

INSIGHTS

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Four keys to unlock IgG

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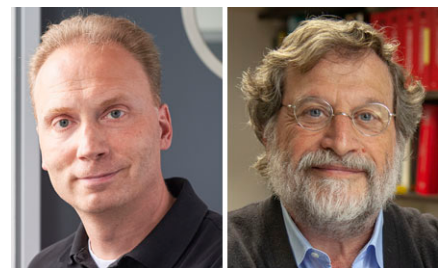
The identification of discrete subclasses within the immunoglobulin G (IgG) isotype by Grey and Kunkel (1964. *J. Exp. Med.* <https://doi.org/10.1084/jem.120.2.253>) provided the framework for our current understanding of differential IgG subclass activity in protective and self-reactive immune responses.

When it comes to our current understanding of immunoglobulin heterogeneity and function, the early sixties may be viewed as a turning point marked by several groundbreaking discoveries. While the diversity of the antigenic specificity of antibodies was recognized early in the study of antibodies, going back to Ehrlich at the turn of the twentieth century, appreciation of isotype diversity was not recognized. Without doubt, one of the most pivotal studies was the paper by Howard Grey and Henry Kunkel published in *JEM* in April 1964, demonstrating that the 7Sγ class of immunoglobulins (known as IgG today) consists of at least three distinct subclasses (Grey and Kunkel, 1964). Together with studies from Lichter and Dray (1964) and Ballieux et al. (1964) in the same year, their paper highlighted that an unexpected additional layer of complexity exists beneath the well-known 7Sγ, 19S (IgM), and b2A (IgA) antibody classes.

IgG antibodies represent the most abundant immunoglobulin isotype in the serum, and a reduction or absence of IgG antibodies predisposes individuals to recurring infections, demonstrating the pivotal role of IgG in host defense. Conversely, self-reactive IgG antibodies (autoantibodies) play a major role in triggering tissue inflammation characteristic of autoimmune diseases such as systemic lupus

erythematosus or inflammatory arthritis. In the treatment of autoimmune diseases and cancer, intact IgG antibodies or IgG-fragment crystallizable (Fc) fusion proteins have become the most widely used platform technology to target autoreactive or cancerous cells either directly or indirectly via modulation of immune responses. Adding to this multitude of IgG activities, polyclonal IgG preparations pooled from the serum of thousands of donors (also known as intravenous immunoglobulins, or IVIg) are able to suppress a wide variety of autoimmune and chronic inflammatory diseases, demonstrating that IgG antibodies can have both pro- and anti-inflammatory activities (Bournazos et al., 2017). The work of Grey and Kunkel provided the conceptual framework for these later studies by demonstrating that heterogeneity within IgG isotypes exists.

In their paper, Grey and Kunkel (1964) made use of the fact that patients with multiple myeloma are characterized by an expansion of a single malignant B cell clone secreting large amounts of one intact IgG molecule or a single antibody light chain (also known as Bence Jones protein), respectively. By injecting purified 7Sγ myeloma proteins obtained from different patients into rabbits, they were able to raise rabbit antisera directed against different human 7Sγ myeloma proteins. Using these



Insights from Falk Nimmerjahn and Jeffrey V. Ravetch.

antisera against a set of 7Sγ myeloma protein preparations in Ouchterlony agar diffusion/precipitation assays, they demonstrated that at least three subgroups of 7Sγ proteins exist and that their heterogeneity is located within the antibody heavy chain or Fc region (panel A in the figure). Their work further demonstrated that these IgG subclasses exist in normal human serum, emphasizing the general importance of their finding beyond multiple myeloma. Moreover, the generation of detection methods (antisera) for distinct IgG subclasses represented a major advance for the field, allowing for the assessment of how select IgG subclasses are generated during different types of immune responses.

