The present invention relates to the combination therapy of antibodies against human CSF-IR with a TLR9 agonist.

Title: COMBINATION THERAPY OF ANTIBODIES AGAINST HUMAN CSF-IR AND TLR9 AGONIST

Abstract: The present invention relates to the combination therapy of antibodies against human CSF-IR with a TLR9 agonist.
Combination therapy of antibodies against human CSF-1R and TLR9 agonist

The present invention relates to the combination therapy of antibodies against human CSF-1R with a TLR9 agonist.

Background of the Invention

CSF-1R and CSF-1R antibodies


CSF-1R is the receptor for CSF-1 (colony stimulating factor 1, also called M-CSF, macrophage colony-stimulating factor) and mediates the biological effects of this cytokine (Sherr, C.J., et al, Cell 41 (1985) 665-676). The cloning of the colony stimulating factor-1 receptor (CSF-1R) (also called c-fms) was described for the first time in Roussel, M.F., et al., Nature 325 (1987) 549-552. In that publication, it was shown that CSF-1R had transforming potential dependent on changes in the C-terminal tail of the protein including the loss of the inhibitory tyrosine 969 phosphorylation which binds Cbl and thereby regulates receptor down regulation (Lee, P.S., et al, Embo J. 18 (1999) 3616-3628). Recently a second ligand for CSF-1R termed interleukin-34 (IL-34) was identified (Lin, H., et al, Science 320 (2008) 807-811).

Currently two CSF-1R ligands that bind to the extracellular domain of CSF-1R are known. The first one is CSF-1 (colony stimulating factor 1, also called M-CSF, macrophage; SEQ ID NO: 86) and is found extracellularly as a disulfide-linked homodimer (Stanley, E.R. et al., Journal of Cellular Biochemistry 21 (1983) 151-159; Stanley, E.R. et al, Stem Cells 12 Suppl. 1 (1995) 15-24). The second one is IL-34 (Human IL-34; SEQ ID NO: 87) (Hume, D. A., et al, Blood 119 (2012) 1810-1820). The main biological effects of CSF-1R signaling are the differentiation, proliferation, migration, and survival of hematopoietic precursor cells to the macrophage lineage (including osteoclast). Activation of CSF-1R is mediated by its CSF-1R ligands, CSF-1 (M-CSF) and IL-34.


Further signaling is mediated by the p85 subunit of PI3K and Grb2 connecting to the PI3K/AKT and Ras/MAPK pathways, respectively. These two important signaling pathways can regulate proliferation, survival and apoptosis. Other signaling molecules that bind the phosphorylated intracellular domain of CSF-IR include STAT1, STAT3, PLCγ, and Cbl (Bourette, R.P. and Rohrschneider, L.R., Growth Factors 17 (2000) 155-166).

CSF-IR signaling has a physiological role in immune responses, in bone remodeling and in the reproductive system. The knockout animals for either CSF-1 (Pollard, J.W., Mol. Reprod. Dev. 46 (1997) 54-61) or CSF-IR (Dai, X.M., et al, Blood 99 (2002) 111-120) have been shown to have osteopetrotic, hematopoietic, tissue macrophage, and reproductive phenotypes consistent with a role for CSF-IR in the respective cell types.


10 TLRs, TLR9 and TLR9 agonists

Different experimental Toll-like receptor agonists for cancer therapy are described (Galluzzi et al, OncolImmunology, 1:5, (2012) 699-716) Toll-like receptors (TLRs) in general are prototypic pattern recognition receptors (PRRs) best known for their ability to activate the innate immune system in response to conserved microbial components such as lipopolysaccharide and double-stranded RNA. Accumulating evidence indicates that the function of TLRs is not restricted to the elicitation of innate immune responses against invading pathogens. TLRs have indeed been shown to participate in tissue repair and injury-induced regeneration as well as in adaptive immune responses against cancer. In particular, TLR4 signaling appears to be required for the efficient processing and cross-presentation of cell-associated tumor antigens by dendritic cells, which de facto underlie optimal therapeutic responses to some anticancer drugs. Thus, TLRs constitute prominent therapeutic targets for the activation/intensification of anticancer immune responses. In line with this notion, long-used preparations such as the Coley toxin (a mixture of killed Streptococcus pyogenes and Serratia marcescens bacteria) and the bacillus Calmette-Guerin (BCG, an attenuated strain of Mycobacterium bovis originally developed as a vaccine against tuberculosis), both of which have been associated with consistent anticancer responses, potently activate TLR2 and TLR4 signaling.

