Characterization of a Mutated IgA2 Antibody of the m(1) Allotype against the Epidermal Growth Factor Receptor for the Recruitment of Monocytes and Macrophages*

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**Background:** IgA constitutes a promising antibody isotype, which requires optimization before immunotherapeutic application.

**Results:** P221R-mutated and wild type IgA2m(1) antibodies were similarly effective in killing tumor cells and in recruiting myeloid effector cells.

**Conclusion:** Improved IgA antibodies constitute promising next generation antibodies for tumor therapy.

**Significance:** These studies support the clinical development of therapeutic IgA antibodies.

IgA antibodies constitute an important part of the mucosal immune system, but their immunotherapeutic potential remains rather unexplored, in part due to biotechnological issues. For example, the IgA2m(1) allotype carries an unusual heavy and light chain pairing, which may confer production and stability concerns. Here, we report the generation and the biochemical and functional characterization of a P221R-mutated IgA2m(1) antibody against the epidermal growth factor receptor (EGFR). Compared with wild type, the mutated antibody demonstrated heavy chains covalently linked to light chains in monomeric as well as in joining (J)-chain containing IgA. Functional studies with wild type and mutated IgA2m(1) revealed similar binding to EGFR and direct effector functions such as EGFR down-modulation and growth inhibition. Furthermore, both IgA molecules triggered similar levels of indirect tumor cell killing such as antibody-dependent cell-mediated cytotoxicity (ADCC) by isolated monocytes, activated polymorphonuclear cells, and human whole blood. Interestingly, the dimeric IgA antibodies demonstrated higher efficiency in direct as well as in indirect effector mechanisms compared with their respective monomeric forms. Both wild type and mutated antibody triggered effective FcεRI-mediated tumor cell killing by macrophages already at low effector to target cell ratios. Interestingly, also polarized macrophages mediated significant IgA2-mediated ADCC. M2 macrophages, which have been described as promoting tumor growth and progression, may convert to ADCC-mediating effector cells in the presence of EGFR-directed antibodies. In conclusion, these results provide further insight into the immunotherapeutic potential of recombinant IgA antibodies for tumor immunotherapy and suggest macrophages as an additional effector cell population.

Monoclonal antibodies and their derivatives constitute a rapidly growing class of biotherapeutics for an increasing number of clinical indications (1, 2). In tumor therapy, all therapeutic antibodies being approved or in clinical trials, are of the human IgG isotype, with clinical experience being available for IgG1, IgG2, and IgG4. At present, many arguments favor human IgG1 as the preferred isotype: first, human IgG1 effectively recruits NK cells and complement for tumor cell killing (3, 4). Second, IgG antibodies display an extended serum half-life, which is mediated by binding to neonatal Fc receptor (FcRn) (5). In addition, production technologies and purification protocols are well established, and regulatory agencies are acquainted with relevant safety issues (6). The importance of antibody stability is underlined by recent observations with human IgG4 antibodies because IgG4 antibodies undergo fragment antibody binding (Fab) arm exchange under experimental and physiological conditions (7, 8). Recent data demonstrated that myeloid effector cells were effectively recruited for ADCC by human IgG2 antibodies (9). However, granulocytes and monocytes were particularly cytotoxic for tumor cells with antibodies of the IgA isotype (4, 10–12), which has not been explored clinically.

IgA antibodies contribute significantly to the humoral arm of the mucosal immune system, which provides important barrier functions to protect the large area of serosal surfaces (13). Three forms of IgA (monomeric, dimeric, and secretory IgA) are distinguished, which have distinct molecular characteristics and serve different functions in the immune system (14). Secretory IgA constitutes a first line of serosal defense against invading pathogens. Secretory IgA is a heavily glycosylated multimeric protein consisting of two monomeric IgA molecules, covalently linked by the joining J-chain and the secretory component (15).
**Effector Cells for a Stabilized IgA2m(1) Antibody**

*In vivo*, secretory IgA is generated when mucosa associated plasma cells produce dimeric IgA, which is transcytosed through epithelial cells via the basolaterally expressed polymeric immunoglobulin receptor (13). Incorporation of the J-chain into dimeric IgA is essential for the covalent interaction of the IgA heavy chain with the secretory component of the polymeric immunoglobulin receptor, which is cleaved proteolytically at the apical side to release secretory IgA (16). The second line of natural defense consists of circulating monomeric IgA, which is produced predominantly by bone marrow-located plasma cells and neutralizes pathogens that passed the mucosal barrier (17). This neutralization is mediated mainly by interacting with the Fc receptor for IgA (FcαRI; CD89), which is the best characterized IgA receptor (18). FcαRI is expressed on monocytes/macrophages, granulocytes, subsets of dendritic cells, and Kupffer cells and binds both monomeric and dimeric IgA isoforms with median affinity. Binding of IgA to FcαRI mediates effector functions such as phagocytosis, oxidative burst, cytokine release, antigen presentation, and ADCC. Thus, monomeric IgA resembles IgG in many structural and functional aspects, except for the recruited effector cell population, whereas J-chain containing dimeric IgA constitutes a tetrameric molecule that offers the potential to target serosal surfaces (19).

