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Immunotherapy with GcMAF revisited - A critical overview of the research of Nobuto Yamamoto

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ABSTRACT

This overview describes the research of Nobutu Yamamoto (Philadelphia) concerning immunotherapy with GcMAF for patients with cancer and for patients infected with pathogenic envelope viruses. GcMAF (Group-specific component Macrophage-Activating Factor) is a mammalian protein with an incredible potency to directly activate macrophages. Since the late 1980s Yamamoto's investigations were published in numerous journals but in order to understand the details of his research, a minute survey of many of his patents was required. But even then, regrettably, a precise description of his experiments was sometimes lacking. This overview tries to summarize all of Yamamoto's research on GcMAF, as well as some selected more recent papers from other investigators, who tried to verify and/or reproduce Yamamoto's reports. In my opinion the most important result of the GcMAF research deserves widespread renewed attention: human GcMAF injections (100 ng per week, intramuscular or intravenous) can help to cure patients with a great variety of cancers as well as patients infected with pathogenic envelope viruses like the human immunodeficiency virus 1 (HIV-1), influenza, measles and rubella (and maybe also SARS-CoV-2). From Yamamoto's data it can be calculated that GcMAF is a near-stoichiometric activator of macrophages. Yamamoto monitored the progress of his immunotherapy via the serum level of an enzyme called nagalase (α-*N*-acetylgalactosaminidase activity at pH 6). I have extensively discussed the properties and potential catalytic site of this enzyme activity in an Appendix entitled: "Search for the potential active site of the latent α-*N*-acetylgalactosaminidase activity in the glycoproteins of some envelope viruses".

Introduction

In 2008/2009 four papers appeared from the group of Nobuto Yamamoto in Philadelphia [1–4] describing successful immunotherapy of cancer and HIV-1 with a mammalian protein GcMAF (Group-specific component Macrophage-Activating Factor). At first glance the results in these four Yamamoto papers looked too good to be true. In a limited number of non-anaemic patients (8 with colon cancer, 16 with breast or prostate cancer, and 15 infected with HIV-1) the serum nagalase level (α-*N*-acetylgalactosaminidase activity at pH 6) was used as a marker for the remaining tumour mass during GcMAF treatment. It was mentioned that after stopping the immunotherapy with GcMAF (which was the sole treatment), when the nagalase level had reached basic levels, all patients remained free of symptoms for many years. For breast cancer patients this was at least 4 years, and even up to 10 years [5]. For patients with colorectal or prostate cancer, or with HIV-1/AIDS, this was at least 7 years. In addition, their nagalase levels did not increase during those years. Mentioning of these symptoms-free survival periods indicated that many of the reported studies were performed around or before the turn of the century. These series of papers sparked my interest and I decided to inspect the experimental bases of Yamamoto's research described in his papers and patents.

Yamamoto and collaborators started in the late 1980s to investigate the mechanisms the human body uses to combat cancer. In the present overview I discuss Yamamoto's proposal for early detection of cancer and for monitoring the changes in malignant tumour burden via the serum levels of nagalase. This activity is here also termed Naga6 to distinguish it from the serum activity of the classical lysosomal enzyme α-*N*-acetylgalactosaminidase (EC 3.2.1.49, Naga; defective in patients with Schindler disease) with a pH optimum at pH 4.3 [6–8], here also called Naga4. I further discuss Yamamoto's reports describing treatment of patients with cancer, as well as with infections with some pathogenic envelope viruses, by applying immunotherapy with GcMAF. Being a retired biochemist, my interest is purely scientific.

For more detailed information on a number of subjects, as indicated

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Abbreviations

DDG	dodecylglycerol
Gal	galactose
α-GalNAc	*N*-acetyl-α-D-galactosaminide, *N*-acetyl-2-deoxy-2-amino-galactose
Gc	Group-specific component
GcMAF	Group-specific component Macrophage-Activating Factor
gp160	monomer of the trimeric spike glycoprotein of HIV-1 with an apparent MW of 160 kDa
gp120	hydrophilic N-terminal part of gp160 released upon cleavage by the host cell furin
gp41	hydrophobic C-terminal part of gp160 formed after cleavage by host cell furin
HIV-1	human immunodeficiency virus 1
Naga	α-*N*-acetylgalactosaminidase
Naga4	(human/chicken) lysosomal Naga activity at pH 4.3
Nagalase	activity of Naga at pH 6.0 (here also called Naga6)
pNP	para-nitrophenol;
SA	sialic acid
SARS-CoV-2	severe acute respiratory syndrome coronavirus
TAM	tumour-associated macrophages.

in this Main text, the reader is encouraged to view the **Supplementary Information** (Sections S1 to S11, Figures S1 to S12, Tables S1 to S2, 87 References).

In an **Appendix** entitled "Search for the potential active site of the latent α-*N*-acetylgalactosaminidase activity in the glycoproteins of some envelope viruses" (Sections A1 to A4, Figures A1 to A10, Table A1, 37 References) I have inspected available 3D structures of glycoproteins (spike proteins) from the pathogenic envelope viruses HIV-1, influenza, Ebola and SARS-CoV-2. None of the structures contained the active site quartet of the amino acid residues Trp-Trp-Asp-Glu indicative for an endo-glycohydrolase. However, they did show characteristic trios of the amino acid residues Trp, Asp and Glu in a conformation similar to the Trp-Asp-Asp active site trio in the lysosomal α-*N*-acetylgalactosaminidase. The W-D-E trio in the HIV-1 and influenza glycoproteins might explain the (latent) nagalase activity reported by Yamamoto. The trios in the spike proteins from Ebola (W-E-E) and SARS-CoV-2 (W-D-E) point to a possible (latent) nagalase activity, but that remains to be established.

Cancer cells have a set of common properties

Genetic mutations in nearly all types of cancer cells affect a set of 12 main cellular signalling pathways and processes. In turn, this leads to the same bioenergetic and immunologic phenotype changes. Because this is of help for a better understanding of Yamamoto's findings, I have discussed this in Section S1 of the **Supplementary Information**.

Yamamoto's discovery: a mammalian protein called GcMAF is the most potent activator of macrophages

As reviewed in 1970 [9], stimulation of the immune system to treat cancer, i.e. immunotherapy, has received attention as far back as the early 1900s. Thereafter, the interest declined until the discovery of tumour-specific antigens in the mid 1950s.

In the late 1980s the group of Nobuto Yamamoto in Philadelphia started research on blood components involved in the activation of macrophages. The basic idea behind these studies was [10] that *''Inflammation induced by either microbial infection or administration of bacterial components results in chemotaxis and activation of macrophages. On this basis, we hypothesize that macrophages within the host recognize a stimulatory signal radiating out from the inflammatory region.''*

Initially, the mechanism of the potent in vivo and in vitro activation of mice macrophages by alkyl-lysophospholipids and their metabolites alkyl-glycerophosphates and alkyl-glycerols was studied [10, 11]. Alkyl-lysophospholipids are among the breakdown products of the walls of many cancer cells. Macrophages were obtained from peritoneal cells of treated and control mice or, for in vitro experiment, from resident peritoneal cells of untreated mice. In both cases nearly all cells (ca. 96%), adherent to glass cover slips, were identified as macrophages by phagocytic and morphological criteria. Activation was determined by ingestion assays, by the production of superoxide and by cytocidal activity against retinoblastoma cells.