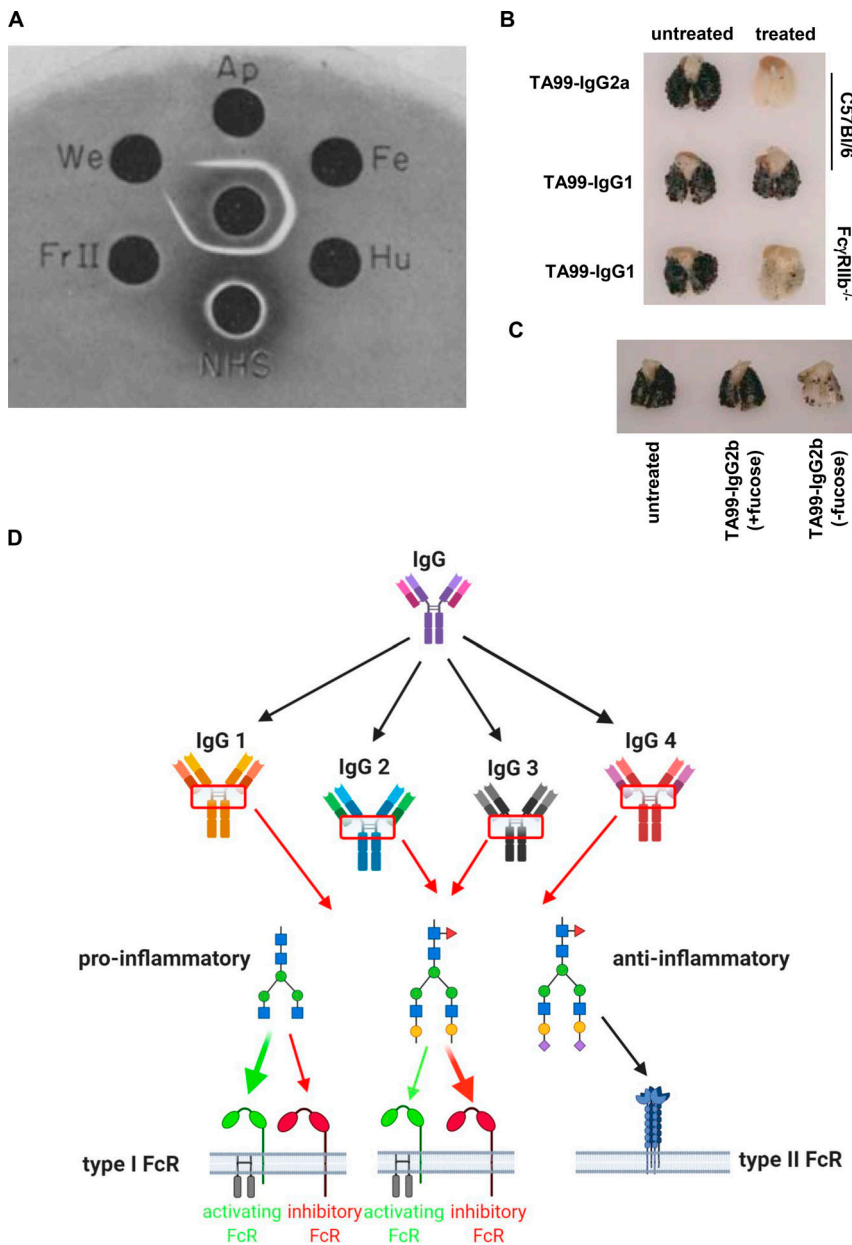
The results of Grey and Kunkel paved the way for identifying similar IgG subclasses in other species, such as mice and nonhuman

