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Amino acid residues involved in the heparin-binding activity of murine IL-12 in the context of an antibody-cytokine fusion protein

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Abstract

An antibody-cytokine fusion protein, composed of the murine single-chain cytokine interleukin-12 (IL-12) genetically fused to a human IgG3 specific for the human tumor-associated antigen HER2/*neu* maintains antigen binding, cytokine, bioactivity, and IL-12 heparin-binding activity. This latter property is responsible for the binding of the cytokine to glycosaminoglycans (GAGs) on the cell surface and the extracellular matrix and has been implicated in modulating IL-12 bioactivity. Previous studies indicate that the p40 subunit of human and murine IL-12 is responsible for the heparin-binding activity of this heterodimeric cytokine. In the present study we used bioinformatic analysis and site-directed mutagenesis to develop a version of the antibody-(IL-12) fusion protein without heparin-binding activity. This was accomplished by replacing the basic arginine (R) and lysine (K) residues in the cluster of amino acids 254–260 (RKKEKMK) of the murine IL-12 p40 subunit by the neutral non-polar amino acid alanine (A), generating an

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AAAEAMA mutant fusion protein. ELISA and flow cytometry demonstrated that the antibody fusion protein lacks heparin-binding activity but retains antigen binding. A T-cell proliferation assay showed IL-12 bioactivity in this construct. However, the IL-12 bioactivity is decreased compared to its non-mutated counterpart, which is consistent with an ancillary role of the heparin-binding site of IL-12 in modulating its activity. Thus, we have defined a cluster of amino acid residues with a crucial role in the heparin-binding activity of murine IL-12 in the context of an antibody-cytokine fusion protein.

Keywords

heparin; glycosaminoglycans; cytokine; interleukin-12; antibody fusion protein; HER2/*neu*

1. Introduction

Interleukin 12 (IL-12) is a glycoprotein with a heterodimeric structure comprised of two disulfide linked subunits of approximately 40 (p40) and 35 (p35) kDa [1–5]. Produced by antigen presenting cells such as macrophages and dendritic cells, IL-12 demonstrates pro-inflammatory and immunoregulatory properties [5], playing an important role in the elimination phase during cancer immunoediting [6]. IL-12 also acts to link the innate and adaptive immune responses by inducing the production of IFN-γ and favoring the differentiation of T_H1 cells [5]. As a potentiator of immune effector functions, IL-12 enhances the cytotoxicity of NK cells and CTL [7, 8]. The resultant increase in the production of IFN-γ by T and NK cells up-regulates MHC class I expression [9, 10], activates macrophages [11, 12], and inhibits angiogenesis by inducing the expression of MIG and IP-10 [13, 14]. Due to the aforementioned properties, the use of IL-12 has been considered an attractive strategy for cancer immunotherapy [15–18]. In fact, systemic injection of IL-12 has facilitated anti-tumor responses in patients with metastatic renal cell cancer or melanoma; however, this strategy has also been associated with severe toxicities that in some cases were fatal [17–22]. Therefore, although IL-12 has great potential for the treatment of cancer, its use has been greatly restricted by its toxicity profile.

Antibody-cytokine fusion proteins have been designed to overcome the limitations associated with IL-12 toxicity by targeting IL-12 to the tumor microenvironment via an antibody [23–25]. The rationale of this approach is to target sufficient quantities of the cytokine to the tumor site, thereby enhancing the anti-tumor activity of both the antibody and the cytokine and eliciting a secondary tumor-specific immune response. We have previously developed an antibody-cytokine fusion protein, composed of the murine single-chain cytokine IL-12 genetically fused to a human IgG3 specific for the human tumor-associated antigen HER2/*neu* [24], which is overexpressed in a subset of breast and ovarian cancers and is associated with poor prognosis [26, 27]. We strategically chose murine IL-12 as a fusion partner since it is biologically active on both murine and human cells, while human IL-12 is not active on murine cells [4, 28]. This antibody fusion protein [anti-HER2/*neu* IgG3-(IL-12)] binds its target antigen, retains cytokine bioactivity, and has proven to be effective against tumors expressing HER2/*neu*, both as a direct anti-tumor agent and as an adjuvant of HER2/*neu* protein vaccination in murine models [24, 29–31].

The murine IL-12 moiety of the antibody-cytokine fusion protein also retains its heparin-binding activity that is responsible for the binding of the cytokine to GAGs [31].

GAGs are linear, negatively charged polysaccharides present on the surface of most animal cells and in the extracellular matrix. They can be non-sulfated, as is hyaluronic acid, or sulfated as are chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin [32–34]. A variety of growth factors, cytokines, and chemokines bind GAGs. This phenomenon is described in general as heparin-binding activity, which can modulate its biological function in different ways depending on the cytokine [32–34]. Binding to GAGs can promote cytokine activity, as has been demonstrated for GM-CSF [35], IL-3 [35], IL-5 [36], and IL-8 [37], or can inhibit cytokine activity, as with IL-7 [38], IFN-γ [39], and IL-10 [40]. GAG binding can also protect the cytokine from proteolytic degradation, as has been shown for basic and acidic FGF [41], IL-6 [42], IL-7 [38], IFN-γ [43]; and/or can facilitate the local accumulation of the cytokine as has been described for IL-2 [44] and IL-6 [42]. The possibility that IL-12 has heparin-binding activity was first proposed by Wolf *et al.* [3], and this activity was found by Hasan *et al.* [45], reporting high affinity binding of IL-12 to heparin/heparan sulfate. This latter group and more recently, Jayanthi *et al.* and Garnier *et al.* suggested that the heparin-binding activity of human and mouse IL-12 involves a cluster of basic amino acids within the C-terminal domain of the p40 subunit of human and mouse IL-12 [45–47].

In the present study we apply structural analysis and site-directed mutagenesis to selectively mutate a cluster of charged residues in the p40 subunit of a single-chain murine IL-12 fused to anti-HER2/*neu* IgG3. This strategy resulted in the development of an antibody fusion protein that lacks heparin-binding activity but binds antigen and exhibits IL-12 bioactivity, although this activity is decreased.

2. Materials and Methods

2.1. Identification of a GAG binding site on murine IL-12

To identify residues in murine IL-12 responsible for GAG binding, we performed a bioinformatic search using the consensus sequences -XBBBXXBX- and -XBBXBX- reported by Cardin and Weintraub [48], within the murine p40 subunit. Based on the structure of the p40 subunit of human IL-23 (PDB ID 3DUH), that is shared with IL-12 [49], we used the SWISS-MODEL suite to generate a homology model of the murine p40 subunit. Surface charges were assessed on the modelled murine p40 subunit using local protein contact potential with vacuum electrostatics as well as the Adaptive Poisson-Boltzmann Solver (APBS)[50], which were in good agreement. Relative potentials on the solvent accessible surface of each were rendered and displayed by the PyMol viewer (Schrödinger, LLC, Portland, OR).