Using dodecylglycerol (DDG, an alkyl-glycerol) to initiate activation of mice macrophages (in vivo and in vitro), it was discovered that various blood components were essential for maximal activation: (a) B lymphocytes, activated by DDG [12]; (b) untreated T lymphocytes [12] and; (c) a soluble serum protein [13] identified as the Gc protein [14, 15]. DDG itself had no activating effect on macrophages.

The Gc protein

The Gc protein (Group-specific component) in blood was identified by Hirschfeld in 1959 (as referred to in [16]) and received its name a year later [17]. Although there are some 120 isoforms of Gc among humans, most people (92 to 100% [16, 18-20]) carry one of the three main polymorphic forms called Gc1f, Gc1s and Gc2 (six allelic combinations possible). The indications 'f' and 's' for the Gc1 proteins stem from their different relative electrophoretic mobility. One (Gc1f) is running faster to the anode (a more negative protein) than the other (Gc1s; s for slow). The difference between these three polymorphic forms of Gc consists of point mutations in the amino-acid sequence at positions 416 and 420 (see Section S2, Table S1). Note that all sequences contain T418.

The molecular mass of Gc is 51.2 kDa (UniProtKB database entry for Gc2: P02774) and the mature protein consists of 458 amino-acid residues. In plasma/serum, it is present in concentrations of 250 to 350 mg/L (ca. 6 μM), but also larger variations (75 to 450 mg/L) have been found in apparently healthy individuals [21]. One of the functions of Gc is to carry the (apolar) vitamin D sterols [22, 23]. It is therefore often referred to as the vitamin-D-binding protein (VDBP or DBP). Gc can also strongly bind actin monomers and a most important function of Gc is to clear extracellular G-actin (globular actin), released from the cytoskeleton of necrotic and/or damaged cells [24, 25]. This prevents the polymerization of these hydrophobic monomers to F-actin (fibrous actin), which can otherwise lead to obstruction of blood capillaries. The plasma half-life of uncomplexed Gc in human blood is 2 to 2.5 days, but the plasma half-life of Gc-actin is only 30 to 60 min. Gc-actin is cleared by the liver.

The daily synthesis of Gc in the liver is ca. 10 mg/kg. Heavy trauma can therefore result in dangerously low levels of Gc and organ failure [26, 27]. Gc in blood of healthy individuals consists to 20 to 44% (mol. mol^{-1}) of Gc-actin [28].

O-Glycosylation of Gc

In 1983 it was reported that the Gc1s protein can contain a single linear tri-saccharide, NeuAc-α- (2→3)-Gal-β-(1→3)-GalNAc-α-(1→0)-, bound to a serine (S) or threonine (T) residue (here also written as SA-Gal-GalNAc-S/T). The O-glycosylation degree was ca. 1% (w/w), whereas Gc contained no N-glycosides [18] (abbreviations: NeuAc, *N*-acetylneuraminic acid, a sialic acid (SA); Gal, galactose; GalNAc, *N*-acetyl-α-D-galactosaminide). In that early study no glycosylation could be detected in Gc2 (presumably due to the detection limit of used methods). As discussed later on, glycosylation can occur on T418, present in all three main forms of Gc, and/or on T420 (absent in Gc2).

GcMAF

Yamamoto and co-workers found that activation of B cells by DDG induced a 3-fold increase of the outersurface-bound β-galactosidase. They suspected that this enhanced activity might play a role in the conversion of Gc into an activator of macrophages. They further assumed that the outersurface-bound neuraminidase (sialidase) activity of untreated T cells was also involved. This led to the idea, that in their animal studies, the known O-glycosylation of the Gc protein [18] was partly deglycosylated by β-galactosidase (of DDG-activated B cells) and sialidase (of T cells) to a Gc product, termed GcMAF [15, 29, 30].

This was substantiated by experiments where purified human Gc1 (a mixture of Gc1f and Gc1s) was treated with purchased β-galactosidase and sialidase immobilized on separate agarose columns. The resultant GcMAF product was as effective as the one produced with B and T cells [15]. When using cells, GcMAF formed only when the Gc protein was first treated with activated B cells and then with T cells; the reverse order did not work. However, with the immobilized enzymes the order of treatment was not important. On that basis it was proposed, but not shown, that the Gc1 protein can contain a branched Gal-(SA)-GalNAc-tri-saccharide group bound to a threonine residue. With human Gc2 an active GcMAF product could directly be obtained with activated B cells, or with column-bound β-galactosidase. Hence it was proposed that Gc2 can contain a Gal-GalNAc- di-saccharide bound to Thr [15]. I have summarized Yamamoto's findings in Fig. 1 and adapted them in the light of the results and suggestions of Borges et al [20, 31, 32]. as discussed in Section S2. In addition I indicated possible active GcMAF forms of Gc1 not mentioned by Yamamoto or Borges.

According to Yamamoto et al., GcMAF was the most potent activator of macrophages described until then [33]. They proposed that GcMAF is the natural activator of macrophages in mammals and that injection of GcMAF, in bypassing involvement of B and T cells, might be used as a potent adjuvant to (non-specifically) enhance the immune response to cancer antigens in humans [30, 33].

The carboxy-terminal part of Gc is responsible for the macrophage-activating effect of GcMAF

Yamamoto discovered that the carboxy-terminal part of the protein was responsible for the macrophage activation [30, 34]. A polypeptide comprising the C-terminal domain (80 amino-acid residues) of Gc1s was prepared by cloning the coding gene fragment and expressing it in insect cells. The purified protein was treated with immobilized β-galactosidase and sialidase and the resulting protein was called CdMAF (Cd from C-terminal domain). It was as active as Gc1s-MAF (even in the treatment of prostate-cancer patients) [35]. Neither of the two MAF proteins caused any adverse effects in humans. Even a MAF protein prepared from a polypeptide with the C-terminal 40 amino-acid residues of Gc1s was mentioned to show activation of macrophages [34]. Peptides with less than 40 amino acids could not be expressed in insect cells. In addition, such small peptides rapidly degrade in blood. The molecular structures of Gc2 and the C-terminal polypeptides discussed here are shown in Fig. 2. Yamamoto's results show that the presence of bound vitamin D in GcMAF is irrelevant for its MAF activity, because CdMAF misses the N-terminal domain that binds vitamin D sterols [36, 37].

Synthetic analogues of GcMAF

There are some reports on synthetic analogues of GcMAF that had an activating effect on macrophages. It was shown that it is the 3D structure of the GalNAc-containing α-helix in Gc that forms the basis for the MAF effect. This is described in Section S3.