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The complexity of IgG subclass activity. (A) Shown is an in-gel antibody precipitation assay (ouchterlony assay) from Grey and Kunkel (1964), demonstrating that a rabbit antiserum generated against one myeloma IgG molecule precipitates some but not all IgG antibodies derived from other multiple myeloma patients (IgG from patient We is not precipitated, whereas IgG from patients Ap, Fe, Hu, and FrII is precipitated). The antiserum also reacted against normal human serum demonstrating the presence of IgG subclasses in healthy individuals. (B) Depicted are lung melanoma metastases in C57BL/6- and FcγRIIb-deficient mice left untreated or treated with IgG subclass switch variants (IgG1 and IgG2a) of the melanoma-specific antibody TA99. (C) Shown are lung melanoma metastases in C57L/6 mice left untreated or treated with a TA99-IgG2b subclass variant carrying a sugar moiety with or without fucose in its Fc domain. (D) Schematic representation of factors influencing IgG activity. Apart from individual IgG subclasses, IgG glycosylation plays a major role in balancing the pro- and anti-inflammatory activities of IgG via modulating the interaction with type I and type II Fc-receptors (FcR). See text for further details.

primates, and allowed for the isolation and biochemical characterization of the four human IgG subclasses as we know them today (named IgG1, IgG2, IgG3, and IgG4; Rowe, 1970). The pivotal studies of Cooper

in the 1970s demonstrating that antibodies switched from one isotype to another added a layer of complexity to the simplistic view that antibodies were only defined by their antigen-binding properties and that which

isotype was associated was significant (Kincade et al., 1970).

The study of Grey and Kunkel stimulated the search for the functional consequences of IgG subclass heterogeneity, triggering investigations into how different IgG subclasses mediate their activity in vivo. This has transformed the way we think about IgG-dependent immune responses and has become the basis for choosing IgG subclasses with a high or low capacity to activate downstream effector functions for therapeutic antibodies. Realizing that IgG subclasses have a differential activity in vivo highlighted that the IgG Fc-domain is far more complex than a simple carrier for the antibody variable region (fragment antigen binding, F(ab)), which has stimulated research into identifying which humoral and cellular effector pathways are responsible for the activity of IgG subclasses in vivo. The diversity of Fc-mediated effector responses far exceeds the four isotypes that were first described by Grey and Kunkel and led to the search for mechanisms to further diversify the Fc structure. A single N-linked glycosylation site on the CH2 domain, first identified by Huber, has provided a structural basis for the functional heterogeneity observed (Arnold et al., 2007). This structural heterogeneity determines the specificity of IgG binding to a diverse set of cellular receptors (Fc receptors) and inflammatory mediators, such as the complement components. Regulation of the glycan composition of the complex, biantennary glycan has been well documented in the IgG response to inflammatory and infectious diseases, highlighting a novel mechanism for diversification of effector responses of IgG. For example, different human (and mouse) IgG subclasses greatly differ in their capacity to activate the classical complement pathway and hence in their ability to induce the production of pro-inflammatory mediators, such as the anaphylatoxins C3a and C5a. Whereas IgG1 and IgG3 efficiently trigger complement activation, IgG2 and IgG4 mostly lack this activity. In a similar manner, IgG1 and IgG3 subclasses are superior in binding to immune cells (termed cytophilic antibodies at the time), an activity of antibodies discovered in the 1960s as well (Berken and Benacerraf, 1966). The molecular basis for antibody cytophilicity was demonstrated by the cloning of the first

Fc-receptors for IgG (FcγRs) in 1986, setting the stage for a series of discoveries which have become the framework of how we think about IgG subclass activity today (Lewis et al., 1986; Ravetch et al., 1986). The broad expression of FcγRs on cells of the innate and adaptive immune system and the presence of activating and inhibitory FcγRs coexpressed on the majority of innate immune cell subsets, suggested that IgG antibody activity is tightly regulated. The generation of knockout mice deficient in all or individual FcγRs confirmed this assumption, demonstrating that a critical determinant of IgG subclass activity is the affinity of each IgG subclass for the three activating FcγRs (FcγRI, IIa, IIIa in humans; FcγRI, III, IV in mice) and for the inhibitory FcγRIIb, also referred to as the A/I ratio (Nimmerjahn and Ravetch, 2005). While Fc subclass and its glycan composition are natural determinants of selective FcR engagement, further refinement has been achieved by engineering the Fc to optimize specific FcR interactions. Thus, for example, development of cytotoxic antibodies for cancer therapy routinely involves considering the binding affinity of IgG subclasses to activating and the inhibitory FcγRs and has stimulated IgG Fc-engineering approaches to further optimize this interaction (panels B and C in the figure).