2.2. Cell lines

The murine myeloma cell lines Sp2/0-Ag14 (ATCC® CRL-1581™) and P3X63Ag8.653 (ATCC® CRL-1580™) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and used to express the antibodies or antibody fusion proteins described

below. These cells were grown in IMDM (Life Technologies, Carlsbad, CA) and 5% heat-inactivated calf serum (Atlanta Biologicals, Norcross, GA). The mouse mammary tumor cell lines D2F2 and D2F2/E2 expressing human HER2/*neu* [51], kindly provided by Dr. Wei-Zen Wei (Wayne State University, Detroit, MI, USA), were cultured in IMDM and 10% heat-inactivated FBS. The murine colon carcinoma cell lines CT26 and CT26-HER2/*neu* expressing human HER2/*neu* [31, 52] were both cultured in IMDM and 10% heat-inactivated FBS. Both D2F2/E2 and CT26- HER2/*neu* were also cultured in the presence of 1mg/mL of G418 (Gemini Bio-Products, Sacramento, CA). All cell lines were cultured in media supplemented with penicillin and streptomycin (Thermo Fisher Scientific, Canoga Park, CA) in 5% CO_2 at 37°C.

2.3. Generation of antibodies and antibody fusion proteins

The isotype control (anti-DNS IgG3) specific for the hapten dansyl [DNS (5-dimethylamino naphthalene-1-sulfonyl chloride)] was reported previously [24, 53]. The construction and expression of anti-HER2/*neu* IgG3-(IL-12) (also described as mscIL-12.her2.IgG3) and the parental antibody without IL-12 (anti-HER2/*neu* IgG3), have been previously reported [24, 29–31]. The anti-HER2/*neu* IgG3-(IL-12) and anti-HER2/*neu* IgG3 are composed of the heavy and light chain variable regions of the humanized antibody 4D5–8 (also known as Herceptin® or trastuzumab) specific for human HER2/*neu*, human κ light chain, and human γ3 heavy chain constant regions. The anti-HER2/*neu* IgG3-(IL-12) has the murine single-chain IL-12 p40 and p35 subunits genetically fused via a flexible linker at the N-terminus of the heavy chain (Fig. 1, Panel A) [24]. The single-chain IL-12 was fused to the N-terminus of the heavy chain in order to preserve the cytokine activity [24]. Murine IL-12 was used because it has activity on both human and murine cells, while human IL-12 has activity only on human cells [4, 28]. Thus, the use of murine IL-12 makes it possible not only to carry out assays using human PBMCs to test biologic activity as described below, but also to perform *in vivo* studies in mice.

To develop the mutant of anti-HER2/*neu* IgG3-(IL-12), the heavy chain expression vector of anti-HER2/*neu* IgG3-(IL-12) was used for site-directed mutagenesis (Retrogen, Inc., San Diego, CA) to generate a mutant fusion protein anti-HER2/*neu* IgG3-(mutIL-12) with modifications in the potential heparin-binding site of IL-12 (Fig. 1, Panel A). To achieve this goal, the murine IL-12 DNA sequence 5'-GCAAGAAAGAAAAGATGAAG - 3' encoding the amino acids RKKEKMK was replaced by the DNA sequence 5'-CCGCGGCAGAAGCGATGGCG - 3' encoding the amino acids AAAEAMA. The mutated heavy chain expression vector was transfected into murine myeloma cells together with the plasmid encoding the anti-HER2/*neu* κ light chain. The procedures of transfection, expression, and purification of the generated antibodies and antibody fusion proteins have been previously described [24, 29, 54].

2.4. Flow cytometry for antigen and GAG binding

One million D2F2/E2 or CT26-HER2/*neu* murine cancer cells or the parental cells not expressing the antigen were incubated at 4°C for 4 h in the presence or absence of 5.5 μg/ml (approximately 1 USP units/ml) of heparin from porcine intestinal mucosa Grade I-A (Sigma-Aldrich, St. Louis, MO) with 4 μg of anti-HER2/*neu* IgG3-(IL-12), anti-HER2/*neu*

IgG3-(mutIL-12), or the equivalent molar amount of anti-HER2/*neu* IgG3 or anti-DNS IgG3 (isotype control), followed by a 1 h of incubation with rabbit anti-human κ antibody conjugated to FITC or PE (BD Biosciences, San Jose, CA). Ten thousand events were recorded for each sample using a FACScan flow cytometer (BD Biosciences, San Jose, CA) in the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility. Histograms were created using the WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA).

2.5. ELISA for heparin and hyaluronic acid binding

Heparin sodium salt from porcine intestinal mucosa Grade I-A (Sigma-Aldrich) or hyaluronic acid sodium salt from bovine vitreous humor (Sigma-Aldrich) were diluted in PBS and added at a concentration of 25 μg/ml on BD heparin-binding plates **(**BD Biosciences, San Jose, CA) and incubated overnight at room temperature [55]. Plates coated with heparin or hyaluronic acid were washed with PBS and blocked with 3% BSA in PBS blocking solution for 1 h at 37°C, followed by washing with PBS. The antibody-cytokine fusion proteins or equivalent molar amount of control IgG3 were incubated in triplicate using serial four-fold dilutions ranging from 0.06 μg/mL to 4 μg/mL for 2 h at 37°C. For the competition study, wild type IgG3-(IL-12) was also incubated in triplicates in the presence of 5.5 μg/ml of heparin or equivalent molar amount of hyaluronic acid. The plates were then washed and incubated with a goat anti-human κ AP-conjugated antibody (Sigma-Aldrich) 1:30,000 in 1% BSA in PBS for 1 h at 37°C. After washing, 1 mg/ml of AP substrate (*p*-nitrophenyl phosphate disodium, Sigma-Aldrich) dissolved in diethanolamine buffer [9.6% diethanolamine (v/v), 0.24 mM $MgCl_2$, in water (pH 9.8)] was added and incubated for 60 min at room temperature before measuring the absorption at 405 nm using a FilterMax F5 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA).

2.6. IL-12 bioactivity assay

The bioactivity of IL-12 was determined by an activated T-cell proliferation assay using human PBMCs as previously described [29] with slight modifications. Briefly, human PBMCs from healthy donors (obtained from the UCLA CFAR Virology Core Lab) were incubated with 25 μg/mL of PHA-P (Sigma-Aldrich) and 10 units/ml of recombinant human IL-2 (Sigma-Aldrich) and cultured in RPMI 1640 (Life Technologies) and 10% heat-inactivated FBS (Atlanta Biologicals) for 3 days. After washing with RPMI 1640, cells were incubated in 96-well plates (10^5 cells/well) for 2 days at 37°C, 5% CO_2 in the presence of an equivalent molar amount of anti-HER2/*neu* IgG3, anti-HER2/*neu* IgG3-(IL-12), or anti-HER2/*neu* IgG3-(mutIL-12) serially diluted 4-fold over a range from 0.0625 ng/mL to 16 ng/mL. Proliferation was measured by [^{3}H]-thymidine (MP Biomedicals, Solon, OH) incorporation assay after 12 h incubation at 37°C in 5% CO2. All measurements were conducted in quintuplicate. Two-way ANOVA statistical analysis was used to determine significant ($p < 0.05$) differences.