According to currently accepted models, TLRs operate as homo- or hetero-dimers and are expressed either at the plasma membrane (TLRs that mainly bind proteolipidic MAMPs, i.e., TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) or in endosomes (TLRs that detect microbial nucleic acids, i.e., TLR3, TLR7, TLR8, TLR9). TLR10, which is the only orphan receptor among human TLRs, has also been shown to co-localize with TLR2 at phagosomes, suggesting that it may share
with TLR2 the ability to bind acylated lipopeptides. Conclusive data on this issue, however, have not yet been reported. TLR1 1-13 are not encoded in the human genome. In mice, TLR1 1-13 are constitutively expressed in the central nervous system and undergo several-fold induction in response to cysticercosis.21 TLR1 can reportedly recognize a profilin-like protein expressed by Toxoplasma gondii and has been localized at the endoplasmic reticulum. TLR13 also appears to be localized intracellularly, where it would specifically detect the vesicular stomatitis virus. So far, the ligand specificity and intracellular localization of TLR12 remain unexplored.

So in summary the different Toll-like receptors have different functions, structure and expression patterns. Consequently also their ligands and agonist have different functions and mode of action. E.g. LPS, the natural ligand of TLR2 and TLR4 also known as endotoxin, has anticancer properties which have been discovered as early as in the 1960s, when the existence of TLRs was not even suspected.

TLR9 is mainly found in the endosomal compartment of B cells, monocytes, macrophages and plasmacytoid Dendritic Cells DCs (Galluzzi et al, OncolImmunology, 1:5, (2012) 699-716). The main ligand of TLR9 is bacterial/viral DNA, differing from its mammalian counterpart for the elevated frequency of unmethylated CpG oligodeoxynucleotides. Indeed, whereas mammalian DNA has no immunostimulatory activity, the administration of bacterial/viral DNA induces a potent Th1 immune response in vivo, which is entirely abrogated in TLR9−/− mice. CpG oligodeoxynucleotides (or CpG ODN) are short single-stranded synthetic DNA molecules that contain a cytidine triphosphate deoxynucleotide ("C") followed by a guanine triphosphate deoxynucleotide ("G"). The "p" refers to the phosphodiester link between consecutive nucleotides, although some ODN have a modified phosphorothioate (PS) backbone instead. When these CpG motifs are unmethylated, they act as immunostimulants (Weiner, GJ; et al, PNAS 94 (1997) 10833-7).

CpG motifs are considered pathogen-associated molecular patterns (PAMPs) due to their abundance in microbial genomes but their rarity in vertebrate genomes (Bauer, S; Current Topics in Microbiology and Immunology 270 (2002) 145-54). The CpG PAMP is recognized by the pattern recognition receptor (PRR) Toll-Like Receptor 9 (TLR9), which is constitutively expressed only in B cells and plasmacytoid dendritic cells (pDCs) in humans and other higher primates (Rothenfusser, S; et al, Human immunology 63 (2002) 1111-9)
Synthetic CpG ODN differ from microbial DNA in that they have a partially or completely phosphorothioated (PS) backbone instead of the typical phosphodiester backbone and a poly G tail at the 3' end, 5' end, or both. PS modification protects the ODN from being degraded by nucleases such as DNase in the body and poly G tail enhances cellular uptake (Dalpeke, AH et al, Immunology 106 (2002) 102-12). The poly G tails form intermolecular tetrads that result in high molecular weight aggregates. These aggregates are responsible for the increased activity the poly G sequence impart; not the sequence itself.

These synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs), such as ODN 1826, have been extensively studied as adjuvants (Steinhagen F. et al, 2011; Vaccine 29(17):3341-55). These CpG motifs are present at a 20-fold greater frequency in bacterial DNA compared to mammalian DNA (Hemmi H. et al, 2000. Nature 408: 740-5). CpG ODNs agonize TLR9, which is expressed on human B cells and plasmacytoid dendritic cells (pDCs), thereby inducing Th1-dominated immune responses (Coffman et al., 2010. Immunity 33(4):492-503). Pre-clinical studies, conducted in rodents and non-human primates, and human clinical trials have demonstrated that CpG ODNs can significantly improve vaccine-specific antibody responses (Steinhagen F. et al, 2011; Vaccine 29(17):3341-55).