In humans, two IgA isotypes, IgA1 and IgA2, and three allotypes, IgA2m(1), IgA2m(2) and IgA2n, have been distinguished (14). The major differences between IgA1 and IgA2 are located (i) in the hinge region, with IgA2 lacking the 13-amino acid elongation with up to five O-glycosylation sites existing in IgA1, (ii) in the N-glycosylation, with IgA2 being more than twice as heavily glycosylated than IgA1, and (iii) in the heavy and light chain linkage, where the Caucasian IgA2m(1) allotype lacks a covalent disulfide bond between heavy and light chains, which is present in IgA1 and in the IgA2m(2) allotype of those of African and Asian descent (14, 20). In previous reports, we demonstrated that IgA antibodies were able to trigger polymorphonuclear cell (PMN)-mediated ADCC more efficiently than IgG antibodies, with IgA2 being more effective than IgA1 (11) and dimeric being more effective than monomeric IgA1 (12). However, the noncovalent linkage of heavy and light chains in the Caucasian IgA2m(1) makes this isotype less attractive for biotechnological production and for a therapeutic antibody as it might lead to an unstable recombinant molecule under certain conditions. This appears particularly relevant for dimeric IgA2, which constitutes a complex molecule consisting of four light and four heavy chains linked by a single J-chain. Thus, dimeric IgA2 may be particularly difficult to produce if the light and heavy chain linkage is not stable. To solve this issue and to create a fully predictable and covalently linked IgA2 molecule, a tetrameric IgA2, which is derived from the IgA2m(2) allotype sequence. This mutation sterically allows the formation of an alternate disulfide bond between the heavy (Cys-241) and the light chain cysteines (Cys-214) (21, 22), similar to that found in non-Caucasian IgA2 allotypes, IgA2m(2) and IgA2n. In the present study, we demonstrate that this mutation results in monomeric and dimeric isoforms of an IgA2m(1) antibody, which demonstrate similar functionality as the respective wild type proteins but possess covalent heavy and light chain linkage. Thus, mutated monomeric or dimeric IgA2m(1) antibodies appear better suited for clinical applications.

The epidermal growth factor receptor (EGFR) constitutes a well established target molecule for tumor therapy, which can be targeted by small molecule tyrosine kinase inhibitors and monoclonal antibodies (23, 24). In contrast to tyrosine kinase inhibitors, which only inhibit EGFR signaling, monoclonal antibodies have a different, dual mode of action against cancer cells (25). Thus, monoclonal EGFR antibodies, similar to cetuximab, zalutumumab, or panitumumab mediate Fab-mediated effector mechanisms, such as blocking ligand binding or signal transduction, growth inhibition or EGFR down-regulation. In addition, their fragment crystallizable (Fc) part can trigger effector mechanisms such as complement-dependent cytotoxicity, phagocytosis, or ADCC. Increasing evidence suggests that ADCC may be a particularly important effector mechanism of EGFR antibodies in patients, whereas the predominant effector cell type (natural killer cells or myeloid cells) is controversial (25). For the CD20 antibody rituximab, the innate mononuclear phagocyte network was demonstrated to be the predominant effector cell population in vivo (26). In tumor biopsies, macrophages constitute a major component of the leukocyte infiltrate, where monocyte-derived M0 macrophages are thought to polarize into the tumor-inhibiting M1 or the tumor-promoting M2 phenotypes (27, 28). In vitro, both M1 and M2 macrophages phagocytosed human IgG1 antibody opsonized tumor cells (29), but the potential of these subpopulations as effector cells for IgA antibodies has not been investigated previously. Here, we demonstrate that M0, M1, and M2 macrophages as well as monocytes and PMN are recruited effectively for ADCC by monomeric and dimeric IgA2 antibodies against EGFR. Both monomeric and dimeric isoforms of a P221R-mutated IgA2m(1) antibody were similarly effective as the respective wild type constructs and constitute promising molecules for next generation antibodies.

**EXPERIMENTAL PROCEDURES**

Experiments reported here were approved by the Ethical Committee of the Christian Albrechts University (Kiel, Germany) in accordance with the Declaration of Helsinki.

**Cell Lines**—Human epidermoid carcinoma cell line A431 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), BHK-21 cells co-transfected with FcαRI (CD89) and Fcγ chain (30) and murine BaF3 cells transfected with human EGFR (30) were kept in RPMI 1640, and the human colon carcinoma cell line DFi (European Collection of Cell Culture, ECACC, Salisbury, UK) was kept in DMEM supplemented with 10% heat-inactivated FCS (medium and FCS from Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (PenStrep, PAA, Pasching, Austria). Selec-

2 The abbreviations used are: EGFR, epidermal growth factor receptor; ADCC, antibody-dependent cell-mediated cytotoxicity; PMN, polymorphonuclear cell; RFI, relative fluorescence intensity; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; M-CSF, macrophage-colony stimulating factor; PD, pixel density; BHK, baby hamster kidney.