3. Results

3.1. The p40 subunit of murine IL-12 contains a putative heparin-binding site.

The heparin-binding properties of murine and human IL-12 were originally reported by Hasan *et al.* [45] who observed that the p40 subunit presented indistinguishable heparin-binding properties compared to the full p70 IL-12, suggesting that the heparin-binding site is located in the p40 subunit. The authors also suggested that the IL-12 heparin-binding site was located close to the C-terminus of the p40 subunit because this region contains a cluster of 6 basic amino acid residues in human and murine IL-12 sequences. Groups of basic residues are found to be at least partially responsible for the heparin-binding properties of IFN-γ [56], IL-8 [37], and other CXC chemokine family members [57]. Probing of the interaction between heparin and IL-8 by NMR has revealed complex interactions between free heparin and several basic residues on IL-8 [58]. Given these observations, we focused on the murine IL-12 p40 subunit and identified amino acid residues potentially involved in this activity in the C-terminal domain (Fig. 1, Panel A). Our predictions were based on 2 criteria: first, sequence conservation using the consensus sequences reported by Cardin and Weintraub (XBBBXXBX and XBBXBX, where B represents basic residues) [48], which have been previously used for the identification of GAG-binding sites even in the absence of a 3D protein structure; second, the distribution of surface charge on the human/murine p40 subunit, which shared between IL-12 and IL-23, and whose structure has been determined crystallographically [59]. The region encoding amino acids 250–262 (VRIQRKKEKMKET) described by Hassan *et al.* [45] contains a cluster of 4 basic amino acid residues: RKKEKM, which is consistent with the consensus site XBBXBX. In addition, this positively charged cluster represents a basic patch on the solvent accessible surface on an exposed loop between beta-strands of the c-terminal domain of the p40 subunit of murine IL-12 (Fig. 1, Panel B). For the above reasons, we identified the residues R254, K255, K256, K258, K260 as members of a cluster of basic amino acids potentially contributing to binding to GAGs exhibited by the p40 subunit of murine IL-12.

3.2. Anti-HER2/neu IgG3-(mutIL-12) retains antigen-binding but did not show heparin-binding activity.

To assess the role of the cluster of basic residues in the murine p40 subunit, we evaluated the binding of the wild type or the mutant of anti-HER2/*neu* IgG3-(IL-12) to cell surface GAGs. We carried out site-directed mutagenesis of the p40 subunit of anti-HER2/*neu* IgG3-(IL-12) to replace basic amino acid residues in the cluster spanning residues 254 to 260, substituting each basic residue in this region with the neutral, non-polar amino acid alanine (A) (Fig. 1, Panel A). This strategy has been used successfully to identify charged amino acid residues responsible for of IL-8 [60] and PF4[61]. The new antibody fusion protein named anti-HER2/*neu* IgG3-(mutIL-12), expressed in murine myeloma cells, is properly assembled and secreted and exhibits the expected molecular weight (data not shown).

We evaluated the ability of anti-HER2/*neu* IgG3-(mutIL-12) to bind human HER2/*neu* expressed on the surface of the murine mammary tumor cell line expressing human HER2/*neu* (D2F2/E2) by flow cytometry compared to the parental D2F2 cell line that does not express the antigen (Fig. 2). As expected, the anti-HER2/*neu* IgG3-(IL-12) shows

superior binding to D2F2/E2 cells compared to D2F2 parental cells not expressing the antigen. The parental antibody without IL-12 (anti-HER2/*neu* IgG3), does not bind D2F2 cells since they do not express the antigen. Thus, the binding of the antibody fusion protein to these cells, detected under these experimental conditions, is through the IL-12 moiety and is consistent with its heparin-binding activity. In fact, this binding is prevented in the presence of free heparin, matching the basal binding level observed with anti-HER2/*neu* IgG3 and suggesting that the wild type antibody fusion protein binds to cells not expressing HER2/*neu* through cell-surface GAGs due to the heparin-binding activity of the IL-12. The binding of anti-HER2/*neu* IgG3-(mutIL-12) to D2F2/E2 cells is less than that of the fusion protein bearing the wild type murine p40 sequence (Fig. 2), which can be explained by its lack of heparin-binding activity. In fact, anti-HER2/*neu* IgG3-(mutIL-12) does not bind to D2F2 cells and showed D2F2/E2 binding equivalent to that of anti-HER2/*neu* IgG3. These results were replicated in another murine carcinoma cell line, CT26-HER2/*neu*, expressing human HER2/*neu* and its parental cell line CT26 (Supplementary Fig. 1). Taken together, these results confirm that anti-HER2/*neu* IgG3-(mutIL-12) retains antigen binding and indicate disruption of its heparin-binding activity.

We confirmed the lack of heparin-binding activity of anti-HER2/*neu* IgG3-(mutIL-12) by ELISA. Fig. 3A shows that while anti-HER2/*neu* IgG3-(IL-12) binds to heparin immobilized on a solid surface in a dose-dependent manner, no binding is detected using anti-HER2/*neu* IgG3-(mutIL-12). We also found that the incubation of anti-HER2/*neu* IgG3-(IL-12) in the presence of free heparin abrogates its ability to bind to heparin coated plates (Fig. 3A), which is consistent with our observations using flow cytometry (Fig. 2). We also found that neither anti-HER2/*neu* IgG3-(IL-12) or anti-HER2/*neu* IgG3-(mutIL-12) bind to hyaluronic acid coated plates (data not shown). Furthermore, free hyaluronic acid does not block the binding of anti-HER2/*neu* IgG3-(IL-12) to heparin coated plates (Fig. 3B). This observation is consistent with the reported lack of binding of human IL-12 to hyaluronic acid [62]. Taken together, these results demonstrate that the cluster of basic amino acid residues 254–260 (RKKEKMK) at the terminal end of murine p40 is crucial for the heparin binding of anti-HER2/*neu* IgG3-(IL-12).

3.3. Anti-HER2/neu IgG3-(mutIL-12) shows decreased IL-12 bioactivity.

We assessed the effect of mutating a basic amino acid cluster in murine p40 subunit on the biological activity of the antibody-cytokine fusion protein by evaluating its ability to stimulate the proliferation of activated human T-cells in PBMCs (Fig. 4). This study is possible because murine IL-12 is active in human cells [4, 28]. Anti-HER2/*neu* IgG3-(mutIL-12) exhibits significant IL-12 bioactivity in a dose dependent manner (Fig. 4), although this activity is decreased compared to that of wild type anti-HER2/*neu* IgG3-(IL-12).

