Afucosylated antibodies increase activation of FcγRIIIa-dependent signaling components to intensify processes promoting ADCC

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Abstract

Antibody-dependent cellular cytotoxicity (ADCC) is a key mechanism by which therapeutic antibodies mediate their antitumor effects. Absence of fucose on the heavy chain of the antibody increases the affinity between the antibody and FcγRIIIa, which results in increased *in vitro* and *in vivo* ADCC compared to the fucosylated form. However, the cellular and molecular mechanisms responsible for increased ADCC are unknown. Through a series of biochemical and cellular studies, we find that human NK cells stimulated with afucosylated antibody exhibit enhanced activation of proximal FcγRIIIa signaling and downstream pathways, as well as enhanced cytoskeletal rearrangement and degranulation, relative to stimulation with fucosylated antibody. Furthermore, analysis of the interaction between human NK cells and targets using a high-throughput microscope-based antibody-dependent cytotoxicity assay show that afucosylated antibodies increase the number of NK cells capable of killing multiple targets and the rate with which targets are killed. We conclude that the increase in affinity between afucosylated antibodies and FcγRIIIa enhances activation of signaling molecules promoting cytoskeletal rearrangement and degranulation, which, in turn, potentiates the cytotoxic characteristics of NK cells to increase efficiency of ADCC.
Introduction

Rituximab is a chimeric therapeutic antibody used for treating individuals with CD20$^+$ hematologic cancers (1), whereas trastuzumab is a humanized therapeutic antibody used for treating individuals with HER2$^+$ cancers (2, 3). Numerous reports have demonstrated that rituximab and trastuzumab can mediate their effects through multiple mechanisms (3, 4). Along with the ability to disrupt survival and growth signaling in cancerous cells, these antibodies can facilitate antibody-dependent cellular cytotoxicity (ADCC) to destroy tumor cells (3, 4). In this context, rituximab and trastuzumab mobilize the innate immune system to attack tumors by promoting the interaction of immune cells and cancer cells expressing CD20 or overexpressing HER2, respectively. This method of cytotoxicity is mediated at the cellular level through the interaction of FcγRIIIa expressed on natural killer (NK) cells or macrophages and the Fc portion of the tumor-bound antibody (3, 4, 5). This directs secretion of lytic molecules toward the antibody-decorated tumor cell, resulting in lysis of the tumor cell (6).

The importance of mobilizing the immune system for ADCC in tumor eradication is documented in numerous preclinical and clinical studies using different therapeutic antibodies. In preclinical in vivo studies, lower antitumor activity with therapeutic antibodies was observed in FcγR chain-deficient mice compared to wild-type mice (7). Similarly, treatment of mice with an “effectorless” form of the therapeutic antibody that does not interact with the Fc receptor also resulted in lower antitumor activity compared to mice treated with unmodified antibody (7). In two clinical studies, follicular or B-cell lymphoma patients with the high affinity allele of FcγRIIIa have a better response rate with rituximab relative to patients with low affinity allele (8, 9), yet a third such study observed no significant variation as a function of the FcγRIIIa allele (10). The relationship between FcγRIIIa affinity and efficacy of trastuzumab in humans is
similarly unclear. One study showed that for metastatic breast cancer patients treated with trastuzumab and a taxane, patients with the high affinity allele had better response rates and progression free survival (PFS), compared to patients with the low affinity allele (11). By contrast, two other studies showed no significant advantages in response rate, PFS, or disease-free survival for trastuzumab-treated metastatic breast cancer patients with the high affinity allele (10, 12, 13). Although explanations for these apparent discrepancies remain to be identified, preclinical data and other evidence that ADCC plays a prominent role in the mechanism of action of some therapeutic antibodies have led to efforts to increase binding of antibodies to FcγRIIIa to augment ADCC (14, 15, 16). To this end, removing fucose from asparagine 297 of the heavy chain results in an increase in binding affinity between the Fc portion of the antibody and FcγRIIIa, which consequently leads to more efficient ADCC (14, 15, 16, 17). Because of these and other related studies, obinutuzumab, an afucosylated anti-CD20 antibody, was developed and shown to have superior efficacy in clinical trials compared to rituximab (14, 18), and is currently approved to treat patients with chronic lymphocytic leukemia. These observations imply that potentiating ADCC via the innate immune system represents a promising approach in the development of therapeutics for oncology indications.

Multiple signaling pathways and components are known to govern cellular processes required for ADCC (5). Stimulation of NK cells through FcγRIIIa begins with phosphorylation of proximal signaling components, including the src family kinase Lck (19), the CD3ζ chain (20, 21), and Zap70 (22). This leads to the activation of multiple protein tyrosine signaling cascades required for NK-cell cytotoxicity (23), including the MAPK and PI3K pathways, which govern degranulation (24, 25, 26), and Vav-1, which regulates actin rearrangement (27, 28, 29). As expected, both actin rearrangement and degranulation are cellular processes required for NK-cell
cytotoxicity (6, 30). Thus, activation of multiple signaling components and pathways that govern actin rearrangement and degranulation through FcγRIIIa is essential for NK cell-mediated ADCC.