**Effectors Cells for a Stabilized IgA2m(1) Antibody**

PMCC (PAA) (12). Single clones were grown in disposable CELLline CL 1000 bioreactors (Sartorius, Goettingen, Germany) under serum-free suspension culture conditions. For control experiments, 225-IgG1 (cetuximab) was bought from Merck (Darmstadt, Germany). 225-IgA was affinity-purified using Capture Select Fab κ chromatography medium (Capture Select, Naarden, The Netherlands) and a prepacked Superdex 200 26 × 600 column (GE Healthcare) (30). Dimeric IgA2 was additionally purified by the C-terminal His tag of the J-chain using His tag-specific columns (GE Healthcare) (12). All purification steps were run on an ÄKTApurity liquid chromatography system (GE Healthcare). UV absorbance at 280 nm, pH, and conductivity of the effluent stream were recorded continuously and analyzed using Unicorn software (version 4.11, GE Healthcare). Purified antibodies were analyzed on analytical size exclusion chromatography using a Superdex 200 10 × 30 column (GE Healthcare). Determination of antibody concentrations and gel electrophoresis was done as described previously (12, 30). For lectin blots, purified antibodies were separated on 3–8% Tris-glycine gels (Invitrogen) and transferred onto PVDF membranes (GE Healthcare). Next, membranes were blocked over-night at 37 °C with buffer A (50 mM Tris/HCl, pH 8, 90 mM NaCl, 2 mM CaCl$_2$, 50 mg/ml BSA, all from Carl Roth, Karlsruhe, Germany) and incubated for 2 h with buffer A supplemented with 0.1% Tween 20 (Merck) and 5 μl of recombinant biotinylated *Sambucus nigra* lectin/Griffonia simplicifolia lectin 1/concanavalin A/Aleuria Aurantia lectin, or *Ricinus communis* agglutinin I (all from Vector Laboratories). After washing three times with buffer B (50 mM Tris/HCl, pH 8, 90 mM NaCl, 2 mM CaCl$_2$, 5 mg/ml BSA, 0.05% Tween 20) for 10 min, deposition of lectin was detected using HRP-labeled NeutrAvidin (Vector Labs). Membranes were developed using enhanced chemiluminescent reaction reagent (Pierce).

**Flow Cytometry and EGFR Down-modulation**—Binding to EGFR and to FcRγ-chain, for EGFR transfected BaF3 cells by adding 1 mg/ml genetin (PAA) for FcRγ, and 20 μM methotrexate (Sigma) for FcRγ-chain, for EGFR transfected BaF3 cells by adding 1 mg/ml genetin and 5% (v/v) supernatant of WEHI-3B (German Collection of Microorganisms and Cell Cultures) cells to supply murine IL-3. Purified human myeloma IgA2m(1) antibody was used as control IgA2 (Meridian Life Science, Memphis, TN). Monomeric and dimeric 225-IgA was produced from the variable regions of the 225 antibody as described earlier (12, 30). Wild type 225-IgA2m(1) is further named 225-IgA2-WT. Generation of a proline 221 to arginine mutant IgA2m(1) antibody (designated as 225-IgA2-P221R) was done using site-directed mutagenesis as reported previously (21, 22). The mutation of the heavy chain was confirmed by sequencing. The mutant IgA2m(1) heavy chain sequence was subcloned into the pEE14.4 glutamine synthetase-expression system and transfected into CHO-K1 cells (Lonza Biologics, Slough, UK). Single clones were additionally transfected with a plasmid encoding a His-tagged human J-chain, and transfectants were selected using puromycin (PAA) (12). Single clones were grown in disposable CELLline CL 1000 bioreactors (Sartorius, Goettingen, Germany) under serum-free suspension culture conditions. For control experiments, 225-IgG1 (cetuximab) was bought from Merck (Darmstadt, Germany). 225-IgA was affinity-purified using Capture Select Fab κ chromatography medium (Capture Select, Naarden, The Netherlands) and a prepacked Superdex 200 26 × 600 column (GE Healthcare) (30). Dimeric IgA2 was additionally purified by the C-terminal His tag of the J-chain using His tag-specific columns (GE Healthcare) (12). All purification steps were run on an ÄKTApurity liquid chromatography system (GE Healthcare). UV absorbance at 280 nm, pH, and conductivity of the effluent stream were recorded continuously and analyzed using Unicorn software (version 4.11, GE Healthcare). Purified antibodies were analyzed on analytical size exclusion chromatography using a Superdex 200 10 × 30 column (GE Healthcare). Determination of antibody concentrations and gel electrophoresis was done as described previously (12, 30). For lectin blots, purified antibodies were separated on 3–8% Tris-glycine gels (Invitrogen) and transferred onto PVDF membranes (GE Healthcare). Next, membranes were blocked over-night at 37 °C with buffer A (50 mM Tris/HCl, pH 8, 90 mM NaCl, 2 mM CaCl$_2$, 50 mg/ml BSA, all from Carl Roth, Karlsruhe, Germany) and incubated for 2 h with buffer A supplemented with 0.1% Tween 20 (Merck) and 5 μl of recombinant biotinylated *Sambucus nigra* lectin/Griffonia simplicifolia lectin 1/concanavalin A/Aleuria Aurantia lectin, or *Ricinus communis* agglutinin I (all from Vector Laboratories). After washing three times with buffer B (50 mM Tris/HCl, pH 8, 90 mM NaCl, 2 mM CaCl$_2$, 5 mg/ml BSA, 0.05% Tween 20) for 10 min, deposition of lectin was detected using HRP-labeled NeutrAvidin (Vector Labs). Membranes were developed using enhanced chemiluminescent reaction reagent (Pierce).