4. Discussion

The interaction between cytokines and GAGs has been documented to modulate their biological activity with physiological importance [33, 34]. It has been postulated that the GAG-binding properties of IL-12 retain this cytokine at sites of secretion establishing local

high concentrations, favoring its paracrine role [45], as has been described for IL-2 [44] and IL-6 [42]. However, the amino acid residues involved in the binding IL-12 to GAGs have not been yet elucidated. We modelled the structure of the p40 subunit of murine IL-12, relying on the on the known structure of human p40 and performed site-directed mutagenesis of a single chain IL-12 construct genetically fused to an anti-HER2/*neu* IgG3. We replaced the basic arginine (R) and lysine (K) residues in the cluster of amino acids 254–260 (RKKEKMK) of the murine IL-12 p40 subunit by the neutral non-polar amino acid alanine (A), generating an AAAEAMA mutant fusion protein.

Given the strongly acidic nature of GAGs, such as heparan sulfate and heparin, the basic amino acids arginine (R) and lysine (K) are expected to be major determinants in the heparin-binding activity of cytokines such as IL-12. A homology model generated from the human p40 subunit predicts that the cluster of basic amino acid residues that we mutated is solvent exposed and generates a basic patch on the surface of both mouse and human p40. This cluster exposes predominantly basic residues on the surface of both human and murine p40. Our selective replacement of lysine (K) and arginine (R) in this cluster with the neutral non-polar residue alanine (A) in murine p40 then changes the overall charge of the cluster. Such a charge change has profound effects: the cytokine fusion protein bearing 5 alanine (A) mutations in p40 (AAAEAMA) shows complete disruption of heparin-binding activity compared to its wild type counterpart. Our finding is consistent with previous studies suggesting that the heparin-binding activity of human and mouse IL-12 involves a cluster of basic amino acids within the C-terminal domain of the p40 subunit of human and mouse IL-12 [45–47]. Importantly, binding to GAGs exhibited by the wild type antibody-(IL-12) fusion protein detected by flow cytometry and ELISA was confirmed by the lack of signal in the presence of free heparin as a competitor. These results are consistent with previous studies in which soluble heparin inhibited binding of IL-12 to an immobilized heparin-BSA complex by ELISA [45]. However, both mutated and wild type antibody-(IL-12) fusion proteins show similar binding to antigen (HER2/*neu*) on the cell surface as demonstrated by flow cytometry. Therefore, the conformational change induced by the mutations of IL-12, fused to the N-terminus of the antibody heavy chain, did not result in steric hindrance that affected antigen binding.

The bioactivity assay shows that anti-HER2/*neu* IgG3-(mutIL-12) is capable of inducing T-cell proliferation despite its lack of heparin-binding activity. These results are consistent with previous studies postulating that the p35 subunit of mouse or human IL-12 is principally responsible for IL-12 receptor binding and signaling [4, 63, 64] and that its heparin-binding region, located on its p40 subunit, does not play a major role in mediating the interaction between IL-12 and its receptor [45]. Nonetheless, studies conducted by Garnier *et al.* have shown that the GAG chondroitin sulfate B is important for the activity of murine IL-12 [65]. However, the role of chondroitin sulfate B may be accessory in facilitating IL-12 signaling since its removal from the cell surface does not completely block the activity of IL-12 [65]. Our results support the possibility that binding of IL-12 to GAGs plans an ancillary role in the cytokine bioactivity since blocking the heparin-binding activity of the IL-12 moiety reduces but does not completely block its ability to stimulate T-cell proliferation. Further studies are needed to better define the properties of the mutated murine IL-12 in the context of the antibody fusion protein.

5. Conclusions

In the present study we selectively mutated a cluster of amino acid residues in the p40 subunit of a single chain murine IL-12 fused to an anti-HER2/*neu* IgG3 antibody, which resulted in a new antibody fusion protein without heparin-binding activity that retains antigen binding and cytokine bioactivity, although the latter is reduced. These studies identified key amino acid residues responsible for the heparin binding activity of murine IL-12 and are consistent with an ancillary role of GAG binding in the cytokine bioactivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AP	alkaline phosphatase
APBS	Adaptive Poisson-Boltzmann Solver
ATCC	American Type Culture Collection
FGF	fibroblast growth factor
BSA	bovine serum albumin
cpm	counts per minute
CTL	cytotoxic T cells
DMEM	Dulbecco's Modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate, HER2/*neu*, epidermal growth factor receptor 2 (HER2)/neuroblastoma-associated gene (*neu*)
GM-CSF	granulocyte-macrophage colony-stimulating factor
GAGs	glycosaminoglycans

IL	interleukin
IFN	interferon
IP-10	interferon-inducible protein 10
IMDM	Iscove's modified Dulbecco's medium
MHC	major histocompatibility complex
MIG	monokine induced by interferon gamma
NK	natural killer
NMR	nuclear magnetic resonance
OD	optimal density
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PF4	platelet factor 4
PHA	phytohemagglutinin
RPMI	Roswell Park Memorial Institute (tissue culture medium)
SD	standard deviation
USP	United States Pharmacopeia

References

[1]. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G, Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes, J Exp Med 170(3) (1989) 827–45. [PubMed: 2504877]

[2]. Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R, Nabavi N, Wolitzky AG, Quinn PM, Familletti PC, et al., Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor, Proc Natl Acad Sci U S A 88(10) (1991) 4143–7. [PubMed: 1674604]

[3]. Wolf SF, Temple PA, Kobayashi M, Young D, Dicig M, Lowe L, Dzialo R, Fitz L, Ferenz C, Hewick RM, et al., Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells, J Immunol 146(9) (1991) 3074–81. [PubMed: 1673147]

[4]. Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK, Gubler U, Cloning and expression of murine IL-12, J Immunol 148(11) (1992) 3433–40. [PubMed: 1350290]

[5]. Trinchieri G, Interleukin-12 and the regulation of innate resistance and adaptive immunity, Nat Rev Immunol 3(2) (2003) 133–46. [PubMed: 12563297]

[6]. Mittal D, Gubin MM, Schreiber RD, Smyth MJ, New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape, Curr Opin Immunol 27 (2014) 16–25. [PubMed: 24531241]

[7]. Gately MK, Warrier RR, Honasoge S, Carvajal DM, Faherty DA, Connaughton SE, Anderson TD, Sarmiento U, Hubbard BR, Murphy M, Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-gamma in vivo, Int Immunol 6(1) (1994) 157–67. [PubMed: 7908534]

[8]. Parihar R, Dierksheide J, Hu Y, Carson WE, IL-12 enhances the natural killer cell cytokine response to Ab-coated tumor cells, J Clin Invest 110(7) (2002) 983–92. [PubMed: 12370276]

[9]. Wong GH, Clark-Lewis I, Harris AW, Schrader JW, Effect of cloned interferon-gamma on expression of H-2 and Ia antigens on cell lines of hemopoietic, lymphoid, epithelial, fibroblastic and neuronal origin, Eur J Immunol 14(1) (1984) 52–6. [PubMed: 6420170]