Immunotherapy with GcMAF

Yamamoto has patented most of his findings, starting in 1993 with a patent on "*In vitro enzymatic conversion of glycosylated human vitamin D binding protein to a potent macrophage activating factor*" [38]. Gc purified

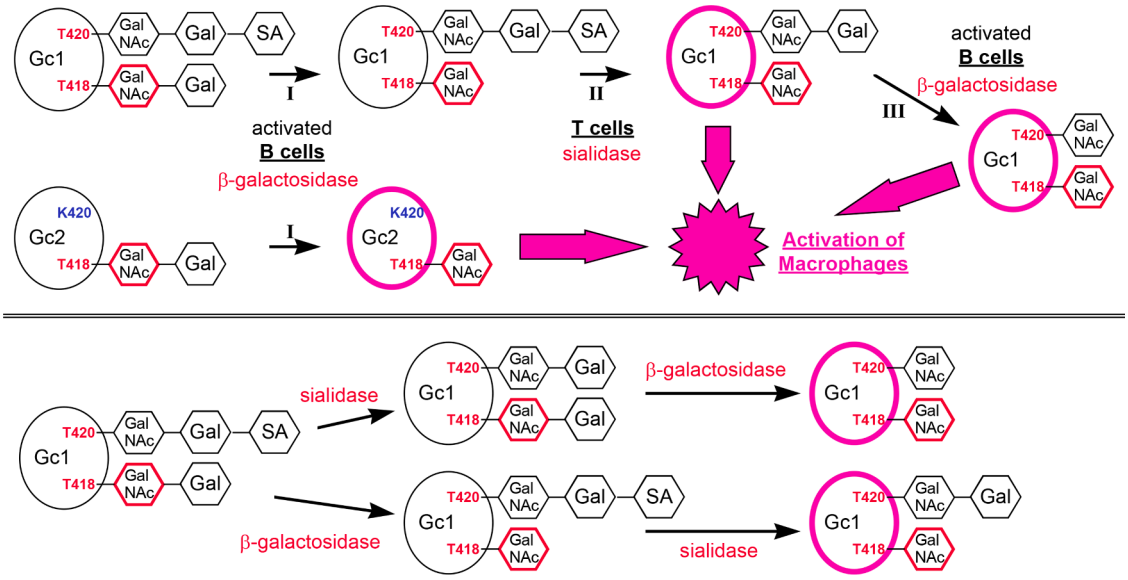

Fig. 1. Schematic representation of the formation of GcMAF from Gc1 or Gc2 according to Yamamoto as adapted by me. Upper panel: Conversion of Gc1 (Gc1f, Gc1s) or Gc2 with activated B cells (reaction I) and T cells (reaction II). Keep in mind that only a small percentage of Gc molecules carries the di- and/or tri-saccharides. For Gc1 the analytical data show that the tri-saccharide is a linear one [18, 31, 32, 47] rather then the branched one proposed by Yamamoto [30]. Apparently the sialidase on T cells does not react with Gc1 if the T418 residue still carries a di-saccharide. Hence Gal-GalNAc-T418 has first to be converted to GalNAc-T418 by β-galactosidase from activated B cells, before sialidase from T cells can react. As explained below, it is likely that Gc1 with GalNAc-T418 plus Gal-GalNAc-T420 is also active (purple oval). In vivo, a further removal of the remaining Gal (Galactose) sugar by activated B cells (reaction III) will result in a product with GalNAc-T418 plus GalNAc-T420. GcMAF prepared from Gc2 only requires the action of β-galactosidase on activated B cells. Reactions I and II can occur in vitro and in vivo; reaction III occurs only in vivo. Lower panel: Preparation of GcMAF from Gc1 with (purchased) β-galactosidase and sialidase. In this case the order of treatment is not important. Note that, dependent on the sequence of treatment with the column-bound enzymes, active GcMAF (purple oval) is produced with either GalNAc-T418 plus GalNAc-T420 or with GalNAc-T418 plus Gal-GalNAc-T420. I support the suggestion of Borges et al [32]. that only the GalNAc sugar on T418 (purple hexagon) is essential for activation of macrophages, provided that T420 (when present) carries no tri-saccharide.

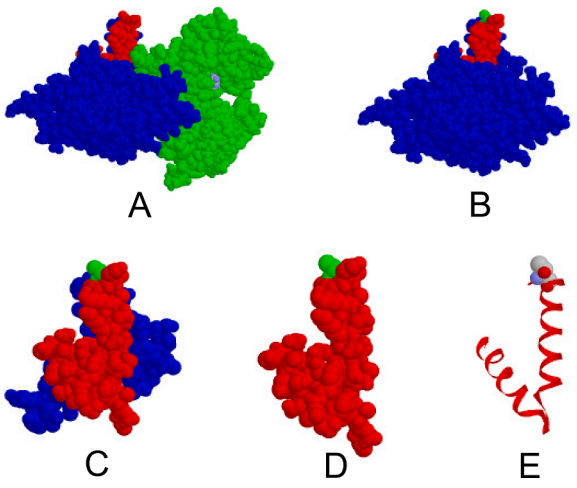

Fig. 2. Molecular structure of human Gc2 with bound actin from rabbit skeletal muscle. (A) The complete X-ray structure of Gc2-actin (PDB entry 1KXP [91]). In this space filling (Van der Waals radii) representation, the Gc2 molecule is in blue, except for the C-terminus, and the actin is in green. The C-terminal 80 amino-acid residues of Gc2 are in red (Lys377- Ile457 in the mature protein, but Lys393 to Ile473 according to the 1KXP file, where the N-terminal leader sequence of 16 amino-acid residues is included). Actin contains a Mg-ATP molecule (Mg in purple, ATP in CPK colours). (B) The same structure, but now the actin molecule has been deleted and T418 in Gc2 is in green. Note that T418 clearly sticks out of the structure. (C) Structure of the C-terminal 80 amino-acid residues. The residues K377 to D416 are in blue, the residues A417 to I457 (the C-terminal 40 amino-acid residues) are in red and T418 is in green. (D) Structure of the C-terminal 40 amino-acid residues in red; T418 in green. (E) Same (D), but now in a ribbon presentation with T418 in CPK colours and space filling. Used CPK colours (elements colours): C, grey; N, blue and O, red.

from blood [39] was treated with column-immobilized β-galactosidase (EC 3.2.1.23) from *Escherichia coli* and sialidase from *Vibrio cholerae* or *Arthrobacter ureafaciens* (all from Boehringer), whereafter the GcMAF preparation was filter sterilized. The saline solution was administered (intramuscular or intravenous; 30 to 35 ng GcMAF every three to five days) to a single individual (Yamamoto or one of his co-workers?) resulting in a significant and lasting level of macrophage activation.

Yamamoto's patients studies

The first patient results were described in 2002 in a patent application [40] entitled "Preparation of potent macrophage activating factors derived from cloned vitamin D binding protein and its domain and their therapeutic usage for cancer, HIV-infection and osteopetrosis.". The patent was filed March 19th 1996, but only published 6 years later (June 25th 2002). To monitor the therapeutic effect, the activity of patient serum to convert pNP-α-GalNAc (para-nitrophenyl-α-GalNAc; colourless) at pH 6.0 into α-GalNAc plus pNP (para-nitrophenol; colourless at pH 6.0, but yellow at pH 10) was measured during the treatment. This exo-glycohydrolase activity was significantly elevated in patients. From 1997 onwards, Yamamoto termed this activity "nagalase" [35].

Another most informative patent [41], entitled "Determination of alpha-N-acetylgalactosaminidase activity", was filed on June 5th 1996, but only published on September 3rd 2003. It described that in order to automate the detection of serum nagalase levels, monoclonal antibodies against purified cancer tissue nagalase or nagalase from serum of HIV-1/AIDS patients were used. The antibodies were produced using the hybridoma technique. This enabled routine monitoring of nagalase protein levels in samples by the sandwich ELISA method. The two monoclonal antibodies did not cross-react, so the nagalase enzyme from cancer patients was presumably different from that of HIV-1/AIDS patients. The ELISA technique, using yet another specific monoclonal antibody, was also applied to monitor nagalase levels in patients with influenza [42–44].