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IgA Production, Purification, and Gel Electrophoresis—Purified human myeloma IgA2m(1) antibody was used as control IgA2 (Meridian Life Science, Memphis, TN). Monomeric and dimeric 225-IgA was produced from the variable regions of the 225 antibody as described earlier (12, 30). Wild type 225-IgA2m(1) is further named 225-IgA2-WT. Generation of a proline 221 to arginine mutant IgA2m(1) antibody (designated as 225-IgA2-P221R) was done using site-directed mutagenesis as reported previously (21, 22). The mutation of the heavy chain was confirmed by sequencing. The mutant IgA2m(1) heavy chain sequence was subcloned into the pEE14.4 glutamine synthetase-expression system and transfected into CHO-K1 cells (Lonza Biologics, Slough, UK). Single clones were additionally transfected with a plasmid encoding a His-tagged human J-chain, and transfectants were selected using puromycin (PAA) (12). Single clones were grown in disposable CELLline CL 1000 bioreactors (Sartorius, Goettingen, Germany) under serum-free suspension culture conditions. For control experiments, 225-IgG1 (cetuximab) was bought from Merck (Darmstadt, Germany). 225-IgA was affinity-purified using Capture Select Fab κ chromatography medium (Capture Select, Naarden, The Netherlands) and a prepacked Superdex 200 26 × 600 column (GE Healthcare) (30). Dimeric IgA2 was additionally purified by the C-terminal His tag of the J-chain using His tag-specific columns (GE Healthcare) (12). All purification steps were run on an ÄKTApurity liquid chromatography system (GE Healthcare). UV absorbance at 280 nm, pH, and conductivity of the effluent stream were recorded continuously and analyzed using Unicorn software (version 4.11, GE Healthcare). Purified antibodies were analyzed on analytical size exclusion chromatography using a Superdex 200 10 × 30 column (GE Healthcare). Determination of antibody concentrations and gel electrophoresis was done as described previously (12, 30). For lectin blots, purified antibodies were separated on 3–8% Tris-acetate gels (Invitrogen) and transferred onto PVDF membranes (GE Healthcare). Next, membranes were blocked over-night at 37 °C with buffer A (50 mM Tris/HCl, pH 8, 90 mM NaCl, 2 mM CaCl$_2$, 50 mg/ml BSA, all from Carl Roth, Karlsruhe, Germany) and incubated for 2 h with buffer A supplemented with 0.1% Tween 20 (Merck) and 5 μl of recombinant biotinylated *Sambucus nigra* lectin/Griffonia simplicifolia lectin 1/concanavalin A/Aleuria Aurantia lectin, or *Ricinus communis* agglutinin I (all from Vector Laboratories). After washing three times with buffer B (50 mM Tris/HCl, pH 8, 90 mM NaCl, 2 mM CaCl$_2$, 5 mg/ml BSA, 0.05% Tween 20) for 10 min, deposition of lectin was detected using HRP-labeled NeutrAvidin (Vector Labs). Membranes were developed using enhanced chemiluminescent reaction reagent (Pierce).

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above. Whole blood (25% v/v) or effector cells and antibodies were added to round-bottomed microtiter plates (Wallac, Turku, Finland). Assays were started by adding effector and target cells at an effector:target ratio of 80:1 (40:1 in the case of macrophages) or as indicated. IgA-FcγRI interaction on effector cells during ADCC was blocked using My43, a murine IgM antibody specific for human FcγRI (Medarex, Annandale, VA). After incubation at 37 °C (3 h for whole blood and GM-CSF-activated PMN (11) assays, 16 h for monocytes/macrophages), aliquots of supernatants were transferred into 96-well plates containing a scintillation mixture (OptiPhase Scintillator Supermix, PerkinElmer Life Science). Percentage of cellular cytotoxicity was calculated as % specific lysis determined in cpm using a scintillation counter (MetaBase TriLux, PerkinElmer Life Science). Percentage of cellular cytotoxicity was measured in cpm using a scintillation counter (MetaBase TriLux, PerkinElmer Life Science). Percentage of cellular cytotoxicity was calculated as % specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100, with maximal 141Cr release determined by adding Triton X-100 (Merck, 1% final concentration) to target cells and basal release as measured in the absence of sensitizing antibodies and effector cells. Antibody-independent cytotoxicity (effectors without target antibodies) or effector-independent (target antibodies without effectors) was not observed.

Data Processing and Statistical Analyses—Data are displayed graphically and analyzed statistically using GraphPad Prism (version 5.0, GraphPad Software, San Diego, CA). Group data are reported as means ± S.E. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student’s t tests. EC50 values were calculated from dose-response curves, reported as means ± S.E. and compared by paired Student’s t test to calculate significant differences between data groups. Significance was accepted when p values were ≤0.05.