[10]. Zhou F, Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation, Int Rev Immunol 28(3–4) (2009) 239–60. [PubMed: 19811323]

[11]. Douvas GS, Looker DL, Vatter AE, Crowle AJ, Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage- associated mycobacteria, Infect Immun 50(1) (1985) 1–8. [PubMed: 3930401]

[12]. Wu C, Xue Y, Wang P, Lin L, Liu Q, Li N, Xu J, Cao X, IFN-gamma primes macrophage activation by increasing phosphatase and tensin homolog via downregulation of miR-3473b, J Immunol 193(6) (2014) 3036–44. [PubMed: 25092892]

[13]. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J, Inhibition of angiogenesis in vivo by interleukin 12, J Natl Cancer Inst 87(8) (1995) 581–6. [PubMed: 7538593]

[14]. Teruya-Feldstein J, Jaffe ES, Burd PR, Kanegane H, Kingma DW, Wilson WH, Longo DL, Tosato G, The role of Mig, the monokine induced by interferon-gamma, and IP-10, the interferon-gamma-inducible protein-10, in tissue necrosis and vascular damage associated with Epstein-Barr virus-positive lymphoproliferative disease, Blood 90(10) (1997) 4099–105. [PubMed: 9354680]

[15]. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK, Antitumor and antimetastatic activity of interleukin 12 against murine tumors, J Exp Med 178(4) (1993) 1223–30. [PubMed: 8104230]

[16]. Colombo MP, Trinchieri G, Interleukin-12 in anti-tumor immunity and immunotherapy, Cytokine Growth Factor Rev 13(2) (2002) 155–68. [PubMed: 11900991]

[17]. Conlon KC, Miljkovic MD, Waldmann TA, Cytokines in the Treatment of Cancer, J Interferon Cytokine Res 39(1) (2019) 6–21. [PubMed: 29889594]

[18]. Floros T, Tarhini AA, Anticancer Cytokines: Biology and Clinical Effects of Interferon-alpha2, Interleukin (IL)-2, IL-15, IL-21, and IL-12, Semin Oncol 42(4) (2015) 539–48. [PubMed: 26320059]

[19]. Car BD, Eng VM, Lipman JM, Anderson TD, The toxicology of interleukin-12: a review, Toxicol Pathol 27(1) (1999) 58–63. [PubMed: 10367675]

[20]. Cohen J, IL-12 deaths: explanation and a puzzle, Science 270(5238) (1995) 908. [PubMed: 7481785]

[21]. Gollob JA, Mier JW, Veenstra K, McDermott DF, Clancy D, Clancy M, Atkins MB, Phase I trial of twice-weekly intravenous interleukin 12 in patients with metastatic renal cell cancer or malignant melanoma: ability to maintain IFN-gamma induction is associated with clinical response, Clin Cancer Res 6(5) (2000) 1678–92. [PubMed: 10815886]

[22]. Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, Sosman JA, Dutcher JP, Vogelzang NJ, Ryan JL, Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production, Blood 90(7) (1997) 2541–8. [PubMed: 9326219]

[23]. Ortiz-Sanchez E, Helguera G, Daniels TR, Penichet ML, Antibody-cytokine fusion proteins: applications in cancer therapy, Expert Opin Biol Ther 8(5) (2008) 609–32. [PubMed: 18407765]

[24]. Peng LS, Penichet ML, Morrison SL, A single-chain IL-12 IgG3 antibody fusion protein retains antibody specificity and IL-12 bioactivity and demonstrates antitumor activity, J Immunol 163(1) (1999) 250–8. [PubMed: 10384123]

[25]. Payés CJ, Daniels-Wells TR, Maffía PC, Penichet ML, Morrison SL, Helguera G, Genetic Engineering of Antibody Molecules, in: Meyers RA (Ed.), Reviews in Cell Biology and

Molecular Medicine, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2015, pp. 1–52.

[26]. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al., Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer, Science 244(4905) (1989) 707–12. [PubMed: 2470152]

[27]. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL, Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, Science 235(4785) (1987) 177–82. [PubMed: 3798106]

[28]. Gately MK, Chizzonite R, Presky DH, Measurement of human and mouse interleukin-12, Curr Protoc Immunol Chapter 6 (2001) Unit 6.16.1–15.

[29]. Helguera G, Dela Cruz JS, Lowe C, Ng PP, Trinh R, Morrison SL, Penichet ML, Vaccination with novel combinations of anti-HER2/neu cytokines fusion proteins and soluble protein antigen elicits a protective immune response against HER2/neu expressing tumors, Vaccine 24(3) (2006) 304–316. [PubMed: 16125282]

[30]. Dela Cruz JS, Lau SY, Ramirez EM, De Giovanni C, Forni G, Morrison SL, Penichet ML, Protein vaccination with the HER2/neu extracellular domain plus anti-HER2/neu antibody-cytokine fusion proteins induces a protective anti-HER2/neu immune response in mice, Vaccine 21(13–14) (2003) 1317–26. [PubMed: 12615426]

[31]. Helguera G, Rodriguez JA, Penichet ML, Cytokines fused to antibodies and their combinations as therapeutic agents against different peritoneal HER2/neu expressing tumors, Mol Cancer Ther 5(4) (2006) 1029–40. [PubMed: 16648575]

[32]. Gandhi NS, Mancera RL, The structure of glycosaminoglycans and their interactions with proteins, Chem Biol Drug Des 72(6) (2008) 455–82. [PubMed: 19090915]

[33]. Coombe DR, Biological implications of glycosaminoglycan interactions with haemopoietic cytokines, Immunol Cell Biol 86(7) (2008) 598–607. [PubMed: 18626488]

[34]. Mulloy B, Rider CC, Cytokines and proteoglycans: an introductory overview, Biochem Soc Trans 34(Pt 3) (2006) 409–13. [PubMed: 16709174]

[35]. Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM, Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis, Nature 332(6162) (1988) 376–8. [PubMed: 2965305]

[36]. Lipscombe RJ, Nakhoul AM, Sanderson CJ, Coombe DR, Interleukin-5 binds to heparin/heparan sulfate. A model for an interaction with extracellular matrix, J Leukoc Biol 63(3) (1998) 342–50. [PubMed: 9500522]

[37]. Webb LM, Ehrengruber MU, Clark-Lewis I, Baggiolini M, Rot A, Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8, Proc Natl Acad Sci U S A 90(15) (1993) 7158–62. [PubMed: 8346230]

[38]. Clarke D, Katoh O, Gibbs RV, Griffiths SD, Gordon MY, Interaction of interleukin 7 (IL-7) with glycosaminoglycans and its biological relevance, Cytokine 7(4) (1995) 325–30. [PubMed: 8589262]