Although the relationship between the increased affinity of afucosylated antibodies for FcγRIIIa and enhanced ADCC (15, 17) is well-established, the cellular and molecular basis for this observed enhancement is unknown. We demonstrate here that human NK cells stimulated with afucosylated trastuzumab or obinutuzmab exhibit an increase in activation of proximal FcγRIIIa signaling components and downstream signaling pathways, including specifically the Vav-1, MAPK, and PI3K pathways. Consistent with these findings, NK cells stimulated by afucosylated antibodies displayed an increase in actin rearrangement and degranulation. Finally, using a high throughput microscope-based cytotoxicity assay developed to characterize certain cellular parameters of ADCC, we observe that afucosylated antibodies enhance the cytotoxic potential of an entire NK-cell population by increasing the frequency of NK cells that can kill multiple targets. Moreover, afucosylated antibodies increase the cytotoxic potential of individual NK cells by decreasing the duration required to kill individual targets. Together, our findings indicate that increased affinity between afucosylated antibodies and FcγRIIIa enhances activation of signaling molecules to promote actin rearrangement and degranulation, and enhances serial killing and more rapid killing, leading to an overall enhancement of ADCC.
Methods

**Immunoblotting.** NK cells were enriched from human blood (all donors used were of FcγRIIIa F/F158 phenotype, except in Supplemental Figure 1, in which samples from V/V158 donors were used) using LSM (MP Biomedicals) followed by labeling with NK isolation kit and sorting with AutoMACs (Miltenyi Biotec). Purity of NK cells is >90%. NK cells were incubated with 1 μg/mL antibody for 30 minutes on ice and washed with media. Anti-human κ light chain antibody (50 μg/mL; Millipore) was added and cells were incubated for indicated timepoints at 37°C. Anti-human κ light chain antibody binds similarly between afucosylated antibody and their respective counterpart (data not shown). Ice-cold media was added to stop the stimulation. Cells were pelleted and lysate was made using RIPA buffer (Sigma) containing phosphatase (Sigma) and protease (Roche) inhibitors. Membranes were probed with antibodies against phosphotyrosine (Millipore), phospho-Lck (Sigma), phospho-CD3ζ chain (Epitomics), phospho-Zap70 (Cell Signaling Technologies), phospho-Vav-1 (Sigma), phospho-Akt (Cell Signaling Technologies), phospho-ERK 1/2 (Cell Signaling Technologies), and pan-actin (Cell Signaling Technologies).

**Intracellular phospho-ERK1/2 staining.** Cells were stimulated as described. After the final wash, cells were pelleted and fixed with 2% paraformaldehyde for 10 minutes, chilled for 1 minute and spun. Ice-cold 90% methanol was added and incubated on ice for 30 min. Cells were washed and stained with phospho-ERK1/2 antibody for 60 minutes. Data were acquired on an LSR flow cytometer (BD Biosciences) and analyzed using FlowJo.
**Actin rearrangement assay.** Cells were stimulated as described. After the final wash, cells were pelleted, fixed with 3.7% paraformaldehyde for 10 minutes at RT, and washed with PBS. Cells were permeabilized with 0.1% Triton X-100/PBS for 5 minutes, pelleted, and stained with 5 units/mL phalloidin (1% BSA/PBS) (Invitrogen) for 20 minutes. Cells were washed with PBS before acquisition.

**CD107a expression assay.** NK cells (1x10^6) were incubated with 1 μg/mL antibody for 30 minutes on ice and washed. Media containing anti-human K light chain antibody (Millipore) and CD107a APC (BD Biosciences) was added and cells were incubated at 37°C. At the indicated timepoints, 4% paraformaldehyde was added and incubated for 10 minutes at RT. Cells were washed with PBS before acquisition.

**Microscope-based ADCC assay.** This assay was modified from that used by Bhat and colleagues (31). NK cells were labeled with PKH26 Red Fluorescent Cell Linker Kit (Sigma) and mixed at 0.4:1 E:T ratio with targets (BT474 for trastuzumab, WIL2-S for anti-CD20 studies), afucosylated or fucosylated antibody, and Sytox Green (Invitrogen) in 10% FBS/RPMI. 5 μL of cell/antibody mixtures were dispensed into 1536-well plates (Aurora) at cell density of 0.0008 cells/μm^2 for trastuzumab or 0.002 cells/μm^2 for anti-CD20 studies. All conditions had replicates. Images were collected on a Leica SP5 confocal microscope in controlled environment (37°C, 5% CO₂) for 14 hours using Leica Matrix software. 40x total magnification was obtained using a 10x dry objective (NA 0.4) and a 4x digital zoom. Scripts were written to generate a movie for each well with random names assigned for blind analysis. Percent cytotoxicity was calculated as: ((number of dead cells at end of sample - number of dead cells at
start) – (number of spontaneous target deaths + number of spontaneous effector deaths))/92 [average number of BT474 cells (or 213 for average number of WIL2-S cells) in field of view]x100.