For cancer patients, the sandwich ELISA method determined the nagalase protein levels via a colour assay in μg antigen (NagAg, purified nagalase from lung cancer tissue) per mL serum/plasma. Curiously, Yamamoto stated [42] that "Serum/plasma α-N-acetylglactosaminidase activity was also expressed as the product of NagAg concentration (μg) and 0.25 nmole/mg/min, because I found that 1 μg of the enzyme (NagAg) in serum has 0.25 nmole/mg/min of the enzyme activity.". An excerpt from Table 1 of that patent is shown in Section S4, Table S2. Thus, the amount of nagalase (as a number without units) determined in 1 mL sample by ELISA was simply multiplied by 0.25 nmole/mg/min. These units (nmol/mg/min) were used in all of Yamamoto's publications although in none of those papers the word 'ELISA' was ever mentioned in the text, only sometimes in the references. As discussed before [8], the nagalase levels mentioned by Yamamoto for healthy persons (in nmol/min per mg serum protein) is ca. two-orders of magnitude higher than those determined in direct activity measurements (in nmol/min per mL of serum).

If I assume, as discussed in Section S11, that Naga6 is simply Naga4 with an altered N-glycosylation and pH optimum, but with similar enzyme kinetics, then an estimate of its serum concentration (in $\mu g.mL^{-1}$) can be made as follows. The normal serum Naga6 activity (with 2,4-dinitrophenol-GalNAc, pH 5.8) is ca. 0.035 $U.mL^{-1}$ [8] and the specific activity of pure recombinant Naga4 (with pNP-GalNac, pH 4.5) is 20.3 $U.mg^{-1}$ [45]. This gives a serum Naga6 protein concentration of ca. 1.7 $\mu g.mL^{-1}$. That value is of the same order of magnitude as the nagalase level mentioned by Yamamoto in serum from healthy individuals (3.9 $\mu g.mL^{-1}$ [42]). Whatever the (odd) units, it is the change in the numerical value of the nagalase level during GcMAF immunotherapy that matters.

A drawback of the ELISA test is that it could not be directly applied to plasma/serum. The antibody binding affinity for Naga6 is apparently inhibited by a highly charged, low-molecular weight substance in serum. Thus, before the ELISA test was carried out, this inhibitor had to be removed from the plasma/serum via treatment with ammoniumsulphate. The precipitate, obtained between 30% and 70% ammoniumsulphate saturation, was redissolved and extensively dialysed before testing with ELISA [41]. In my opinion, the epitope of the used monoclonal antibody might well consist of a combination of protein and carbohydrate. In that case, the inhibitor could be a carbohydrate-rich, major component in serum, e.g. proteoglycans like chondroitin 6-sulphate, keratan sulphate, heparin, dermatan sulphate or hyaluronate. A treatment with ammoniumsulphate had no effect on the directly-determined Naga6 activity in serum [8].

Yamamoto used the ELISA method for patients with a great variety of cancers as well as for healthy individuals. That means that the monoclonal antibody raised against purified nagalase from lung cancer tissue [42] reacted specifically with nagalase from all these sources. I therefore conclude that nagalase in plasma/serum from all cancer patients must be one and the same protein and that this is also present in serum from healthy individuals. However, Yamamoto always reported, but did not show, that the activity level in serum of healthy individuals is due to α-galactosidase and not to nagalase. He stated [1] "This is the enzyme activity of α-galactosidase that can catabolize the chromogenic substrate (i.e., p-nitrophenyl N-acetyl-α-D-galactosaminide) for Nagalase.". This is incorrect, because it was later shown that recombinant human α-galactosidase (α-Gal, EC 3.2.1.22) did not show any activity with pNP-α-GaNAc [45].

The curative effects of GcMAF in patients with prostate, breast or colon cancer, leukaemia or HIV-1, for whom conventional treatments (surgery, γ-irradiation and/or chemotherapy) were no longer effective, were first published as a series of figures [42] using the serum nagalase levels as monitor. A more elaborate set of figures, from a study with over 500 patients, appeared in 2002 [40], although that patent was filed on March 19th 1996. The patent stated that "After 25 weekly administrations of 100 ng GcMAF the majority (>90%) of prostate and breast

cancer patients exhibited insignificantly low levels of the serum enzyme. A similar result was also observed after 35 GcMAF administrations to colon cancer patients. Similar curative effects of GcMAF on lung, liver, stomach, brain, bladder, kidney, uterus, ovarian, larynx, esophagus, oral and skin cancers were observed."

Responses in the scientific world and in the public media

For the majority of the academic community the effects of GcMAF in cancer patients as described in the 2002 patent [40] will have passed unnoticed. In addition there was no apparent interest of pharmaceutical companies for this or other patents of Yamamoto. This led the principal investigator, perhaps in a desperate attempt for attention, to publish these findings from the early 1990s in four regular papers in 2008/2009 [1–4]. An example of a figure from the 2002 patent (filed March 19th 1996; published June 25th 2002) is shown in Fig. 3 where it is compared with a figure of a 2008 paper [1]. Similar comparisons can be made for the figures concerning patients with colorectal or breast cancer, or with HIV-1/AIDS. Indeed, the four 2008/2009 papers received quite some world-wide media attention. However, after some years the papers were strongly criticised by Ugarte et al [46]. with comments like ''*No key opinion leader has validated its use in oncology.*'' and ''*These results cannot be scientifically validated as they contradict established tenets in oncology.*'' My comment to this is that there are numerous examples of major discoveries in science that were rejected using such kind of arguments, because they did not fit into the current ideas of the leading scientific community. In addition the authors mentioned some suspected irregular administrative and technical reasons. This resulted in the retraction by the publishers of three of these Yamamoto papers. Ugarte et al [46]. made no efforts to mention any of Yamamoto's earlier papers.

GcMAF is a near-stoichiometric activator of macrophages

In converting the amounts of GcMAF protein into the actual number of molecules of this activator, I came to the conclusion that GcMAF is indeed a fantastic and highly specific activator of macrophages. An amount of 100 ng GcMAF, with an apparent molecular weight (M_r) of 51.2 kDa in 5.5 L blood (volume of blood in adults), gives a concentration of 0.355 pM. Assuming a degree of GalNAc glycosylation at T418 of up to 2% (mol.mol^{-1}) [18, 20, 31, 47], the concentration of active GcMAF molecules is 7.1 fM (0.0071 pM). With Avogadro's Number (1 Mol contains $6.0221.10^{23}$ molecules), this amounts to $4.276.10^{9}$ molecules of active GcMAF in one L blood. Blood contains $0.15.10^{9}$ to $0.6.10^{9}$ monocytes per litre, so this would give 7 to 29 active GcMAF molecules per monocyte. Even when the GalNAc glycosylation of T418 would be only 1% (mol.mol^{-1}) [18, 47], there would still be 4 to 14 molecules of active GcMAF per monocyte. This example also demonstrates the incredible specificity of GcMAF: 3.5 to 7 fM can bind and activate macrophages in a background of 6 μM Gc, i.e. a difference of nine orders of magnitude. Even administration of 30 to 35 ng GcMAF induced a noticeable activation of systemic macrophages [38].

The effect of administered GcMAF in cancer patients is rapid; it interacts with systemic macrophages within ca. 30 min [35]. In addition, intramuscular injection led to a 40-fold increase in the systemic activated macrophages count in 4 days [1], while with intravenous administration this count increased to more than 100-fold in 2 days [5].