[39]. Daubener W, Nockemann S, Gutsche M, Hadding U, Heparin inhibits the antiparasitic and immune modulatory effects of human recombinant interferon-gamma, Eur J Immunol 25(3) (1995) 688–92. [PubMed: 7705397]

[40]. Salek-Ardakani S, Arrand JR, Shaw D, Mackett M, Heparin and heparan sulfate bind interleukin-10 and modulate its activity, Blood 96(5) (2000) 1879–88. [PubMed: 10961890]

[41]. Gospodarowicz D, Cheng J, Heparin protects basic and acidic FGF from inactivation, J Cell Physiol 128(3) (1986) 475–84. [PubMed: 3528177]

[42]. Mummery RS, Rider CC, Characterization of the heparin-binding properties of IL-6, J Immunol 165(10) (2000) 5671–9. [PubMed: 11067924]

[43]. Lortat-Jacob H, Baltzer F, Grimaud JA, Heparin decreases the blood clearance of interferon-gamma and increases its activity by limiting the processing of its carboxyl-terminal sequence, J Biol Chem 271(27) (1996) 16139–43. [PubMed: 8663206]

[44]. Wrenshall LE, Platt JL, Stevens ET, Wight TN, Miller JD, Propagation and control of T cell responses by heparan sulfate-bound IL-2, J Immunol 170(11) (2003) 5470–4. [PubMed: 12759423]

[45]. Hasan M, Najjam S, Gordon MY, Gibbs RV, Rider CC, IL-12 is a heparin-binding cytokine, J Immunol 162(2) (1999) 1064–70. [PubMed: 9916734]

[46]. Garnier P, Mummery R, Forster MJ, Mulloy B, Gibbs RV, Rider CC, The localisation of the heparin binding sites of human and murine interleukin-12 within the carboxyterminal domain of the P40 subunit, Cytokine 110 (2018) 159–168. [PubMed: 29753267]

[47]. Jayanthi S, Koppolu B, Smith SG, Jalah R, Bear J, Rosati M, Pavlakis GN, Felber BK, Zaharoff DA, Kumar TK, Efficient production and purification of recombinant human interleukin-12 (IL-12) overexpressed in mammalian cells without affinity tag, Protein Expr Purif 102 (2014) 76–84. [PubMed: 25123642]

[48]. Cardin AD, Weintraub HJ, Molecular modeling of protein-glycosaminoglycan interactions, Arteriosclerosis 9(1) (1989) 21–32. [PubMed: 2463827]

[49]. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA, Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12, Immunity 13(5) (2000) 715–25. [PubMed: 11114383]

[50]. Jurrus E, Engel D, Star K, Monson K, Brandi J, Felberg LE, Brookes DH, Wilson L, Chen J, Liles K, Chun M, Li P, Gohara DW, Dolinsky T, Konecny R, Koes DR, Nielsen JE, Head-Gordon T, Geng W, Krasny R, Wei GW, Holst MJ, McCammon JA, Baker NA, Improvements to the APBS biomolecular solvation software suite, Protein Sci 27(1) (2018) 112–128. [PubMed: 28836357]

[51]. Wei WZ, Shi WP, Galy A, Lichlyter D, Hernandez S, Groner B, Heilbrun L, Jones RF, Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA, Int J Cancer 81(5) (1999) 748–54. [PubMed: 10328228]

[52]. Penichet ML, Challita PM, Shin SU, Sampogna SL, Rosenblatt JD, Morrison SL, In vivo properties of three human HER2/neu-expressing murine cell lines in immunocompetent mice, Lab Anim Sci 49(2) (1999) 179–88. [PubMed: 10331548]

[53]. Tan LK, Shopes RJ, Oi VT, Morrison SL, Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins, Proc Natl Acad Sci U S A 87(1) (1990) 162–6. [PubMed: 2296577]

[54]. Helguera G, Penichet ML, Antibody-cytokine fusion proteins for the therapy of cancer, Methods Mol Med 109 (2005) 347–74. [PubMed: 15585931]

[55]. Mahoney DJ, Whittle JD, Milner CM, Clark SJ, Mulloy B, Buttle DJ, Jones GC, Day AJ, Short RD, A method for the non-covalent immobilization of heparin to surfaces, Anal Biochem 330(1) (2004) 123–9. [PubMed: 15183770]

[56]. Lortat-Jacob H, Grimaud JA, Interferon-gamma binds to heparan sulfate by a cluster of amino acids located in the C-terminal part of the molecule, FEBS Lett 280(1) (1991) 152–4. [PubMed: 1901275]

[57]. Witt DP, Lander AD, Differential binding of chemokines to glycosaminoglycan subpopulations, Curr Biol 4(5) (1994) 394–400. [PubMed: 7922353]

[58]. Joseph PR, Mosier PD, Desai UR, Rajarathnam K, Solution NMR characterization of chemokine CXCL8/IL-8 monomer and dimer binding to glycosaminoglycans: structural plasticity mediates differential binding interactions, Biochem J 472(1) (2015) 121–33. [PubMed: 26371375]

[59]. Lupardus PJ, Garcia KC, The structure of interleukin-23 reveals the molecular basis of p40 subunit sharing with interleukin-12, J Mol Biol 382(4) (2008) 931–41. [PubMed: 18680750]

[60]. Kuschert GS, Hoogewerf AJ, Proudfoot AE, Chung CW, Cooke RM, Hubbard RE, Wells TN, Sanderson PN, Identification of a glycosaminoglycan binding surface on human interleukin-8, Biochemistry 37(32) (1998) 11193–201.

[61]. Mayo KH, Ilyina E, Roongta V, Dundas M, Joseph J, Lai CK, Maione T, Daly TJ, Heparin binding to platelet factor-4. An NMR and site-directed mutagenesis study: arginine residues are crucial for binding, Biochem J 312 (Pt 2) (1995) 357–65. [PubMed: 8526843]

[62]. Jayanthi S, Koppolu BP, Nguyen KG, Smith SG, Felber BK, Kumar TKS, Zaharoff DA, Modulation of Interleukin-12 activity in the presence of heparin, Sci Rep 7(1) (2017) 5360. [PubMed: 28706183]

[63]. Ling P, Gately MK, Gubler U, Stern AS, Lin P, Hollfelder K, Su C, Pan YC, Hakimi J, Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity, J Immunol 154(1) (1995) 116–27. [PubMed: 7527811]

[64]. Zou JJ, Schoenhaut DS, Carvajal DM, Warrier RR, Presky DH, Gately MK, Gubler U, Structure-function analysis of the p35 subunit of mouse interleukin 12, J Biol Chem 270(11) (1995) 5864–71. [PubMed: 7890716]

[65]. Garnier P, Gibbs RV, Rider CC, A role for chondroitin sulphate B in the activity of interleukin 12 in stimulating gamma-interferon secretion, Immunol Lett 85(1) (2003) 53–8. [PubMed: 12505197]

Highlights

We generated an antibody-(murine IL-12) fusion protein lacking heparin-binding activity.