Analysis of NK cells performing multiple killing events. Movies were interrogated for NK cells killing 2 or more targets using Imaris software (Bitplane Scientific Software). Percent of total NK cells performing multiple killing events was calculated as: (number of NK cells killing multiple targets)/[average number of NK cells in field of view (36 for trastuzumab, 86 for anti-CD20 studies) x100. Still images were captured with Imaris.

Duration of cytotoxicity analysis. Movies of brightfield and fluorescent merged images were interrogated for cytotoxic events in which the entire process could be visualized. Duration of cytotoxicity was calculated by counting the number of frames from initial stage of killing, defined as frame or frames with NK/target engagement preceding first visible Sytox green, to full detachment between target and NK cell, then multiplying by 8 minutes (duration between frames).
Results

Afucosylated antibody-stimulated NK cells exhibit increased activation of tyrosine kinases and proximal FcγRIIIa signaling

Early studies of FcγRIIIa signaling demonstrated that herbimycin A, a broad spectrum tyrosine kinase inhibitor, abrogates ADCC antibody-stimulated NK cells, suggesting that tyrosine kinase activation and tyrosine phosphorylation are required for cytotoxicity (23). To determine whether afucosylated antibodies alter tyrosine kinase-dependent signaling, the phospho-protein repertoire of antibody-stimulated NK cells was assessed by preparing cell extracts and subjecting them to immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 1A, we observed an increase in total protein tyrosine phosphorylation in NK cells treated with afucosylated trastuzumab (Fig. 1A) or obinutuzumab (Fig. 1B) relative to their fucosylated counterparts. In addition, NK cells bearing the high affinity form (V/V158) of FcγRIIIa also exhibited more protein tyrosine phosphorylation when stimulated with afucosylated antibodies (Supplemental Figure 1).

Afucosylated antibodies do not appear to alter the kinetics of representative tyrosine phosphorylated proteins when compared to their respective fucosylated partners. Specifically, the phosphorylation kinetics of the 85 kDa and 60 kDa phospho-proteins were comparable following treatment with both forms of trastuzumab (Fig. 1D). Similar results were observed when comparing obinutuzumab and rituximab treated NK cells (Fig. 1E). Together, these findings imply that the increase in binding affinity between FcγRIIIa and antibody enhances FcγRIIIa-dependent signal strength, as reflected by the total phospho-tyrosine content, but not phosphorylation kinetics.
To estimate the amount of fucosylated trastuzumab required to elicit similar phosphotyrosine signals compared to trastuzumab, the phosphorylated tyrosine protein repertoire of NK cells from 4 different donors stimulated with titrating amounts of trastuzumab were compared to NK cells stimulated with a single concentration of afucosylated trastuzumab. Based on densitometry of all of the phosphorylated tyrosine protein bands, we observed that an average of approximately 8.5-fold more trastuzumab was required to elicit the same signaling as afucosylated trastuzumab (range of approximately 1.5-20 fold, depending on the donor; Supplemental Figure 2).

To further characterize and compare signaling elicited by afucosylated antibodies and their fucosylated counterparts, changes in proximal FcγRIIIa signaling were assessed. After stimulating NK cells, more phosphorylated Lck was observed to accumulate in cells activated with afucosylated trastuzumab (Fig. 2A). We also observed in these cells a relative increase in phosphorylated CD3ζ chain (Fig. 2B) and Zap70 (Fig. 2C), which are both targets of Lck. Similar findings were observed when comparing NK cells stimulated with obinutuzumab and rituximab (Fig. 2D). Together, these data imply that afucosylated antibodies enhance activation of proximal FcγRIIIa signaling.

Afucosylated antibody stimulated-NK cells exhibit increased activation of signaling components and pathways required for FcγRIIIa-mediated cytotoxicity

After activation by Lck, Zap70 phosphorylates linker of activated T cells (LAT) to initiate numerous downstream signaling pathways (32), including the Vav-1 pathway that is required for actin rearrangement (27, 28, 29), and the MAPK and PI3K pathways that are essential for degranulation (24, 26). To further characterize the enhanced signaling mediated by
Afucosylated antibodies, activation of these pathways was assessed by immunoblot analysis of the phosphorylated components of proteins in these pathways. Notably, an increase in phosphorylated Vav-1 was observed in NK cells stimulated with afucosylated trastuzumab relative to stimulation by fucosylated trastuzumab (Fig. 3A). Similarly, we observed an increase in phospho-Akt in NK cells stimulated with afucosylated trastuzumab and obinutuzumab compared to their fucosylated controls (Fig. 3B, 3F). Furthermore, MAPK pathway activation was also enhanced in afucosylated trastuzumab- and obinutuzumab-stimulated cells (Fig. 3C, 3G).

To quantify the activation status on a per cell basis, we used flow cytometry to assess the expression of intracellular phospho-ERK1/2. In these experiments, we observed that phospho-ERK1/2 expression was evident in a larger fraction of afucosylated trastuzumab-stimulated NK cells (Fig. 3D), and the phospho-ERK1/2 MFI was higher in these cells (Fig. 3E). These findings indicate that afucosylated trastuzumab activates more cells than fucosylated trastuzumab, and that the cells in the population are, on average, activated to a greater extent. These observations are consistent with the conclusion that an increase in affinity between FcγRIIIa and antibody promotes stronger FcγRIIIa signaling.