Yamamoto has reported that the effect of 500 ng injections in patients did not much differ from that of 100 ng [48]. With in vitro experiment it was observed that the macrophage activating effect of GcMAF clearly diminished at higher concentrations and even became strongly inhibiting (Table III of [14]). This effect has been confirmed by other investigators [49–51]. I have visualized Yamamoto's results in Fig. 4. A plot of the activating effect against the logarithm of the GcMAF concentration shows a bell-shaped curve. As a biochemist, this reminded me to the typical substrate inhibition pattern in an enzymatic assay [52]. Thus, at high concentrations GcMAF can apparently bind at a second site on macrophages (weak binding) where it completely inhibits the activating effect of bound GcMAF at the primary site (strong binding).

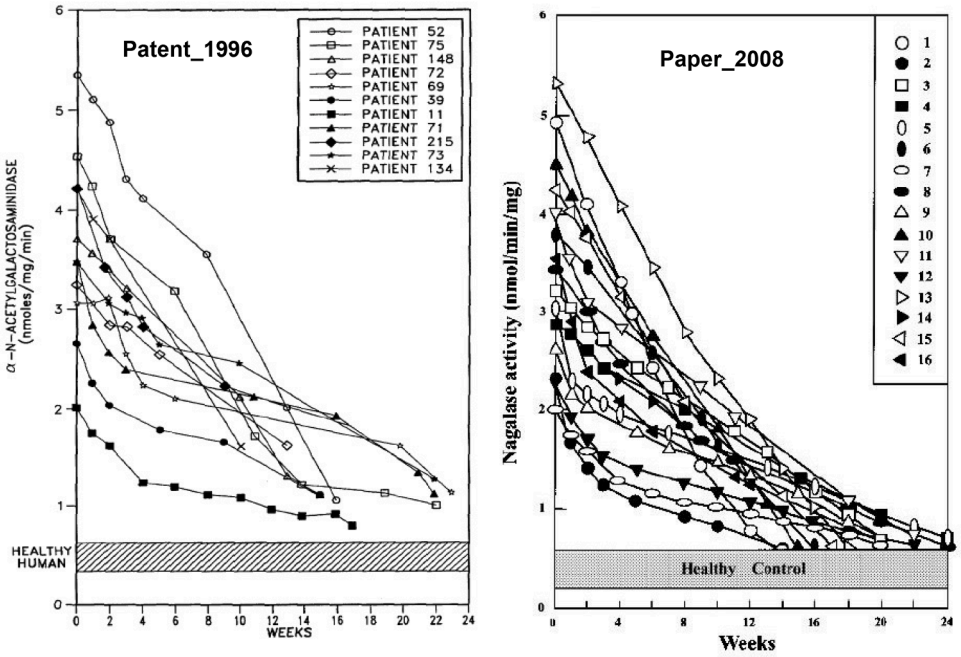

Fig. 3. Comparison of nagalase time courses from prostate cancer patients during immunotherapy with GcMAF as reported by Yamamoto in 2002 (filed in 1996) [40] (left; 11 patients) and 2008 [1] (right; 16 patients). The x-y axes have been scaled for direct comparison. The nagalase "activity" was given in the units nmoles/mg/min (left) or nmol/min/mg (right). Note that not the activity of nagalase was measured, but the amount of nagalase protein in the patient's serum (ELISA).

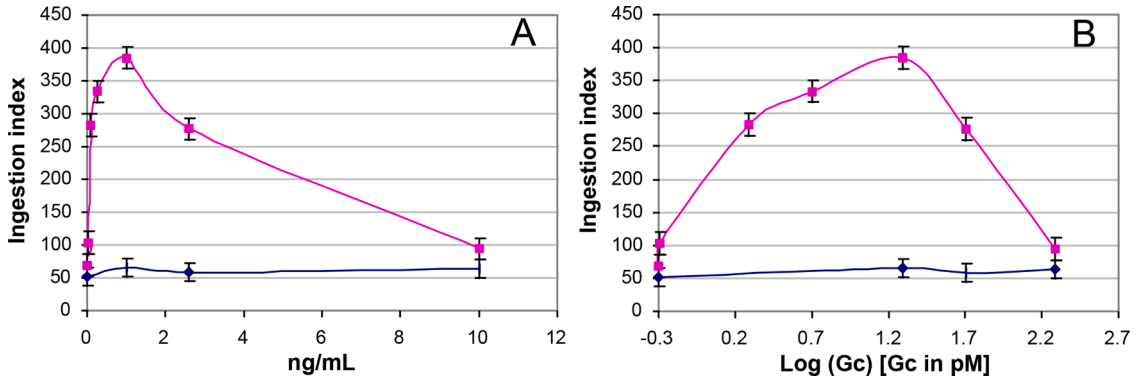

Fig. 4. Correlation between the concentration of GcMAF and its activating effect on macrophages (in vitro). The figure is a graphical representation of Table III from [14]. In this paper the term "GcMAF" was not yet used. Short description of the experiment: mouse splenic B cells were incubated with lyso-PC (lysophosphatidylcholine) for 30 min (activation of B cells, stimulated expression of outersurface-bound β-galactosidase). The cells were then washed, resuspended and incubated with various amounts of purified human Gc for 2 h (removal of Gal from Gc). Subsequently, this mixture was used to culture untreated (mouse) T cells during 2 h (removal of SA, formation of GcMAF (see Fig. 2). The resultant medium was diluted (30 to 100 fold) and tested for phagocytic activity. (A) Plot of the macrophage activation (ingestion activity) against the amounts of human Gc (ng/mL). (B) Plot of the ingestion activity against the logarithm of the Gc concentration (pM). Purple lines, Gc treated with B and T cells; black line, untreated Gc.

Malicious tumours evade the immune system by local disabling of the inflammatory response

Old observations in animals

In my view, in a classical animal-model experiment of 1962 it was shown that the inflammatory response to a foreign body, viz. a black cotton sewing thread implanted in rodent transplantable tumours in mice or rats, was insignificant when compared to the response of such a thread implanted in other tissues in the same animal [53] (see Section S5.1). Thus, the immune response was disabled solely within the tumour and in its immediate environment.

Yamamoto's explanation: tumours evade the immune system because they excrete nagalase which degrades the precursor of GcMAF (but not injected GcMAF)

Yamamoto et al [29, 54-57] proposed that elevated serum levels of nagalase inactivate all precursor molecules of GcMAF in the blood by an endo-glycohydrolase activity. See Section S5.2 for details. However, Borges et al. [20, 31]., analyzing serum of cancer patients with mass spectrometry, showed this to be incorrect (see Section S9).

My interpretation of degradation of the "*precursor*" is rather that the locally produced GcMAF by B and T cells around a tumour is greatly inactivated by the high levels of excreted nagalase (exo-glycohydrolase activity) within and around the tumours. Nagalase in the blood has no effect on GcMAF injected elsewhere in the body for reasons discussed in Section S.5.2.

What is required is a series of clear enzymologic experiments with purified Naga6, using Gc2 and purified GcMAF (from recombinant Gc2 with a known degree of glycosylation at T418) as substrates. This may answer questions on the endo- vs. exo-glycohydrolase activity and on the true K_m and V_{max} values of Naga6 for Gc2 and GcMAF.

Surgical removal of tumours and metastases rapidly decreases nagalase levels and increases GcMAF "precursor" levels in serum

Yamamoto demonstrated [56] that surgical removal of the primary tumour resulted in a major drop of the serum nagalase level within one day. At the same time the "*precursor activity*" increased considerably. This is described in Section S5.3, Fig. S9. The nagalase concentration in serum is thus an equilibrium between the rates of production by the tumour plus metastases and its removal from blood by the body. On that basis one can estimate that the concentration of nagalase (or any other factor leading to inactivation of locally produced GcMAF) within and around a tumour, having a diameter of 1 cm, is some four orders of magnitude higher than the concentration in serum (in an adult with 5.5 L blood). This apparently enables the tumour (and metastases) to degrade the locally produced GcMAF. However, macrophages activated elsewhere in the body by injected GcMAF will not be inactivated that way.