We mutated the RKKEKMK amino acid cluster of IL-12 p40 to AAAEAMA to eliminate heparin binding.

The mutated IL-12 fused to the antibody shows cytokine bioactivity although it is reduced.

The mutated antibody-(IL-12) fusion protein retains antigen binding.

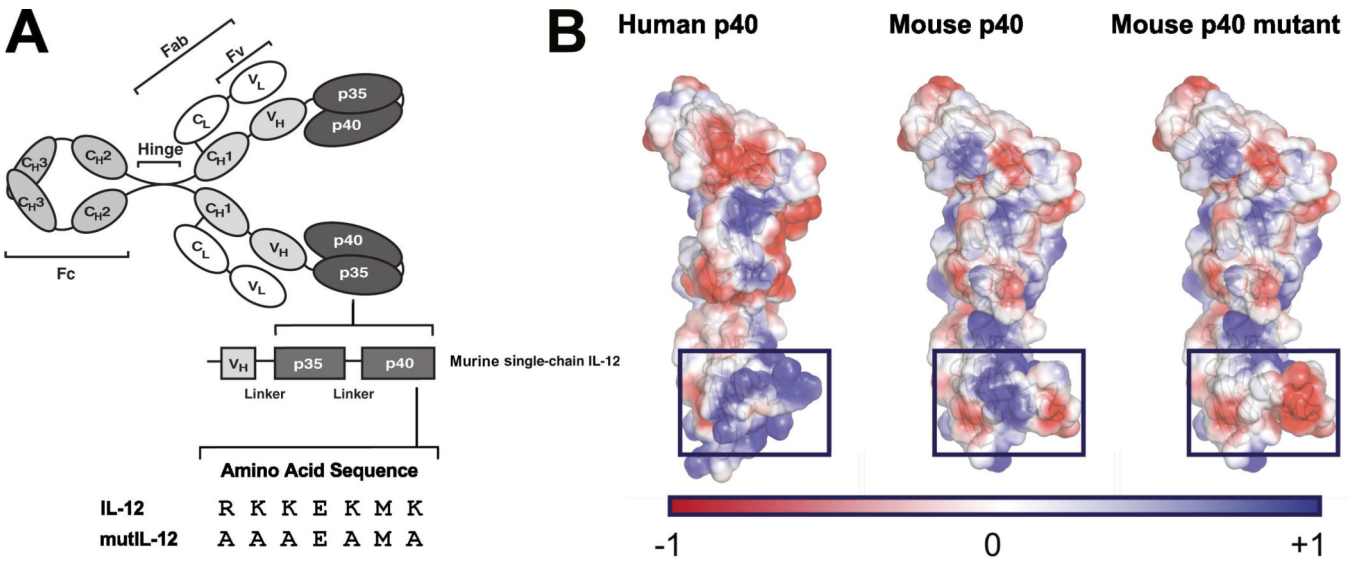

Figure 1: Schematic representation of anti-HER2/*neu* IgG3-(IL-12) and mutations in the cluster of basic amino acids in the p40 subunit of murine IL-12.

Panel A. Single-chain IL-12 in which p40 and p35 subunits joined together via a flexible linker is attached to the N-terminus an IgG3 heavy chain via another flexible linker. The cluster of amino acids 254–260 (RKKEKMK), numbered according to the secreted polypeptide, of the murine IL-12 p40 subunit was modified by exchanging the basic residues, arginines (R) and lysines (K) for the neutral non-polar amino acid alanine (A) resulting in the mutant sequence AAAEAMA. Panel B. Surface charges displayed on solvent accessible surface renderings of the atomic structures of the p40 subunit of human IL-23 (left; PDB 3DUH), a homology model of the wild type murine p40 subunit (middle), and the homology model of the generated AAAEAMA mutant murine p40 (right). Transparent ribbon model of the proteins overlaid over the surface models are also shown for reference. Boxed regions highlight the location of a cluster of basic residues in the p40 subunit of the human and mouse proteins, and the absence of basic charges at this site in the mutant protein. Relative electrostatic charge is displayed with negative shown in red and positive in blue.