Afucosylated antibody stimulated-NK cells exhibit increased actin rearrangement and degranulation

Two key cellular mechanisms required for NK cell cytotoxicity are actin rearrangement and degranulation (6). To determine whether afucosylated antibodies are more effective than fucosylated antibodies in eliciting actin rearrangement, we assessed F-actin accumulation in antibody-stimulated NK cells by phalloidin staining and flow cytometry. Consistent with the
increase in phospho-Vav-1, we observed an increase in F-actin content in NK cells stimulated with afucosylated trastuzumab (Fig. 4A). Furthermore, at all timepoints examined, the MFI ratio of F-actin content was higher (Fig. 4B) in cells stimulated with afucosylated trastuzumab, indicating that the afucosylated antibody promoted more actin rearrangement in NK cells relative to the antibody of the fucosylated form.

To determine whether afucosylated antibodies enhance the efficiency of degranulation, we used flow cytometry to measure the relocalization of the lysosomal marker, CD107a, to the cell surface. We observed a greater fraction of NK cells expressing surface CD107a when stimulated with afucosylated trastuzumab (Fig. 4C). Similarly, a higher percentage of obinutuzumab-activated NK cells expressed CD107a compared to rituximab-stimulated cells (Fig. 4D). We also observed that the MFI of the CD107a was greater on NK cells stimulated with afucosylated trastuzumab (Fig. 4E) and obinutuzumab (Fig. 4F) relative to their fucosylated antibody counterparts. These observations together indicate that stimulation by an afucosylated antibody leads to more degranulating NK cells and elicits, on average, a greater amount of degranulation per cell.

Microscope-based cytotoxicity assay detects increased afucosylated antibody-mediated ADCC at low E:T ratio

Conventional assays that measure ADCC use high E:T ratios (i.e. 10:1 to 25:1), which can be considered non-physiologic given that NK cells are a rare cell type in solid tumors, relative to tumor cells (33, 34). Conventional ADCC assays also do not allow for visualization of the interaction between NK cells and targets, thus prohibiting the identification and characterization of changes in the duration required for cytotoxicity or individual NK-cell
cytotoxic potential. To address these deficiencies, we adapted a microscope-based cytotoxicity assay (31) to visualize the interaction between NK cells and targets, thus enabling us to quantify target killing, and to characterize other parameters of ADCC under more physiologic conditions. As shown in Fig. 5A, continuous monitoring of interactions between live NK cells and their live targets allowed us to observe the progression of killing at time points spanning a 6-hour interval. Quantitatively, cultures containing antibody yielded substantially more cell death compared to negative control cultures without antibody, demonstrating that the assay assesses ADCC (Fig. 5B). Using this assay, we observe more cytotoxicity in the presence of afucosylated trastuzumab relative to cytotoxicity in the presence of fucosylated trastuzumab (Fig. 5C). Similarly, obinutuzumab-treated NK cells killed more WIL2-S CD20-expressing target cells relative to target cells killed by NK cells stimulated with rituximab (Fig. 5D). However, obinutuzumab is a type II antibody that induces more apoptosis in target cells without the need for NK cells, relative to rituximab (14), a type I antibody. Nonetheless, the increase in the number of dead cells observed with obinutuzumab treatment is not simply a reflection of more potent antibody-dependent, immune cell-independent cytotoxicity, since targets with antibody-only conditions (NK cell-independent killing) were included as controls and factored into the equation for percent cytotoxicity (see methods). Therefore, our analysis of percent cytotoxicity accounts for any apoptosis attributable to killing mediated solely by antibody. As the data show, ADCC is enhanced with obinutuzumab (Fig. 5E), indicating that the increase in cytotoxicity observed with obinutuzumab, relative to rituximab, is not due to the epitope-binding specific differences between these type I and type II antibodies.
Afucosylated antibodies increase frequency of multiple killing events and decrease time required for target cell lysis

To address the possibility that afucosylated antibodies increase ADCC by increasing the cytotoxic potential of the NK-cell population, experiments were performed to address multiple killing events. We found that serial killing events are mediated by single NK cells in assays containing either afucosylated (Fig. 6A, top) or fucosylated trastuzumab (Fig. 6A, bottom). However, we observed that the frequency of NK cells killing multiple targets is higher in assays containing afucosylated trastuzumab relative to assays containing the fucosylated antibody (Fig. 6C).

We also sought to determine whether the time required to lyse a target cell is modulated by the fucosylation state of the antibody, thereby leading to potentiation of ADCC. To assess the duration required for cytotoxicity, live-cell microscopy videos were interrogated for cytotoxic events in which target engagement, lysis, and release were visualized and measured (Fig. 6B). As shown in Figure 6B and D, NK cells in cultures containing fucosylated trastuzumab took twice as long to kill target cells relative to cultures containing the afucosylated form (50 vs. 100 min, Fig. 6D). These findings are consistent with the earlier observation that there are more dead cells at early time points in assays containing afucosylated trastuzumab relative to the same time point in assays containing fucosylated trastuzumab (Fig. 5A). Considered together, these observations indicate that NK cells in assays containing afucosylated trastuzumab kill faster and more often.