RESULTS

Production and Purification of Mutant 225-IgA2-P221R Antibody—CHO-K1 cells, growing under serum-free suspension culture conditions, were transfected with vectors coding for the appropriate heavy and light chain genes by seeding them in DMEM-select medium to gain re-adherence (30). Twenty-four hours after transfection, the cells were placed under 1-methionine sulfoximine selection and re-adapted to serum-free conditions by growing in CD-CHO-select medium (30). During the next weeks, single clones were produced by limiting dilution cloning and screening for antibody concentration in supernatants by IgA-specific ELISA. To produce dimeric IgA2 antibodies, well producing 225-IgA2-WT and 225-IgA2-P221R transfecctomas were transfected additionally with a plasmid encoding the human His-tagged J-chain (12). Best producing clones were cultured in special cell line CL1.1000 production flasks. This system allowed a production of 5.9 ± 2.8 and 4.7 ± 2.7 mg/week/flask and a median antibody concentration of 296 ± 141 and 239 ± 127 μg/ml for the monomeric mutant and wild type IgA2, respectively. For dimeric 225-IgA2-WT and 225-IgA2-P221R a median yield of 4.6 ± 1.8 and 4.6 ± 2.5 mg/week/flask and a median antibody concentration of 229 ± 91 and 231 ± 125 μg/ml were obtained, respectively. Purification of monomeric and dimeric IgA2 was performed as described previously (12, 30). In Fig. 1A, affinity-purified IgA2m(1) antibodies were subjected to size exclusion chromato-
mediate Fab-mediated effector functions. First, both antibodies were compared for their binding with EGFR-expressing A431 cells (Fig. 2). In these experiments, 225-IgG1, wild type, and P221R mutant 225-IgA2 antibodies proved to bind with similar avidity as confirmed by calculating EC50 values (Table 1 and Fig. 2A). However, the respective IgG1 antibody bound to EGFR with slightly higher avidity. Consequently, growth of EGFR-expressing DiFi colon carcinoma cells was inhibited at similar
concentrations by 225-IgA2-WT, 225-IgA2-P221R, and the respective IgG1 (Table 1, Fig. 2B). Next, we investigated their ability to mediate EGFR down-modulation using EGFR-expressing BaF3 cells. Both IgA2 and the IgG1 antibody were able to induce EGFR down-modulation with similar efficiency within 4 h, which was further increased after 24 h. Both dimeric 225-IgA2 antibodies were more effective than their respective monomeric equivalents (p < 0.05 indicated by #, Fig. 2C).

**Effector Cell Recruitment by EGFR-directed IgA2 Antibodies**—The ability of 225-IgA2 antibodies to bind the IgA Fc receptor, FcαRI, was analyzed by indirect immunofluorescence analyses using FcαRI/FcRγ-chain-co-transfected BHK cells (31). On these transfectants, the mutant 225-IgA2-P221R antibody demonstrated significantly lower binding than the wild type 225-IgA2 (p < 0.05), in monomeric as well as in dimeric form, whereas a control IgA2 antibody showed intermediate binding (Table 1, Fig. 3A).

In ADCC assays with human whole blood as effector source, both monomeric and dimeric 225-IgA2-WT performed similarly effective ADCC of A431 tumor cells as their respective P221R mutants and outperformed the respective IgG1 antibody (p < 0.001, Table 1, Fig. 3B). Next, we isolated FcαRI-expressing mono- and polymorphonuclear effector cells from blood of healthy donors to assess their contribution to ADCC. In ADCC assays with GM-CSF-activated PMN effector cells, which mainly consist of neutrophils, monomeric and dimeric 225-