Comparison of serum levels of nagalase and traditional tumour markers during immunotherapy with GcMAF

Yamamoto has reported a series of studies where the levels of nagalase and one or more traditional cancer markers have been monitored in serum from breast cancer patients. The patients received a weekly injection with 100 ng GcMAF as the only treatment [3]. I have visualized one of the tables from that paper in Fig. 5. It can be seen that the treatment resulted in a simultaneous decrease of nagalase and tumour markers levels. This agrees with Yamamoto's conclusion that nagalase is a measure of the tumour burden.

The situation is different for prostate cancer. In that case, the presently used marker is PSA (prostate-specific antigen; the Ser-endopeptidase kallikrein-3; EC 3.4.21.77). PSA is synthesized within the prostate epithelium [58, 59], but has also been detected in salivary glands, brain, breast and other tissues, albeit with a much lower (two orders of magnitude) concentration [60]. Its concentration in human semen fluid (ejaculate) is 0.5-2 mg/mL [58, 61], which is over five orders of magnitude higher than the PSA level (0-4 ng/mL) in serum of healthy men. Tiny leaks from the prostate induced by e.g. mechanical pressure, infection (prostatitus) or benign prostate hyperplasia, can result in elevated serum levels of PSA. Therefore it has been questioned whether PSA is a reliable marker for prostate cancer. PSA levels of 8-12 ng/mL have been found in many men without any signs of cancer [62, 63] (see Fig. S5 in Section S6).

Yamamoto has followed nagalase and PSA levels in serum from prostate cancer patients during monotherapy with 100 ng GcMAF per week (Fig. 6). When the prostate was removed (prostatectomy) before commencing therapy (Fig. 6, lower graphs), the nagalase and PSA levels decreased like in Fig. 5. The decreasing nagalase levels were presumably due to metastases, because tumour removal leads to normal levels within 24 h [56] (see Fig. S4 in Section S5.3). However, when the prostate was not removed, the nagalase level decreased as usual, but the PSA level did not (Fig. 6, upper graphs). Apparently, the Naga6-producing tumour cells were attacked and removed by activated macrophages, but other changes and/or damage caused by the tumours were still there and PSA kept leaking out of the prostate. Note the high absolute averaged PSA levels in Fig. 6 for the patients without prostatectomy: for A1 26.5 ng/mL, for A2 17.8 ng/mL and for A3 61.0 ng/mL. According to Yamamoto the latter patients showed no further cancer symptoms for at least 7 years after the GcMAF treatment was stopped. This reminded me of what Warburg noted in 1955 [64] where he stated:

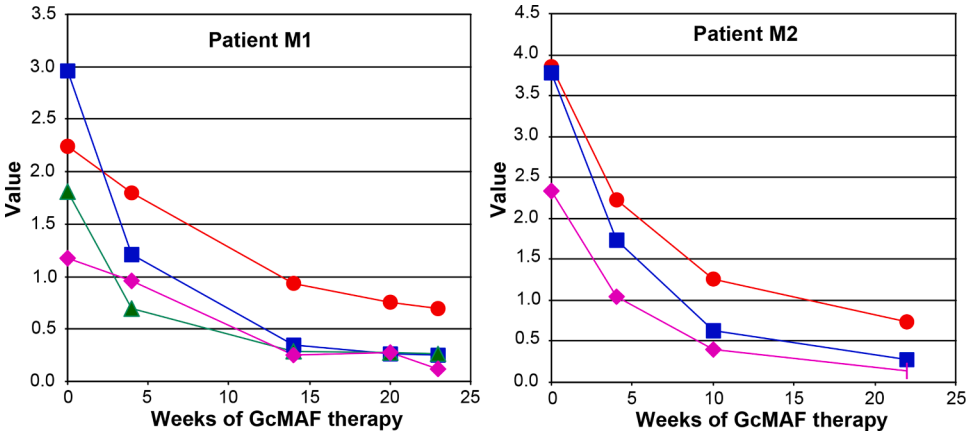

Fig. 5. Serum levels of nagalase (red) and the cancer tumour markers CA 27.29 (blue), CA 15-3 (green) and CEA (purple) from two breast cancer patients during GcMAF immunotherapy. The figure is a graphical representation of data from two of the four patients described in Table III from [3]. The units for CA 27.29 and CA 15-3 are U/mL and CEA is in ng/mL. In order to get all numerical values on the same y-axis scale, the values for CA 27.29 and CA 15-3 were divided by 100, and those for CEA were divided by 10. The units for nagalase were nmol/min/mg. Values for healthy individuals were: 0.35 to 0.69 for nagalase (n=6), <30 for CEA or CA 15-3 and 37.5 for CA 27.29.

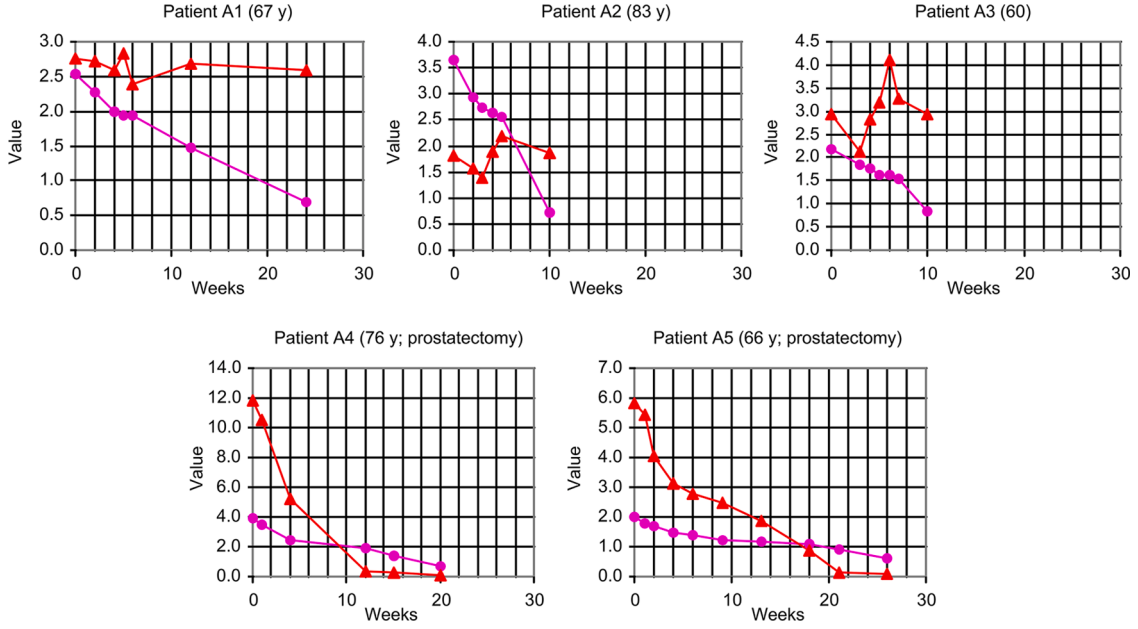

Fig. 6. Correlation between the nagalase and PSA levels in serum from prostate cancer patients during a time course study with GcMAF immunotherapy (100 ng once a week, intramuscular) for 10 to 26 weeks. The figure is a graphical representation of Table 3 from [1]. Upper panels: Nagalase (purple) and PSA (red) levels from three patients without prostatectomy. To get all numerical values on the same y-axis scale, the values for PSA were divided by 10 (A1 and A2) or 20 (A3). The age of the three patients (years) is indicated. Lower panels: Nagalase and PSA levels for two patients with prostatectomy before the start of the treatment. The nagalase levels were in nmol/min/mg and the PSA levels in ng/mL.