Similar results were obtained using this assay to compare obinutuzumab and rituximab, when using the target cell line WIL2-S. In assays containing obinutuzumab, NK cells achieved more multiple killing events (Fig. 6E) and killed faster (Fig. 6F) relative to cells in assays
containing rituximab. Together, these data indicate that afucosylated antibodies promote ADCC by enhancing the cytotoxic potential of the whole NK-cell population by increasing the frequency of cells that can kill multiple targets and decreasing the interval required by NK cells to lyse their targets.
Discussion

Rituximab and trastuzumab are naturally fucosylated antibodies that are key therapeutics in the treatment of various cancers (1, 2, 3, 4). Their mechanism of action is thought to include ADCC. In preclinical studies, lower antitumor activity with these antibodies was observed in FcγR chain-deficient mice or when wild-type mice were treated with a mutated therapeutic antibody that prevented interaction with the Fc receptor (7). In clinical studies, a better response rate with rituximab or trastuzumab was observed in patients with the high affinity allele of FcγRIIIa (8, 9), further supporting the conclusion that ADCC is a significant component of the therapeutic mechanism of action. However, the apparent added benefit afforded by the high affinity allele is not evident in all indications or circumstances since patients with the high and low affinity FcγRIIIa alleles responded equally well to rituximab or trastuzumab for their respective indications in other studies (10). Although mechanisms to account for these apparent discrepancies are unknown, evidence that ADCC is an important mechanism of action was sufficiently strong that efforts to modify antibodies to increase binding to FcγRIIIa to augment ADCC have been pursued (14, 15, 16).

Because ADCC is mediated by antibody binding to FcγRIIIa (8, 9) expressed on NK cells (10), studies were focused on modifying the Fc portion of the antibody to increase ADCC. It was found that removal of the alpha-1, 6 fucose moiety on the N-glycan at Asn-297 of the heavy chain resulted in enhanced interaction between antibody and FcγRIIIa (8, 9, 15, 17). More importantly, binding enhancement from the removal of fucose directly led to more efficient ADCC (15, 17) and better efficacy in preclinical models (17, 35). Consistent with those data, our studies also show removal of fucose is sufficient to drive increased NK-cell signaling and activation.
One of the biologics borne out of these efforts is obinutuzumab, an anti-CD20 afucosylated antibody that exhibited increased ADCC in preclinical studies and is currently approved for CD20^+ hematologic cancers (14). Obinutuzumab showed increased efficacy in patients, as measured by better overall response rate and higher reported complete remission, with no increase in adverse side effects compared to its fucosylated counterpart rituximab (18). Thus, removal of fucose on therapeutic antibodies is a promising technology to improve mobilization of the innate immune system by enhancing NK cell-mediated ADCC (36).

However, cellular mechanisms that account for the enhanced ADCC observed with afucosylated antibodies have not been defined. A better understanding of these cellular mechanisms may help design approaches to make afucosylated antibodies even more effective, or keep them maximally effective, especially in light of new focuses on combination therapies (37). With development of inhibitors against components of the MAPK, PI3K, and other pathways that cancer cells utilize for growth and survival, these compounds may inadvertently antagonize signaling in immune cells to inhibit therapeutic antibody-mediated ADCC. It is also known that certain chemotherapeutic agents, such as paclitaxel and docetaxel, negatively affect NK-cell cytotoxicity (38). Therefore, our findings highlight the idea that developing a biologic that potentiates signaling molecules and pathways required for ADCC is extremely important.

Our current studies center on the molecular and cellular processes as well as the cytotoxic properties responsible for the improvement in afucosylated antibody-mediated ADCC. In this series of experiments, we observe an increase in phosphorylation of Lck, CD3ζ, and Zap70 in NK cells stimulated with afucosylated trastuzumab and obinutuzumab. In addition, these afucosylated antibodies augment activation of the Vav-1, MAPK and PI3K pathways, which are regulators of actin rearrangement and degranulation, respectively. Consistent with this result,
afucosylated trastuzumab- and obinutuzumab-activated NK cells exhibited more actin rearrangement and degranulation. In fact, not only were more NK cells degranulating, but also degranulating more on a per cell basis. The former observation suggests the increased binding affinity between FcγRIIIa and antibody enabled more cells to reach the signaling threshold required for activation while the latter implies signaling enhancement in cells that have already reached the signaling threshold.

We also developed a high-throughput microscope-based cytotoxicity assay to assess changes in cytotoxic potential and properties. Analysis of the data showed that afucosylated antibodies increase the cytotoxic potential of individual NK cells by decreasing the time required for lysis. In addition, afucosylated antibody treatment led to an increase in the number of NK cells that could lyse multiple targets, thereby increasing the cytotoxic potential of the NK-cell population as a whole. These properties can contribute to the increase in ADCC mediated by afucosylated antibodies, and are consistent with the enhanced molecular and cellular mechanisms observed. Together, our findings support a model in which the increased binding affinity between FcγRIIIa and afucosylated antibody results in enhanced activation of proximal FcγRIIIa signaling molecules and their downstream components. Consequently, there is an increase in actin rearrangement and degranulation, thereby potentiating cytotoxic characteristics to augment NK cell-mediated ADCC.