Numerous sequences have been shown to stimulate TLR9 with variations in the number and location of CpG dimers, as well as the precise base sequences flanking the CpG dimers. This led to the creation of classes or categories of CpG ODN, which are all TLR9 agonist based on their sequence, secondary structures, and effect on human peripheral blood mononuclear cells (PBMCs). The three main classes of CpG ODNs are class A, B and C, which differ in their immune-stimulatory activities (Krug A. et al., 2001, Eur J Immunol, 31(7): 2154-63). Furthermore, CpG ODNs activate TLR9 in a species-specific manner (Bauer, S. et al, 2001, PNAS, 98(16):9237-42). One of the first Class A ODN, ODN 2216, was described in 2001 by Krug et al (see above) This class of ODN was distinctly different from the previously described Class B ODN (i.e., ODN 2006) in that it stimulated the production of large amounts of Type I interferons, the most important one being IFNa, and induced the maturation of pDCs.

Class A ODN are also strong activators of NK cells through indirect cytokine signaling. Class A ODN typically contain 7 to 10 PS-modified bases at one or both ends that resist degradation by nucleases and increase the longevity of the ODN.
The above rules strictly define the class, but variability of the sequence within these "rules" is possible. It should also be noted that changes to the sequence will affect the magnitude of the response. For example, the internal palindrome sequence can be 4 to 8 base pairs in length and vary in the order of bases, however the pattern, 5'-Pu Pu CG Pu Py CG Py Py-3', was found to be the most active when compared to several other sequences. The poly G tail found at either end of the DNA strand can vary in length and even number, but its presence is critical to the activity of the molecule.

Class B ODN (i.e. ODN 2007) are strong stimulators of human B cell and monocyte maturation. They also stimulate the maturation of pDC but to a lesser extent than Class A ODN and very small amounts of IFNa. The strongest ODN in this class have three 6mer sequences. Class B ODNs have been studied extensively as therapeutic agents because of their ability to induce a strong humoral immune response, making them ideal as a vaccine adjuvant.


Moreover, the administration of type B CpG oligonucleotides (alone or combined with chemotherapeutics or peptide vaccines) to tumor-bearing rodents reportedly exerts potent anticancer effects. Initial Phase I/II clinical trials to test the safety and efficacy of CpG-7909 for oncological indications were launched in April 2000. Approximately in the same period, CpG-7909 begun to be extensively investigated as an adjuvant for cancer-unrelated indications (mainly antiviral vaccines), showing no severe side effects and encouraging efficacy.

During the last decade, the safety and anticancer potential of CpG-7909 (as a standalone agent or in combination with chemotherapy and/or vaccination approaches) have been investigated in a large number of Phase I/II clinical trials, including studies with leukemia, lymphoma, basal cell carcinoma, melanoma,
esophageal squamous cell carcinoma, NSCLC, renal cell carcinoma, and prostate cancer patients. Several TLR9 agonist are known and currently developed in clinical testing Agatolimod (tricosasodium salt of a synthetic 24-mer oligonucleotide containing 3 CpG motifs ; Pfizer) GNKG168 (CpG ODN; SBI Biotech) , IMO-2055 (synthetic oligonucleotide containing unmethylated CpG dinucleotides; Idera Pharmaceuticals), MGN-1703 (Mologen). Typically these TLR9 agonist are used in the treatment of different cancers:

Schroder K et al, ( J Leukoc Biol. 81(6) (2007) 1577-90 relates to TLR agonist (unmethylated CpG-containing DNA (CpG DNA)) the regulation of mouse T1R9 expression and defines a molecular mechanism by which IFN-gamma amplifies mouse macrophage responses to CpG DNA.

**Summary of the Invention**

The invention comprises the combination therapy of an antibody binding to human CSF-1R (including antibodies binding to domains D1-D3 and antibodies binding to domains D4-D5, preferably antibodies binding to domains D4-D5 as described herein) with a Toll-like receptor 9 (TLR9) agonist for use in the treatment of cancer.

One embodiment is an antibody which binds to human CSF-1R characterized in binding to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-1R for use in

a) the inhibition of cell proliferation in CSF-1R ligand-dependent and/or CSF-1 ligand-independent CSF-1R expressing tumor cells;

b) the inhibition of cell proliferation of tumors with CSF-1R ligand-dependent and/or CSF-1R ligand-independent CSF-1R expressing macrophage infiltrate;

c) the inhibition of cell survival (in CSF-1R ligand-dependant and/or CSF-1R ligand-independent) CSF-1R expressing monocytes and macrophages; and/or

d) the inhibition of cell differentiation (in CSF-1R ligand-dependent and/or CSF-1R ligand-independent) CSF-1R expressing monocytes into macrophages,
wherein the anti-CSF-IR antibody is administered in combination with TLR9 agonist.