Along the same lines, studying the mechanism of checkpoint control and immunomodulatory antibodies targeting molecules such as CTLA-4, PD-1, or CD40 has revealed that different strategies need to be considered to make full use of the potent immunomodulatory activities of these antibodies in immunotherapy of cancer. While CTLA-4-specific antibodies required interaction with activating FcγRs to deplete CTLA-4-expressing intratumoral regulatory T cells, CD40-specific antibodies were critically dependent on the presence of the inhibitory FcγRIIb on B cells or other innate immune cell subsets (Beers et al., 2016; Bournazos et al., 2017). Mechanistically, FcγRIIb seemed to act as a platform for cross-linking the Fc portion of CD40-specific antibodies bound to dendritic cells, allowing an optimal induction of activating signaling pathways to induce antigen-

presenting cell activation. In contrast, the optimal IgG Fc-domain for preventing the inactivation of PD-1-expressing tumor-specific T cells with PD-1-specific antibodies is an IgG subclass with low or absent FcγR-binding ability to prevent the depletion of tumor-specific T cells, highlighting the importance of understanding the activity of individual IgG antibodies in detail.

To engineer IgG antibodies with low or absent FcγR and complement binding abilities, systematic mutational studies were performed, revealing that the sugar domain attached to each IgG heavy chain in the CH2 region plays a critical role for IgG activity (Bournazos et al., 2017). More importantly, variations in the composition of this sugar domain, which can exist in more than 30 variations per individual IgG heavy chain, were demonstrated to have major effects on IgG activity (Arnold et al., 2007). Thus, IgG antibodies lacking the penultimate fucose residue showed a selective increase in binding to the activating FcγRIIIa, resulting in enhanced cytotoxic antibody activity (panels C and D in the figure). In contrast, IgG antibodies with high levels of terminal sialic acid residues had an active anti-inflammatory activity, and depleting this sugar moiety from IVIg preparations impaired the ability of IVIg to suppress autoantibody induced tissue inflammation (panel D in the figure). Thus, apart from classical engineering approaches targeting the amino acid backbone of antibodies, glycoengineering allows for optimizing IgG subclass activity (Bournazos et al., 2017). Both afucosylated and hypersialylated IgG glycosylation variants have either already been approved for clinical application or have successfully passed early clinical testing, respectively.

Identifying how the vast array of potential IgG subclass glycosylation variants mediate their activity and which signals instruct B cells to produce select IgG glycosylation variants will be major challenges to optimize vaccine strategies and therapeutic antibodies, and to understand autoantibody-induced tissue pathology during autoimmune diseases in the future. Evidence supporting the critical need for a more in-depth understanding of IgG subclass glycosylation comes from the more recent identification that some

of the unwanted effects IgG antibodies can trigger during viral infections may be explained by select IgG glycovariants. Thus, antibody-dependent enhancement of virus infections, characteristic for dengue virus infections, was shown to correlate with low levels of virus-specific antibody fucosylation (Wang et al., 2017). Moreover, SARS-CoV-2 patients producing afucosylated antibody responses against viral surface proteins were characterized by more severe inflammatory responses and tissue pathology, emphasizing that both IgG subclass and glycosylation modulate beneficial and detrimental effects of antibody responses (Chakraborty et al., 2021; Larsen et al., 2020). The study by Grey and Kunkel (1964) was the starting point for these studies and was prescient in recognizing that isotype heterogeneity was an essential aspect of the antibody response. We now recognize that this heterogeneity is responsible for both the protective and pathogenic properties of antibodies and for the orchestration of innate and adaptive pathways by selective Fc receptor engagement. After nearly 60 years, we are still exploring this complexity that Grey and Kunkel set in motion.

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