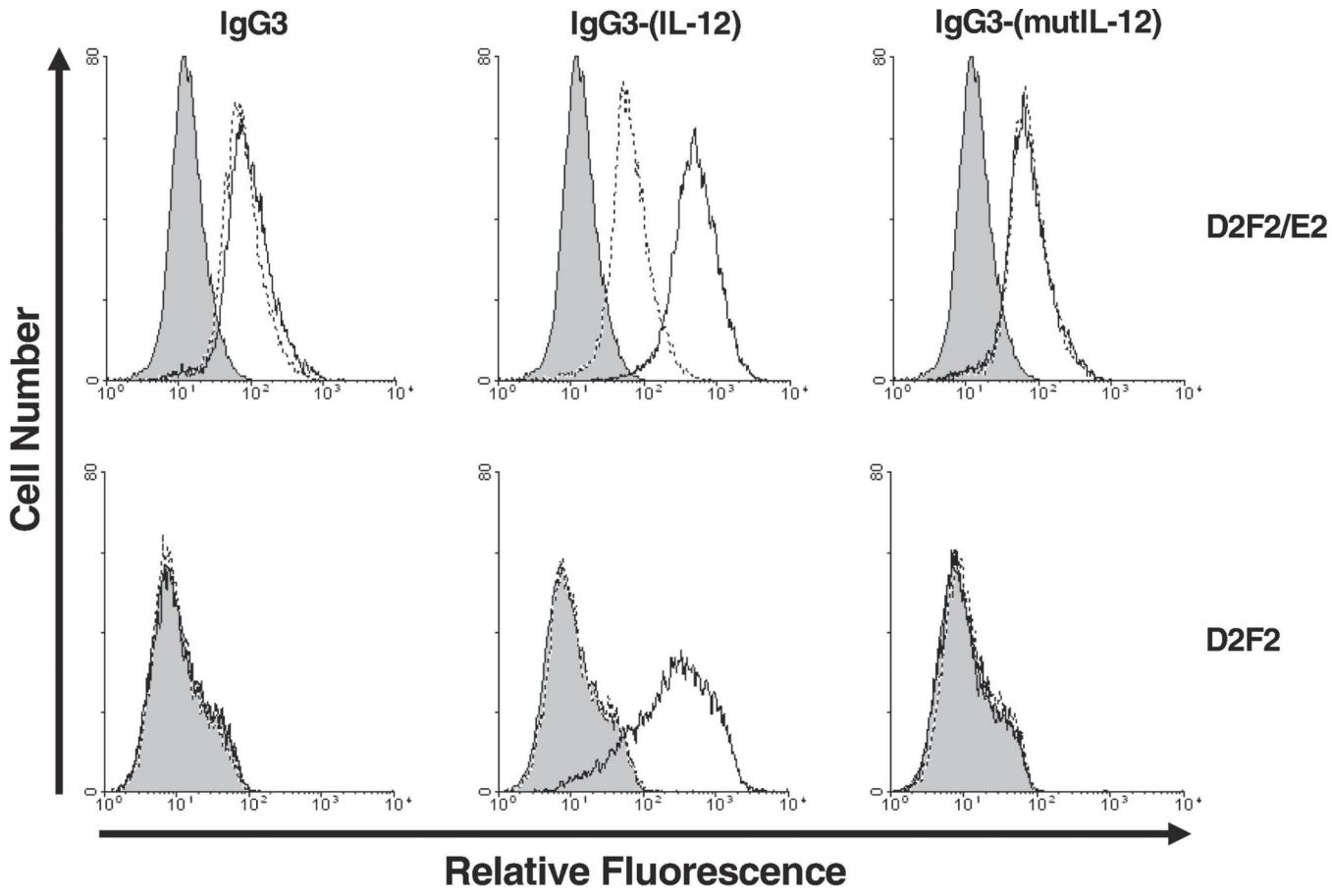

Figure 2. Heparin and antigen binding properties of anti-HER2/*neu* IgG3-(IL-12) and anti-HER2/*neu* IgG3-(mutIL-12) assessed by flow cytometry.

D2F2/E2 murine mammary cancer cells expressing human HER2/*neu* and the parental D2F2 not expressing the antigen were incubated with anti-DNS IgG3 isotope control (filled histogram) or with the human HER2/*neu* specific IgG3, IgG3-(IL-12) or IgG3-(mutIL-12) (open histograms) in the presence (dotted line) or absence (solid line) of heparin. Antibody binding was detected using FITC-conjugated rabbit anti-human κ antibody and flow cytometry. Results are representative of four independent experiments.

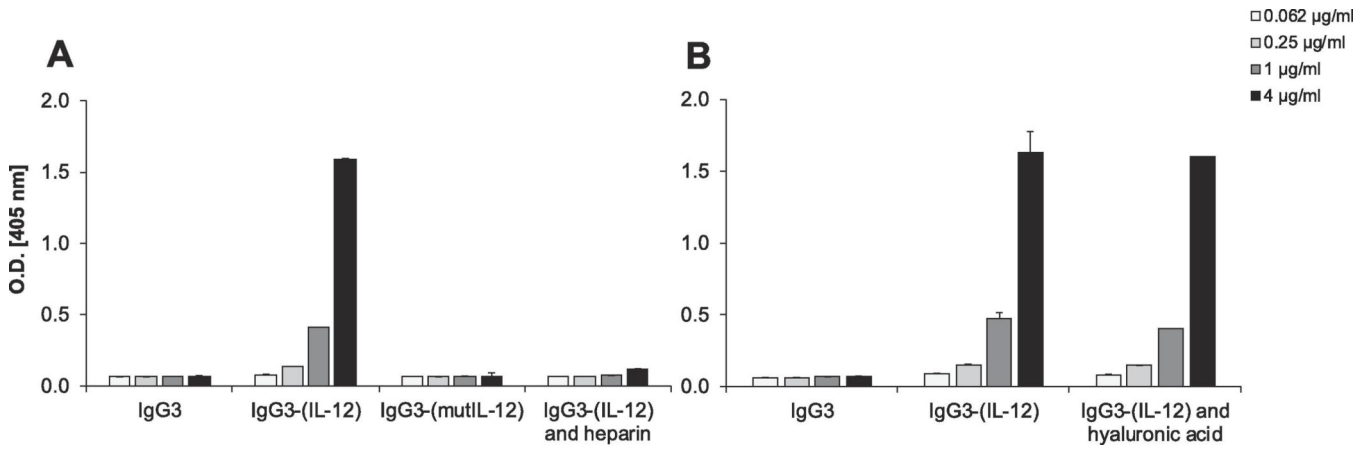

Figure 3. Heparin binding properties of anti-HER2/*neu* IgG3-(IL-12) and anti-HER2/*neu* IgG3-(mutIL-12) assessed by ELISA.

Heparin coated plates were incubated with different concentrations of human HER2/*neu* specific IgG3-(mutIL-12), IgG3-(IL-12), IgG3-(IL-12) with free heparin, or the equivalent molar amount of human HER2/*neu* specific IgG3 (Panel A) or human HER2/*neu* specific IgG3-(IL-12), IgG3-(IL-12) with free hyaluronic acid, or the equivalent molar amount of human HER2/*neu* specific IgG3 (Panel B). Binding was detected using a goat anti-human κ antibody conjugated to AP. The absorbance was measured at 1 h of incubation with substrate. Error bars are mean ± SD of triplicate measurements. Results in Panel A are representative of four independent experiments.

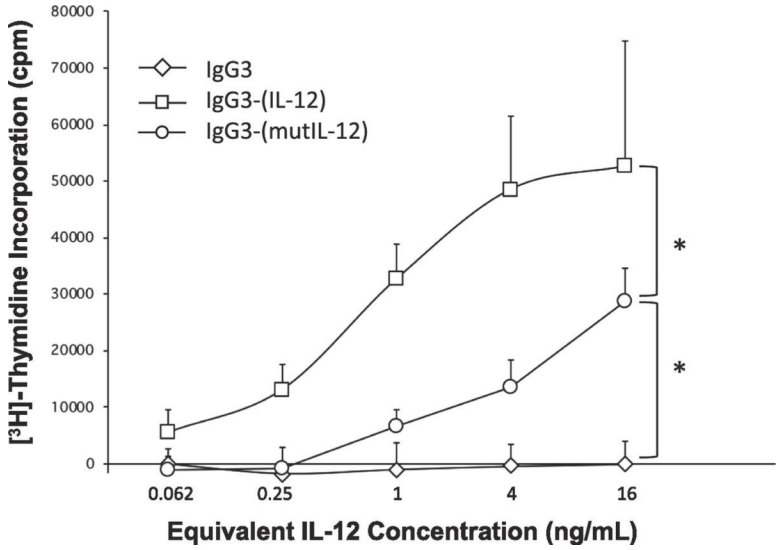

Figure 4. IL-12 bioactivity of anti-HER2/*neu* IgG3-(IL-12) and anti-HER2/*neu* IgG3-(mutIL-12) assessed in a T-cell proliferation assay.

Activated PBMCs were incubated for 2 days in the presence of human HER2/*neu* specific IgG3-(IL-12), IgG3-(mutIL-12), or IgG3. Proliferation was measured by [^{3}H]-thymidine incorporation assay. The equivalent IL-12 concentration (ng/mL) of the human HER2/*neu* specific IgG3-(IL-12) and IgG3-(mutIL-12) is shown in a logarithmic scale. Since the human HER2/*neu* specific IgG3 does not contain IL-12, the molar amount of this antibody equivalent to that of the antibody fusion proteins was used. Error bars are mean ± SD of quintuplicate measurements. * $p < 0.05$. Results are representative of four independent experiments.