In one embodiment the TLR9 agonist is characterized by induction of IFN-alpha, IL-6, and/or IL-12 (elevating the levels of IFN-alpha, IL-6, and/or IL-12) in plasmacytoid dendritic cells (pDCs). In one embodiment the TLR9 agonist is characterized by elevating the level of IFN-alpha in human plasmacytoid dendritic cells (pDCs) (as measured by sandwich ELISA).

In one embodiment of the invention the TLR9 agonist of the invention is a oligodeoxynucleotides containing a) cytosine-phosphate-guanosine (CpG) motifs (CpG ODNs) b) pyrimidine -phosphate-guanosine (YpG) motifs (YpG ODNs) or c) cytosine-phosphate -purine (CpR) motifs (CpR ODNs).

In one embodiment of the invention the TLR9 agonist of the invention is a oligodeoxynucleotides containing cytosine-phosphate-guanosine (CpG) motifs (CpG ODNs).

CSF-IR antibodies binding to domains D1-D3 of human CSF-IR are described e.g. in WO 2009/026303 and WO 2009/112245 relate to certain anti-CSF-IR antibodies binding to CSF-IR within the first three subdomains (D1 to D3) of the Extracellular Domain (CSF-1R-ECD). WO2011/123381(A1) relates to antibodies against CSF-IR. and Sherr, C.J., et al, Blood 73 (1989) 1786-1793 (typically these antibodies are characterized by inhibiting CSF-IR ligand-dependent but not CSF-IR ligand-independent CSF-IR proliferation and/OR signaling).

CSF-IR antibodies binding to domains D4-D5 of human CSF-IR are described e.g. within the present invention, in WO2011/070024, in PCT/EP2012/075241 and Sherr, C.J., et al, Blood 73 (1989) 1786-1793 (typically these antibodies are characterized by inhibiting CSF-IR ligand-dependent and CSF-IR ligand-independent CSF-IR proliferation and/OR signaling).

In one embodiment is an antibody which binds to human CSF-IR is characterized in binding to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-IR.

In one embodiment of the invention the anti-CSF-IR antibody is characterized in that the antibody binds to human CSF-IR fragment delD4 (SEQ ID NO: 65) and to
human CSF-IR Extracellular Domain (SEQ ID NO: 64) with a ratio of 1:50 or lower.

In one embodiment of the invention the antibody is characterized in that the antibody does not bind to human CSF-IR fragment delD4 (SEQ ID NO: 65).

In one embodiment of the invention the antibody is characterized in that the heavy chain variable domain is SEQ ID NO: 7 and the light chain variable domain is SEQ ID NO: 8,

b) the heavy chain variable domain is SEQ ID NO: 15 and the light chain variable domain is SEQ ID NO: 16;

c) the heavy chain variable domain is SEQ ID NO: 75 and the light chain variable domain is SEQ ID NO: 76;

d) the heavy chain variable domain is SEQ ID NO: 83 and the light chain variable domain is SEQ ID NO: 84;

or a humanized version thereof.

In one embodiment of the invention the antibody is characterized in that the heavy chain variable domain is SEQ ID NO: 7 and the light chain variable domain is SEQ ID NO: 8,

b) the heavy chain variable domain is SEQ ID NO: 15 and the light chain variable domain is SEQ ID NO: 16;

or a humanized version thereof.

In one embodiment of the invention the antibody is characterized in that the heavy chain variable domain is SEQ ID NO: 23 and the light chain variable domain is SEQ ID NO: 24, or

b) the heavy chain variable domain is SEQ ID NO: 31 and the light chain variable domain is SEQ ID NO: 32, or

c) the heavy chain variable domain is SEQ ID NO: 39 and the light chain variable domain is SEQ ID NO: 40, or

d) the heavy chain variable domain is SEQ ID NO: 47 and the light chain variable domain is SEQ ID NO: 48, or
e) the heavy chain variable domain is SEQ ID NO:55 and the light chain variable domain is SEQ ID NO:56.