"*Ebenso gehören hierher die ruhenden Krebszellen der menschlichen Prostata, die nach HAMPERL im hohen Alter in fast 100% der untersuchten Fälle gefunden werden, ohne daß sie klinisch in Erscheinung treten.*".

Decreasing nagalase levels in Dutch cancer patients during GcMAF immunotherapy

I have received a series of data from a Dutch physician who has tried to cure cancer in 33 of his patients by applying GcMAF immunotherapy. The nagalase time plots from 8 of these patients are compared with a plot of Yamamoto's studies (see Fig. S6 in Section S7). These data clearly confirmed the efficiency of Yamamoto's GcMAF therapy.

Serum from healthy individuals and cancer patients both show a clear Naga6 activity

I have shown that serum from human individuals contains four Naga activities differing in their pH optimum (pH 4, 5.2, 5.8 and 8) [7, 8]. There were clear kinetic differences between the activity at pH 4 (Naga4) and the other ones. This is elaborated in Section S8. My conclusion was that the activity at pH 5.8 (which I call Naga6) is nagalase.

Sources of nagalase in tumour tissue and pathogenic envelope viruses according to Yamamoto

As described above, Yamamoto showed beyond doubt that malicious tumours are the source of elevated levels of Naga6. However, Yamamoto also reported that the serum of HIV-1/AIDS patients often had increased Naga6 activity [65]. In addition, he showed that in that case the source of the activity was hidden (latent) in the envelope glycoprotein gp160 of the HIV-1 virus (presently often called the spike protein). That protein is directly involved in the attachment of this envelope virus to the target cell. Once bound, the gp160 protein is cleaved (by the endoprotease furin from the target cell) into two proteins: gp120 and gp41. The latter, bound to the virus membrane, thereby causes a fusion with the

membrane of the target cell enabling insertion of the viral genome into the target cell. The soluble gp120 protein is released into the blood of the patient.

It was shown that purchased recombinant gp160 protein had no Naga6 activity, but when treated with trypsine a significant activity was detected [65]. Recombinant gp120 also possessed a clear nagalase activity (assayed as exo-glycohydrolase), while the gp41 protein did not.

Yamamoto further reported that similar envelope glycoproteins (spike proteins) from various other pathogenic viruses also had latent nagalase activities. Thus, the HA (haemagglutinin) protein of influenza [44], the fusion (F) protein of measles and rubella (unpublished) all carried (latent) nagalase in their envelope glycoproteins. Their nagalase activities could be unmasked by proteolytic cleavage, e.g. the HA1 protein, cleaved from the HA protein, showed activity [44, 65]. These findings are in agreement with the original reports of Yamamoto, that there is no cross-reaction of monoclonal antibodies raised against the (purified) nagalase activities from cancer patients and patients infected with HIV-1, Epstein-Barr virus or herpes [42]. Thus, the nagalase activities from cancer patients and from patients infected by the different envelope viruses are all due to different proteins.

Pasquato et al [66]. reviewed the mechanism of infection of various human pathogenic envelope viruses and compared the domain arrangement of several viral glycoproteins. Many of these viruses possess similar fusion-active envelope glycoproteins that must be activated by specific proprotein convertases (Ca^{2+}-dependent Ser endoproteases; EC 3.4.21) from the target cell. Note that the SARS-CoV-2 virus (severe acute respiratory syndrome coronavirus) is also an envelope virus with spike-like glycoproteins.

In order to find a possible clue for the latent Naga6 activity, I have compared the amino-acid sequences of the binding proteins (which are released after infection) from six different envelope viruses in more detail (with Clustal X [67]). However, in agreement with findings by Rey and Lok [68], there were absolutely no similarities. Hence the solution of this question may be found in the 3D structures of these binding proteins. I have extensively explored this possibility and the results are described in the Appendix. My main conclusion is that a putative nagalase active site can be uncovered in the glycoproteins of a number of envelope viruses.

Mass-spectrometric analyses of glycosylations in Gc and GcMAF

Yamamoto has always described that cancer patients, or patients infected with pathogenic envelope viruses, had reduced levels of the "*precursor*" of GcMAF in their serum. The term "*precursor*" was used for the glycosylated forms of Gc in serum. It was proposed, but not shown, that deglycosylation of this precursor by the elevated serum activity of nagalase in patients (assumed to be an endo-glycohydrolase) led to the failure of macrophage activation. After 2008 this has been investigated by several groups (described in Section S9). The conclusion was that the detectable degree of Gc glycosylation in serum from cancer patients did not differ from that in healthy individuals.

Recent research on GcMAF immunotherapy

In Section S10 I have summarized some recent, renewed interest in the application of cancer immunotherapy with GcMAF. Yamamoto reported that GcMAF also helps to fight infections with a number of envelope viruses. This is now being tested for SARS-CoV-2 as mentioned in Section S10.

Discussion

Is nagalase produced by cancer cells, by tumour-associated macrophages (TAMs) or by both?

Yamamoto's studies leave no doubt that malignant tumours are the source of nagalase. In the meantime it has been established that emerging tumours often recruit immune cells and that solid malignant tumours can consist of acquired immune cells for up to 50% of their mass. Most of these recruited immune cells are macrophages. In 2002 Yamamoto published that in tumour-bearing hosts, GcMAF activates tumoricidal macrophages. In addition, results were presented suggesting that GcMAF might also inhibit angiogenesis induced by endothelial cells. It was further assumed that tumour-associated macrophages (TAMs) may induce angiogenesis [69].

It is now assumed that the expression profile of TAMs has been altered by cancer cells in such a way that they assist the tumour to grow and promote angiogenesis. Likewise they induce secretion of substances (a/o cytokines) that inactivate the immune system in the immediate environment of the tumour. In my view, this is also the cause of the inactivation of locally produced GcMAF by B and T cells. Presently, tumoricidal macrophages are called M1-like macrophages, while TAMs are also termed M2-like macrophages [70–73]. The properties of macrophages (e.g. expression profile, transcriptosome) can be highly diverse and constitute a continuum of many types of macrophages ranging from M1 (anti-tumour) to M2 (pro-tumour) macrophages. This plasticity has mostly been investigated in in vitro experiments. A variety of proteins can determine, whether monocytes convert into M1-type or M2-type macrophages. In vitro, lipopolysaccharides (LPS) plus interferon-gamma (IFNγ) can convert monocytes into M1-type macrophages, whereas interleukine-4 or interleukine-13 can convert them into M2-like ones. In addition, macrophage colony-stimulating factors (CSFs) can switch monocytes into M1-type macrophages (CSF-2) or M2-type ones (CSF-1), see e.g [72, 74].. The plasticity of macrophages is well known in wound healing (see e.g [75].) and has long been compared with their actions in tumours [76].