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**FIGURE 2.** **Fab-mediated effector functions are not affected by the P221R mutation.** In A, binding to EGFR-expressing A431 tumor cells was analyzed by indirect immunofluorescence using FITC-labeled human κ-light chain-directed antibody for staining. For 225-IgA2-WT and 225-IgA2-P221R, similar RFI were observed for monomeric (left graph) and dimeric (right graph) IgA antibodies, whereas a respective 225-IgG1 antibody yielded higher RFI compared with IgA antibodies. Growth inhibition of DiFi colon carcinoma cells was analyzed in MTS assays (B). Concentrations above 50 or 10 nM of monomeric IgA2 or IgG1 (left panel) and 2.5 nM of dimeric IgA2 (right panel) antibodies were sufficient for a reduction in cell viability of 50%, respectively. In C, EGFR down-modulation by IgA2 antibodies was investigated by incubating EGFR-transfected BaF3 cells with EGF or respective EGFR antibodies. Residual EGFR was detected using a FITC-labeled non-cross-reactive EGFR antibody. Mutant and wild type IgA2m(1) reduced the level of residual EGFR significantly if cells were incubated for 4 or 24 h with monomeric or dimeric antibodies, respectively. The respective 225-IgG1 demonstrated lower efficiency compared with IgA2m(1) antibodies. Results (n ≥ 4) are presented as mean ± S.E. of EGFR binding (RFI) (A), % cell viability (B), and % EGFR down-modulation. C, significant effects (p ≤ 0.01) compared with control IgA2 (A) and untreated cells (B and C) are indicated by black asterisk for mutant IgA2m(1) and gray asterisk for wild type IgA2m(1) antibodies.
IgA2-P221R triggered similar effective ADCC than their respective wild type counterparts but significantly higher levels of killing than 225-IgG1 \((p < 0.001,\) Table 1, Fig. 3C). With isolated monocytes as effector cells, both monomeric and dimeric 225-IgA2-WT were similarly effective in triggering tumor cell killing compared with their respective P221R mutant forms and to 225-IgG1 (Table 1, Fig. 3E). Next, we investigated whether wild type and mutant IgA2m(1) were equally resistant to denaturing temperature conditions (Fig. 2E). Both monomeric 225-IgA2 antibodies were incubated for 5 min at different temperatures, and functionality was analyzed in ADCC assays using freshly isolated PMN as effector and A431 as target cells. Wild type 225-IgA2m(1) and the respective 225-IgG1 were significantly less efficient in mediating ADCC after incubation at 72–84 °C than the 225-IgA2m(1)-P221R (Fig. 4A). In addition, both 225-IgA2 antibodies as well as the IgG1 were incubated at 78 °C for different time intervals (Fig. 4B). Also, in this case, the P221R-mutated 225-IgA2m(1) displayed higher resistance under these denaturing conditions than its wild type IgA2m(1) and the IgG1 counterpart. However, above 84 °C, also the P221R-mutated 225-IgA2 was denatured and failed to induce ADCC.

**Macrophages Are a Potent Effector Cell Population for IgA2 Antibodies**—Next, we investigated whether macrophages could be recruited by EGFR-directed IgA2 antibodies to mediate ADCC against A431 cells. Both monomeric and dimeric EGFR-specific 225-IgA2 antibodies and 225-IgG1 were similarly effective in mediating macrophage-dependent ADCC of A431 tumor cells (Table 1, Fig. 5, A1 and A2). In ADCC assays with different effector:target ratios, both wild type and mutant IgA2 antibodies were similarly effective in mediating ADCC by macrophage recruitment even at low effector:target ratios (Fig. 5B). Maximal effectiveness of macrophages-mediated ADCC was reached at effector:target ratios of 40:1 and higher. For the respective IgG1, similar levels of cytotoxicity were detected. Subsequently, we investigated whether IgA-mediated ADCC was dependent on FcαRI interaction. Therefore, we performed ADCC assays with a constant concentration of EGFR-specific IgA2 or IgG in the presence of My43 to specifically block IgA-FcαRI interactions (Fig. 5C). Addition of My43 blocked ADCC mediated by both 225-IgA2-WT and 225-IgA2-P221R antibodies in a dose-dependent manner, whereas IgG-mediated ADCC was not affected. Specific lyses decreased from initially 48.9 ± 7.4 and 46.0 ± 7.3% to 7.4 ± 4.7 and 6.8 ± 2.7%.

**Effects of Macrophage Polarization on Cytokine and Chemokine Release and ADCC**—To investigate the influence of macrophage polarization on their capability to mediate ADCC, macrophages were polarized into M1 or M2 phenotypes by supplementing medium with IFNγ and LPS or IL-4 for 24 h, respectively. In response to cytokine treatment, macrophages changed their cytokine and chemokine expression profile: M1 macrophages, treated with IFNγ and LPS, strongly expressed chemokines (CXCL10, CXCL11, CCL2, CCL5), whereas M2 macrophages, treated with IL-4, and M0 (untreated) macrophages did not (Fig. 6B). Concerning cytokines, M1 macrophages expressed IFNγ at high levels, TNFα at higher and IL-1ra and IL-8 at lower levels compared with M2 and M0 macrophages. M0 macrophages displayed lower expression of cytokines (IL-1ra, IFNγ) and of chemokines (CXCL10, CCL2). Next, we analyzed whether treatment of macrophages with cytokines influences their capability to mediate ADCC of human A431 tumor cells by EGFR-specific antibodies. Interestingly, M1 and M2 macrophages showed similarly efficient killing by all three EGFR antibodies compared with M0 macrophages (Fig. 6C).

**DISCUSSION**

**Advantages and Disadvantages of IgA Iso- and Allotype Selection**—IgA antibodies serve important functions in the mucosal immune system (13–15) and have been demonstrated to mediate potent tumor cell killing in vitro (11, 12). During these in vitro evaluations, monomeric IgA2m(1) antibodies proved more effective in triggering ADCC than IgA1 antibodies (11), which might be related to the different Fab arm orientation of IgA1 versus IgA2 antibodies (31). In view of a clinical application, the IgA2 isotype has other potential advantages. First, IgA1 antibodies contain an elongated heavily O-glycosylated hinge region (32), which is more susceptible to bacterial proteases than the IgA2 hinge (33). This may cause stability issues in vivo, particularly at bacterially colonized sites. Additionally, O-glycosylation is typically diverse and difficult to control during biomolecule production, which limits regulatory and safety experience (6). Importantly, aberrantly hypogalactosylated natural IgA1 antibodies are critically involved in the development of IgA nephropathy, one of the most common causes leading to renal failure (34). Thus, IgA2 represents the preferred isotype for further immunotherapeutic development.