While these studies focused on the interaction of therapeutic antibodies and FcγRIIIa expressed on NK cells to mediate ADCC, macrophages also express FcγRIIIa (39) and have been implicated to have a role in therapeutic antibody-mediated tumor clearance in vivo (7). Furthermore, results from recent studies suggest that macrophage-mediated ADCC and antibody-dependent phagocytosis as potential modes of action (40), and these effector mechanisms are
enhanced in the presence of afucosylated antibodies (41). Although it is beyond the scope of this study to address the changes in the molecular and cellular processes afforded by afucosylated antibodies in macrophages, our current experiments could lend insight into future studies designed to explore them since macrophages share similar signaling pathways (42) required for ADCC and antibody-dependent phagocytosis with NK cells.

The fucosylated antibodies trastuzumab and rituximab vary substantially with respect to the efficiency with which they generate NK cells capable of performing serial killing (Figure 6C, E). One possible explanation is the difference in antibody affinity for FcγRIIIa; trastuzumab binds FcγRIII 5 times stronger than does rituximab (43). This, again, is consistent with the observation that stronger binding affinity enhances certain cytotoxic characteristics. Another explanation is the type of targets the NK cells are attacking. Cytotoxic properties also may be modulated by differences in factors such as the expression level of the target molecule (44), repertoire and expression levels of activating and/or inhibitory NK-cell ligands by the tumor cells (45, 46), and/or tumor-borne adhesion molecules (47) on different types of cancer. A better understanding of these differences will help guide development of a given therapeutic antibody toward indications where it could be more efficacious.

Finally, studies have shown that serum antibodies can compete with fucosylated therapeutic antibodies for FcγRIIIa (48), resulting in very few productive NK/tumor cell interactions. However, because afucosylated antibodies have a substantially higher affinity for FcγRIIIa, afucosylated antibodies are likely to be unaffected by this competition with endogenous antibodies and remain bound to the receptor on the NK cell (48). By being exempt from this competition, the dwell time between the afucosylated therapeutic antibodies and FcγRIIIa will be increased, thereby eliciting the signaling events required to activate the NK cell.
This ability to remain engaged to the receptor and drive productive signaling is particularly important in scenarios in which expression of the antigen is low because ADCC correlates with antigen density (49). Low antigen expression constrains the number of immune complexes formed on the target cell and limits the number of FcγRIIIa engaged because FcγRIIIa cannot stably bind monomeric antibody (50). Consequently, in such circumstances, the dwell time is short and there is a lack of productive signaling. However, the increase in affinity with afucosylated antibodies and FcγRIIIa would overcome such limitations and thus permit the signaling threshold to be reached, resulting in more activated NK cells and consequently ADCC. Furthermore, in vitro studies have shown that trastuzumab efficacy increases with antigen expression level (44), identifying corresponding therapeutic implications for the use of afucosylated antibodies; patients with low expressing antigens, and who would not be responsive to fucosylated therapeutics, may experience efficacy when treated with afucosylated antibodies.

Results from our studies are also consistent with those of previous studies regarding the quantitative gain afforded by using afucosylated antibodies over fucosylated ones. In a study of 4 different NK-cell donors, we determined that, on average, approximately 8.5 times as much fucosylated trastuzumab is required to elicit the same phospho-tyrosine protein signature as afucosylated trastuzumab. This observation is consistent with previous studies demonstrating a 2-11 fold-change in the EC₅₀ between afucosylated and fucosylated trastuzumab in ADCC assays (17).

Together, results from these studies serve to advance the understanding of the molecular and cellular mechanisms dictating NK-dependent cytotoxic characteristics by which afucosylated antibodies potentiate ADCC to increase efficacy. They may also guide strategies for combination therapies in oncology indications; the greatest patient benefits may be achieved by
avoiding combination therapies that compromise NK cell-dependent cytotoxicity, or by including therapies in which NK cell-dependent cytotoxicity either is not compromised or is actually enhanced.
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References


Figure Legend

Figure 1. **NK cells stimulated by afucosylated antibodies exhibit an increase in activation of tyrosine kinases, but the kinetics of activation are unaltered.** NK cells were stimulated by incubating with afucosylated antibody or its fucosylated counterpart for 30 minutes on ice and then cross-linked with anti-human secondary antibody for indicated timepoints. (A) Cell lysate of NK cells from F/F158 donors stimulated with afucosylated or fucosylated trastuzumab or anti-human secondary antibody alone were probed with antibodies against phosphotyrosine. (B) Similarly, cell lysate of NK cells from F/F158 donors stimulated with obinutuzumab or rituximab were probed with anti-phosphotyrosine antibody. (C) The densitometry ratio of the band at approximately 85 (left) and 60 (right) kDa relative to the corresponding actin band from cell lysate of NK cells stimulated with afucosylated (square, solid line) or fucosylated (circle, dashed line) trastuzumab. (D) The densitometry ratio of the 85 (left) and 60 (right) kDa band relative to actin of NK cells stimulated with obinutuzumab (square, solid line) or rituximab (circle, dashed line). The bands of interest are denoted in (A) and (B) with arrows. Figures are representative of at least 3 independent experiments and donors.