This then leads to the question whether elevated levels of Naga6 in serum, reported by Yamamoto for a wide variety of cancer patients, is due to secretion by cancer cells, by recruited immune cells, or by both. More insight into these questions can possibly be obtained by studying secretion profiles (focussing on Naga4 and Naga6) of cancer cell-lines and of M2-like macrophages. It has to be remembered, however, that the properties of macrophages, derived from monocytes in in vitro studies can differ from one experiment to another. In addition, it has been reported that the knowledge, obtained from these in vitro experiments, can widely differ from those of in vivo experiments [77].

My suggestion would be to perform histochemical experiments with tumour coupes using X-GalNAc (X, 5-bromo-4-chloro-3-indoxyl) at pH 5.8. This may yield more detailed information on the exact sites of Naga6 activity within the various tumour cells. The technique was introduced long ago [78] and has been widely used to localize the sites of enzymes like glycosidases, esterases or phosphatases within cells and viruses [79].

My thesis: nagalase (Naga6) is Naga4 with an altered N-glycosylation

Yamamoto has never disclosed the properties of the purified nagalase preparation from lung cancer tissue, that he used to prepare monoclonal antibodies. He must have used SDS-PAGE to follow the purification procedure, so the M_r of nagalase was known to him. The same holds for the nagalases purified from serum of patients infected with pathogenic envelope viruses. In Section S11 I have presented data that lead me to the thesis that Naga6 might just be a Naga4 enzyme with an aberrant N-glycosylation that affects some of its main properties as well as its excretion. Sequence and glycosylation analysis of a purified Naga6 enzyme may solve this question.

Immunotherapy with GcMAF is, in my view, superior to any other immunotreatment for beginning cancers

In the present literature, the term 'immunotherapy' (or biological therapy) for the general treatment of cancer includes immune

checkpoint inhibitors, immune cell therapy, therapeutic antibodies and vaccines as well as immune-modulating agents (e.g. Bacillus Calmette-Guérin, BCG) [80–82]. Nearly all of these therapies have potentially serious adverse effects. In 2020, mRNA vaccines against the SARS-CoV-2 virus opened a new chapter in human immunotherapy. With animals, there is more experience with the mRNA method [83]. With human applications it has still to be awaited whether there are possible short and/or long term adverse effects. Immunotherapy with GcMAF is free of any adverse effects [4, 80]. It is also considered for fighting SARS-CoV-2 [84] and already in study with 97 patients in Italy and 600 patients in the Ukraine (www.clinicaltrials.gov; NCT04845971 and NCT04762628, respectively).

In the last decade, the existence of cancer stem cells (CSCs) has been demonstrated [85–87]. This may explain why after conventional cancer therapies, recurrence is often observed: the CSCs have the capacity to initiate tumour growth anew. Yamamoto's results (especially those reported in the retracted papers from 2008/2009) strongly suggest that the GcMAF-stimulated immune system may be capable to remove also the last residual cancer stem cells.

Concluding remarks

(a) It should be firmly verified whether elevated nagalase levels in cancer patients, as determined by Yamamoto via an indirect but sensitive and specific protein quantification (ELISA), can also be detected via direct activity measurements, e.g. by the method described in 2017 [7, 8]. This can only be performed with patients before commencing any treatment, or in patients where all treatments have been halted for at least 4 weeks. The direct activity measurement can be used indiscriminately, i.e. for cancer patients or for patients infected with a variety of pathogenic envelope viruses (HIV-1, influenza, rubella, measles and maybe SARS-CoV-2). For the ELISA method, monoclonal antibodies against the purified Naga6 activity of each individual type of disease have to be prepared with the hybridoma technique.

(b) If (a) holds true, then it is advisable to regularly (every 6 to 12 months) determine the serum Naga6 activity of adults by the direct method. Especially in elderly people, elevated Naga6 levels are indicative for emerging, unwanted neoplasm's that try to evade the immune system and for infections with pathogenic envelope viruses. Immunotherapy with GcMAF will directly activate systemic macrophages which will attack the unwanted sources of elevated nagalase levels indiscriminately. In addition, such activated macrophages may destroy cancer stem cells.

(c) The MAF activity of GcMAF relies on the presence of a single GalNAc residue attached to T418 in Gc. When Gc is produced by recombinant methods in *E. coli*, the resulting Gc protein cannot be converted into active GcMAF [88], because *E. coli* cannot glycosylate proteins. Expression in hosts that can perform glycosylation of cloned Gc, e.g. insect cells, *Pichia pastoris* yeast, or human embryonic kidney cells, may result in a glycosylated Gc protein that can be successfully converted to GcMAF. Still better would be the expression of Gc2 in ExpiCHO-S cells which are able to directly produce GcMAF [89]. It is of vital importance to determine the percentage and type of glycosylation on T418 in order to predict the expected MAF activity of the product. Thus far, this has never been mentioned in any of Yamamoto's or other studies, except maybe by Borges and Rehder for a purchased GcMAF preparation [32].

(d) Also bovine Gc can be converted to GcMAF, which can activate human macrophages [15]. That protein is less suitable for injection in humans, but it has been applied via oral intake to (indirectly) stimulate the immune system via direct targeting of tissue-resident macrophages of e.g. the oral cavity (Waldeyer's tonsillar ring) or the Peyers's patches in the gastro-interstitial tract (gut-associated lymphoid tissue). In the form of a spray it can activate resident macrophages in the lungs (bronchus-associated lymphoid tissue) [90]. It is worthwhile to try whether a spray with bovine GcMAF might help to fight infection with the SARS-CoV-2 virus. It is hoped that Governments Health Departments will soon allow the sale of oral GcMAF in drugstores as a regular nutrient for optimal immune function just like the vitamins C and D_3.

(e) MAF proteins, as active as GcMAF, can also be prepared as a recombinant polypeptide comprising the C-terminal 80 amino acids of Gc (and even the C-terminal 40 amino acids). Such peptides do not contain the domains of Gc that are responsible for binding of actin, vitamin D or fatty acids [36, 37].

(f) Patients (cancer and non-cancer) with elevated plasma levels of Naga6 often have low levels of ascorbate (vitamin C) and calcidiol (25 $(OH)D_3$, vitamin D_3). Optimal performance of immune cells relies on sufficient plasma levels of ascorbate and calcidiol. In fact, I have proposed that the effects of these two compounds on immune cells are mutually dependent (Albracht, S.P.J. (2021) Hypothesis: mutual dependency of ascorbate and calcidiol for optimal performance of the immune system, Medical Hypothesis, under review). Hence it is necessary that during immunotherapy with GcMAF sufficient supplemental ascorbic acid and calcidiol are taken to keep plasma levels to 70 to 80 μM ascorbate (intake 200 to 500 mg per day) and 80 to 200 nM calcidiol (intake 50 to 70 μg per day).

(h) GcMAF immunotherapy may be of help to cancer patients where traditional cancer treatments failed. However, their immune system should be intact; GcMAF will not be effective in anaemic patients. Likewise, because the immune system has only a limited capacity to remove malignant tumours, the bulk tumour mass should first be removed by surgery and/or destroyed by γ-irradiation. Any chemotherapeutic treatment should have been finished for at least 4 weeks, to enable a sufficient recovery of the immune system.

(i) In most cases, organ destruction caused by malignant tumours will not be cured by GcMAF immunotherapy. Therefore the onset of any unwanted malignant neoplasms detectable by elevated serum levels of Naga6 (or otherwise) should be immediately treated by immunotherapy with GcMAF plus ascorbate and calcidiol. It is my opinion, from literature and own experience, that elevated Naga6 levels, whatever the cause, will virtually always decrease by such a treatment.

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S.P.J. Albracht is the sole author

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

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