**Stabilizing IgA2m(1) by a Single Mutation**—In humans, three different allotypes have been described for IgA2: IgA2m(1), m(2), or IgA2n (14). Although immunogenicity of allotypes has not been an important issue with IgG1 antibodies (35), this may be different for IgA antibodies, which target more immunologically active sites such as the mucosal tissue. Thus, the ethnicity of potential patient populations may impact the selection of appropriate antibody allotypes. However, the most common Caucasian allotype, IgA2m(1), contains an unusual pairing of
light and heavy chains. This explains why this alloform dissociates into heavy and light chain homodimers under denaturing, nonreducing conditions despite strong non-covalent interactions between light and heavy chains in the IgA2m(1). Although heavy and light chains in non-Caucasian IgA2 allootypes IgA2m(2) and IgA2n are covalently assembled as in IgG1 or IgA1 antibodies, we decided to improve the well characterized and functionally well performing 225-IgA2m(1) antibody by a single point mutation instead of developing a completely new antibody by changing the allotype. Mutations in the Cα1 domain of the IgA2 heavy chain (proline to arginine at position 221 and proline to serine at position 212) were described previously.

FIGURE 3. Both 225-IgA2-WT and 225-IgA2-P221R are similarly effective in mediating ADCC. In A, monomeric (left graph) and dimeric (right graph) antibodies were compared for binding to FcγRI/FcγRIIa chain co-transfected BHK-21 cells using an FITC-labeled human κ-light chain-directed antibody in indirect immunofluorescence. Although 225-IgA2-WT displayed similar RFI as a control IgA2 antibody, the P221R mutant showed significantly reduced RFI. B, in whole blood assays, 225-IgA2-WT and its P221R mutant triggered similarly effective killing of A431 tumor cells. Monomeric and dimeric 225-IgA2-P221R mediated similarly effective ADCC with GM-CSF-activated PMN (C) and isolated monocytes (D) as effector cells compared with their respective wild type counterparts. The specific lysis induced by 225-IgG1 was significantly lower than by 225-IgA2 antibodies using whole blood and PMN, but similar using monocytes as effector cells. Results (n = 4) are shown as mean ± S.E. of FcγRI binding (RFI) (A) and % specific lysis (B–D). Asterisks indicate significant differences (p < 0.01) between IgA and control antibody (225-IgG1 in A, control IgA2 in B–D) using black asterisks for mutant IgA2m(1) and gray asterisks for wild type IgA2m(1) antibodies in A and B, and black asterisks for 4 h and gray asterisks for 24-h values of the specific IgA2m(1) antibody compared with control IgA2 in C. Significant differences (p < 0.05) between wild type and mutant 225-IgA2 are indicated by a plus sign.
Previously to prevent the protein from degradation (21, 22). These mutations sterically enable the formation of an alternate disulfide bridge between the heavy and light chains in the IgA2m(1) allotype, similar to the IgA2m(2) allotype. Our results show that a single P221R amino acid exchange derived from the IgA2m(2) sequence is sufficient to prevent dissociation into heavy and light chain homodimers in both monomeric and dimeric isoforms. Furthermore, the P221R mutation enhanced the stability of the protein as its resistance to long term storage and denaturizing temperature was increased significantly. Thus, a P221R-mutated 225-IgA2m(1) antibody might be better suited for bioindustrial production and for clinical applications.

Functional Evaluation of Mutated 225-IgA2m(1) Antibody—

Next, we investigated whether the P221R mutation influenced the capability of the IgA2 antibodies to activate Fab- and Fc-mediated effector functions. The 225-IgA2-P221R antibody was equally efficient as 225-IgA2-WT in Fab-mediated functions, such as binding to EGFR, growth inhibition, and down-modulation of EGFR in tumor cells. Although mutant 225-IgA2-P221R was less efficient than wild type IgA2 in binding to FcRγ1-directed antibody My43 was supplied in a dose-dependent manner, whereas ADCC mediated by the respective 225-IgG1 was not affected. Results (n ≥ 5) are shown as mean ± S.E. of % specific lyses. Asterisks indicate significant differences (p ≤ 0.01) of EGFR-specific to control IgA (A and B) or between IgA and IgG antibodies (C) using black asterisks for mutant IgA2m(1) and gray asterisks for wild type IgA2m(1) antibodies. E:T, effector:target ratio.

E: T, effector: target ratio.