Figure 2. **Components of early FcγRIIIa signaling are intensified in NK cells stimulated with afucosylated antibodies.** NK cells were stimulated by incubating with afucosylated or fucosylated antibodies for 30 min on ice and then cross-linked with anti-human secondary antibody for indicated times. (A-C) Cell lysates of NK cells stimulated with afucosylated or fucosylated trastuzumab were made and run on a 4-12% gel, followed by transfer onto PDVF membranes and probed with an antibody against phospho-Lck (A), phospho-CD3ζ chain (B) or phospho-Zap70 (C). (D) Cell lysate of NK cells stimulated with obinutuzumab and rituximab were also made and run on a 4-12% gel, followed by transfer onto PDVF membranes and probed.
with an antibody against phospho-CD3ζ chain. Figures are representative of at least 3 independent experiments and donors.

Figure 3. NK cells stimulated by afucosylated antibodies exhibit an increase in phosphorylation of Vav1, Akt, and ERK1/2. (A-E) NK cells were stimulated as described above. Cell lysates were made and run on a 4-12% gel, followed by transfer onto PDVF membranes. Membranes were probed with antibodies against (A) phospho-Vav1, (B) phospho-Akt, and (C) phospho-ERK1/2. (D, E) Trastuzumab stimulated NK cells were also permeabilized, stained for intracellular phospho-ERK1/2, and assessed for expression by flow cytometry. (D) Percent of NK cells stimulated with afucosylated (square, solid line) or fucosylated (circle, dashed line) trastuzumab expressing phospho-ERK1/2. (E) Phospho-ERK1/2 mean fluorescent intensity (MFI) ratio of time point to time 0 of NK cells stimulated with afucosylated (white bar) or fucosylated (light gray) trastuzumab. Bars represent SEM of multiple experiments. Asterisks represent statistical significance based on two-tailed unpaired Student’s t test (*, p<0.05; **, p < 0.005). (F, G) Cell lysate from obinutuzumab or rituximab stimulated NK cells were assessed for phospho-Akt (F) and phospho-ERK1/2 (G). Figures are representative of at least 3 independent experiments and donors.

Figure 4. NK cells stimulated by afucosylated antibodies exhibit an increase in actin rearrangement and degranulation. NK cells were stimulated as described above. (A, B). Actin rearrangement was assessed in stimulated NK cells by intracellular staining with phalloidin and flow cytometry. (A) Phalloidin staining flow profiles of afucosylated (left) and fucosylated (right) trastuzumab stimulated NK cells at time 0 (shaded) and 5 minutes (open). Figure is
representative of 4 independent experiments and donors. (B) MFI ratio of time point to time 0 of NK cells stimulated with afucosylated (square, solid line) or fucosylated (circle, dashed line) trastuzumab, or secondary antibody alone (triangle, dotted line) was calculated. Bars represent the SEM of 4 independent experiments. Asterisks represent statistical significance based on two-tailed unpaired Student’s t test (*, p<0.05; **, p < 0.005). (C-F) Degranulation was assayed in stimulated NK cells by CD107a staining and flow cytometry. (C) Percent of NK cells stimulated with afucosylated (white) or fucosylated (light gray) trastuzumab or secondary antibody alone (dark gray) expressing CD107a at time 0 and 4 hr. (D) Percent of CD107a⁺ NK cells after activation with obinutuzumab (white), rituximab (light gray), or secondary antibody (dark gray). Panels C and D are representative of at least 4 independent experiments and donors. (E) MFI of CD107a staining (normalized to secondary antibody alone) on NK cells stimulated with afucosylated (white) or fucosylated (light gray) trastuzumab at time 0 and 4 hr. (F) MFI of CD107a staining (normalized to secondary antibody alone) on NK cells stimulated with obinutuzumab (white) or rituximab (light gray). Bars in panels E and F represent SEM of at least 4 independent experiments. Asterisks represent statistical significance based on two-tailed paired Student’s t test (*, p<0.05; **, p < 0.005).

Figure 5. Microscope based cytotoxicity assay can detect differences in afucosylated and fucosylated antibody mediated ADCC at 0.4:1 E:T ratio. (A) Still images from time 0 to 360 min of BT474 target cells co-cultured with NK cells treated with 10 ng/mL of afucosylated (top), fucosylated (middle), or no antibodies (bottom). NK cells are red, while dead or dying cells are green. Numbers of dead/dying cells are denoted in white in the bottom left-hand corner. Solid white bar in right bottom corner of first image for each condition denotes 100 μm scale bar. (B)
Raw number of dead cells at time 0 and 14 hour in samples treated with 10 ng/mL of afucosylated (white and dark gray bars) or fucosylated (light gray and black bars) trastuzumab. (C) Percent cytotoxicity of BT474 targets mediated by NK cells treated with increasing concentration of afucosylated (square, solid line) or fucosylated (circle, dashed line) trastuzumab. (D) Raw number of dead cells at time 0 and 6 hour in samples treated with 10 ng/mL of obinutuzumab (white and dark gray bars) or rituximab (light gray and black bars). (E) Percent cytotoxicity of WIL2-S targets mediated by NK cells treated with increasing concentration of obinutuzumab (square, solid line) or rituximab (circle, dashed line). Asterisks represent statistical significance based on two-tailed unpaired Student’s t test (*, p < 0.05; **, p < 0.005). Bars represent the SEM of at least 3 samples per condition. Figures are representative of at least 3 independent experiments and donors.

