binding of FcγRIIa to IgG2κ was further confirmed by biosensor analysis, showing that both FcγRIIa-H131 and -R131 bind human IgG2 and have preferential binding to IgG2κ. As expected, FcγRIIa-H131 bound IgG2 with higher affinity, with the R131 allotype having negligible affinity to IgG2λ.

In conclusion, although the different IgG effector functions are normally assigned to the different IgG subclasses, we now show that the light chain isotype can also significantly affect phagocytosis, particularly for the IgG2 subclass, through differential interaction with FcγRIIa. IgG2κ interacts with FcγRIIa with higher affinity and induces a stronger phagocytic response than IgG2λ. Why the light chain of IgG2 in particular affects this binding is still unknown. Perhaps it is because of its unique hinge, which is truncated compared to the other IgG subclasses and lacking some key-amino acids required for binding to FcγR in the other IgG subclasses, making the interaction between IgG2 and FcγRIIa somewhat unique. An alternative explanation could be that the Fab fragments of IgG2 are particularly close to the Fc-part of the IgG molecules due to its extremely short hinge region, blocking access to FcγR-binding determinants (lower hinge and upper CH2 domain).

Our data suggest that IgG2κ responses may be more beneficial than IgG2λ responses against, for example, encapsulated bacteria where IgG2 responses are dominating, while IgG2λ responses may cause less inflammation and therefore be more beneficial in other responses where immune activation is not desired or for development of anti-inflammatory drugs.
REFERENCE LIST


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Supplementary figure 1. Comparison of IgG2κ and IgG2λ GDob1-mediated phagocytosis of S.pneumoniae in either a homozygous H131 (HH) or R131 (RR) donor in a single experiment. While the H131 donor demonstrated stronger phagocytosis overall, IgG2κ–mediated phagocytosis in the R131-donor approached that of IgG2λ in the H131-donor.