FIGURE 4. Thermal stability of recombinant IgA2m(1) antibodies. Monomeric antibodies were incubated for 5 min at different denaturating temperatures (A), or at 78 °C (B) for different time periods. Maintenance of functionality was analyzed in ADCC assays using PMN as effector and A431 as target cells. Relative specific lysis was reduced for both wild type and mutant IgA2m(1) antibodies, with the latter demonstrating a significantly slower reduction. Results (n = 5) are presented as mean ± S.E. of relative specific lysis (%). Significant differences (p ≤ 0.001) between wild type and mutant IgA2m(1) are indicated by number. In B, curves for IgG1 and IgA2m(1)-WT are superimposed.
BHK double transfectants might not fully reflect all dynamic states of the FcεRI receptor on human leukocytes. Unfortunately, binding of IgA antibodies to FcεRI on human effector cells could not be reliably investigated by immunofluorescence analyses due to the low FcεRI expression on these cells and the low affinity interaction with the ligand. Both wild type and mutated 225-IgA2 antibodies demonstrated higher efficiency in Fab- and Fc-mediated assays when dimeric IgA2 was compared with equimolar concentrations of the respective monomeric molecules, which was also the case for the respective 225-IgG1. Similar results have been reported previously for monomeric versus dimeric IgA1 (12) and are probably explained by the tetravalency compared with bivalency of dimeric versus monomeric IgA.

Macrophages as Effector Cell Population for 225-IgA2m(1) Antibodies—Myeloid cells such as monocytes and PMN have been described previously to be recruited effectively for ADCC by EGFR antibodies of IgA1 isotype in vitro (12). In tumor sections, myeloid cells, including tumor-associated macrophages, constitute the major component of the leukocyte tumor infiltrate (27, 28); however, their potential role for tumor progression or surveillance is controversial. For example, experiments with cytokine-transfected tumor cells demonstrated inhibitory effects of the recruited myeloid cells on tumor growth (41, 42).

More recently, tumor-associated macrophages were divided into the tumor growth inhibiting M1 and the tumor promoting M2 phenotype, which are both thought to derive from a common M0 precursor (43). Also for tumor-associated neutrophils, corresponding N1 and N2 phenotypes have been proposed (44). However, a programmatic change of myeloid cells, e.g., on IL-12 exposure has been reported (45), and most of these studies investigated the role of tumor associated myeloid cells in the absence of tumor-directed antibodies. In the presence of antibodies, both M1 and M2 macrophages were demonstrated to kill rituximab-opsonized tumor cells in vitro (29), related to the contribution of myeloid cells for the therapeutic efficacy of rituximab in vivo (26). Recently, cetuximab was demonstrated to activate M2 macrophages in vitro (46), but the contribution of this activation on in vivo growth of EGFR-expressing tumors was not investigated in these experiments. To the best of our knowledge, our study is the first to investigate the impact of IgA antibodies on tumor cell killing by selectively differentiated macrophages. Interestingly, M0, M1, and M2 macrophages were similarly effective in killing of IgG- and IgA-coated tumor cells (Fig. 5C). Because macrophages are phagocytes internalizing rapidly cross-linked IgA, the results of the cytotoxicity assays might underestimate the potency of IgA (47). Whether these in vitro experiments also relate to in vivo efficacy of EGFR

FIGURE 6. Polarized macrophages display different cytokine and chemokine profiles but similar capacity to trigger IgA-mediated ADCC. Monocytes were treated with M-CSF for 7 days to induce differentiation into macrophages. Next, cells were left untreated (M0) or treated for 24 h with IFNγ+LPS or IL-4 for final polarization into M1 or M2 phenotypes, respectively. A, cytokines and chemokines released into fresh medium were analyzed using a commercial array kit. Pixel density of dot plots was calculated using ImageJ software. Results (n ≥ 2) are shown as mean ± S.E. of pixel density (% to PC). B, both 225-IgA2 antibodies and the respective 225-IgG1 induced similar specific lysis by recruiting M0, M1, and M2 macrophages to mediate ADCC of A431 tumor cells. Results (n ≥ 4) are shown as mean ± S.E. of % specific lysis. Significant differences (p ≤ 0.01) between 225-IgA2m(1) and control IgA2 antibody were indicated with respectively colored asterisks.
antibodies of IgA isotype needs to be determined in further studies. However, these studies are complicated by the lack of a FcαRI orthologue in mice (48), a limitation that can be overcome by using human FcαRI transgenic mice (17). First, *in vivo* studies in these mice demonstrated an unexpectedly short serum half-life of our IgA antibodies, which may be related to the lack of sialylation. Thus, exposed terminal galactose may mediate binding of both IgA2m(1) antibodies to the hepatically expressed asialoglycoprotein-receptor (49), which rapidly internalizes these antibodies. These *in vivo* data are indicating the requirement for further improvements of our current molecules.

**Conclusion**—In conclusion, we have demonstrated that macrophages, in addition to monocyes and PMN, may constitute another potent effector cell population for EGFR-directed IgA antibodies. Interestingly, M0, M1, and M2 macrophages displayed similar ADCC activity, suggesting that also tumor-associated M2 macrophages can be recruited against cancer cells by tumor-specific IgA antibodies. Furthermore, a P221R-mutated version of the Caucasian IgA2m(1) allotype demonstrated the typical heavy and light chain pairing of other antibody isotypes and proved functionally as effective as its wild type counterpart. Together, these results further promote the concept of employing IgA antibodies in tumor therapy.

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