Figure 6. Afucosylated antibodies lead to more frequent multiple killing events and decreased time required for target cell lysis. (A, C, E) Movies were interrogated for NK cells that could kill 2 or more targets. (A) Still images of a NK cell treated with 10 ng/mL afucosylated (top) or fucosylated (bottom) trastuzumab killing multiple targets. Number denotes targets lysed. (C) The percent of total NK cells performing multiple killing events was calculated for afucosylated (square) and fucosylated (circle) treated NK cells. (E) Percent of obinutuzumab (square) and rituximab (circle) stimulated NK cells performing killing multiple events. (B, D, F) Movies were interrogated for NK cells in which the whole cytotoxic process was visualized to determine time required for cytotoxicity. (B) Still images of a NK cell treated with 10 ng/mL afucosylated (top) or fucosylated (bottom) trastuzumab killing a single target as a function of time. E denotes NK cell, while T denotes target being lysed. (D) The time required to perform cytotoxicity
between 10 ng/mL afucosylated (square) and fucosylated (circle) treated NK cells. (F) Duration required to perform cytotoxicity between 1 ng/mL obinutuzumab (square) and rituximab (circle) treated NK cells. Bars represent the SEM. p-values were calculated based on two-tailed unpaired Student’s t test in panels C-F. Figures are representative of at least 3 independent experiments and donors. Solid white bar in right bottom corner of first images in panel A and B denote 15 μm scale bar.
Figure 1

A. Afucosylated Fucosylated Secondary only

IB: anti phospho-tyrosine

IB: anti actin

B. Obinutuzumab Rituximab

IB: anti phospho-tyrosine

IB: anti actin

C. Densitometry (pp85/actin)

D. Densitometry (pp60/actin)
Figure 2

A. 

<table>
<thead>
<tr>
<th></th>
<th>Afucosylated</th>
<th>Fucosylated</th>
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<tbody>
<tr>
<td>min:</td>
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<td>0 2</td>
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</tbody>
</table>

IB: anti phospho-lck
IB: anti actin

B. 

IB: anti phospho-CD3ζ
IB: anti actin

C. 

<table>
<thead>
<tr>
<th></th>
<th>Afucosylated</th>
<th>Fucosylated</th>
<th>Secondary only</th>
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</thead>
<tbody>
<tr>
<td>min:</td>
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<td>0 1 2 5 10</td>
<td>0 1 2 5 10</td>
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</table>

IB: anti phospho-Zap70
IB: anti actin

D. 

<table>
<thead>
<tr>
<th></th>
<th>Obinutuzumab</th>
<th>Rituximab</th>
</tr>
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<tbody>
<tr>
<td>min:</td>
<td>0 1 2 5 10</td>
<td>0 1 2 5 10</td>
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</table>

IB: anti phospho-CD3ζ
IB: anti actin
Figure 3

A. Afucosylated | Fucosylated | Secondary only
  min: 0 1 2 5 10 0 1 2 5 10 0 1 2 5 10

IB: anti phospho-Vav-1
IB: anti actin

B. Afucosylated | Fucosylated | Secondary only
  min: 0 1 2 5 10 0 1 2 5 10 0 1 2 5 10

IB: anti phospho-Akt
IB: anti actin

C. pERK1 ▶️ pERK2 ▶️
IB: anti phospho-Erk1/2
IB: anti actin

D.

Percent phospho-ERK1/2+ (%)

Time (min)

E.

MFI ratio (stim/t=0)

Time (min)

F. Obinutuzumab | Rituximab
  min: 0 1 2 5 10 0 1 2 5 10

IB: anti phospho-Akt
IB: anti actin

G. Obinutuzumab | Rituximab
  min: 0 1 2 5 10 0 1 2 5 10

pERK1 ▶️ pERK2 ▶️
IB: anti phospho-Erk1/2
IB: anti actin
Figure 4

A. Afucosylated and Fucosylated F-actin

B. MFI ratio (stim/0time): Afucosylated, Fucosylated, Secondary only

C. Percent CD107a+ (%): Afucosylated, Fucosylated, Secondary only

D. Percent CD107a+ (%): Obinutuzumab, Rituximab, Secondary only

E. MFI ratio (trastuzumab/secondary): Afucosylated, Fucosylated

F. MFI ratio (obi or rit/secondary): Obinutuzumab, Rituximab
Afucosylated antibodies increase activation of FcγRIIIa-dependent signaling components to intensify processes promoting ADCC

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