In one embodiment of the invention the antibody is characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO: 2, and a CDR1 region of SEQ ID NO:3, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5, and a CDR1 region of SEQ ID NO:6, or

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 9, a CDR2 region of SEQ ID NO: 10, and a CDR1 region of SEQ ID NO: 11, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 12, a CDR2 region of SEQ ID NO: 13, and a CDR1 region of SEQ ID NO: 14, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO:21, and a CDR1 region of SEQ ID NO:22, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

f) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ
ID NO:43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO:45, and a CDR1 region of SEQ ID NO:46, or

g) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 49, a CDR2 region of SEQ ID NO: 50, and a CDR1 region of SEQ ID NO: 51, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:52, a CDR2 region of SEQ ID NO: 53, and a CDR1 region of SEQ ID NO: 54; or

h) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:69, a CDR2 region of SEQ ID NO: 70, and a CDR1 region of SEQ ID NO:71, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 72, a CDR2 region of SEQ ID NO:73, and a CDR1 region of SEQ ID NO:74, or

i) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:77, a CDR2 region of SEQ ID NO: 78, and a CDR1 region of SEQ ID NO: 79, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:80, a CDR2 region of SEQ ID NO: 81, and a CDR1 region of SEQ ID NO: 82.

One embodiment of the invention is an antibody binding to human CSF-IR, for use in the treatment of a patient having a CSF-IR expressing tumor or having a tumor with CSF-IR expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-IR ligand and wherein the anti-CSF-IR antibody is administered in combination with a TLR9 agonist.

In one embodiment of the invention the antibody is of human IgGl subclass or of human IgG4 subclass.

The invention further comprises the use an of an CSF-IR antibody according to the invention for the manufacture of a medicament for treatment of a CSF-IR mediated disease in combination with a TLR9 agonist.

The invention further comprises the use an of an CSF-IR antibody according to the invention for the manufacture of a medicament for treatment of cancer in combination with a TLR9 agonist.
The invention further comprises the use of an CSF-IR antibody according to the invention for the manufacture of a medicament for treatment of bone loss in combination with a TLR9 agonist.

The invention further comprises the use of an CSF-IR antibody according to the invention for the manufacture of a medicament for treatment of metastasis in combination with a TLR9 agonist.

The invention further comprises the use of an CSF-IR antibody according to the invention for the manufacture of a medicament for treatment of inflammatory diseases in combination with a TLR9 agonist.

The invention further comprises an CSF-IR antibody according to the invention for treatment of a CSF-IR mediated disease in combination with a TLR9 agonist.

The invention further comprises an CSF-IR antibody according to the invention for treatment of cancer in combination with a TLR9 agonist.

The invention further comprises a CSF-IR antibody according to the invention for treatment of bone loss in combination with a TLR9 agonist.

The invention further comprises a CSF-IR antibody according to the invention for treatment of metastasis in combination with a TLR9 agonist.

The invention further comprises an CSF-IR antibody according to the invention for treatment of inflammatory diseases in combination with a TLR9 agonist.

The combination therapies of the antibodies described herein show benefits for patients in need of a CSF-IR targeting therapy.

The antibodies according to the invention show efficient antiproliferative activity against ligand-independent and ligand-dependent proliferation and are therefore especially useful in the treatment of cancer and metastasis in combination with a TLR9 agonist.

The invention further provides a method for treating a patient suffering from cancer, comprising administering to a patient diagnosed as having such a disease (and therefore being in need of such a therapy) an effective amount of an CSF-IR antibody according to the invention in combination with a TLR-9 agonist.
Surprisingly it has been found that addition of TLR9 agonist to anti-CSF-IR antibody therapy resulted in a statistically significant more than additive improvement of median time to progression compared to anti-CSF-IR antibody monotherapy or TLR9 agonist monotherapy (see Example 13 and Figure 1).

**Description of the Figures**

**Figure 1** In vivo efficacy of a combination of a <CSF1R> antibody with a TLR9 agonist in the MC38 mouse CRC in vivo model - Median time to progression. Addition of TLR9 agonist to anti-CSF-IR antibody therapy resulted in a statistically significant more than additive improvement of median time to progression (46 days) compared to anti-CSF-IR antibody monotherapy or TLR9 agonist monotherapy.

**Figure 2** Growth inhibition of BeWo tumor cells in 3D culture under treatment with different anti-CSF-IR monoclonal antibodies at a concentration of l^g/ml.

X axis: viability normalized mean relative light units (RLU) corresponding to the ATP-content of the cells (CellTiterGlo assay).

Y axis: tested probes: Minimal Medium (0.5% FBS), mouse IgGl (mlgGl, ^g/ml), mouse IgG2a (mlgG2a ^g/ml), CSF-1 only, Mab 2F1 1, Mab 2E10, Mab2H7, MabGlO and SC 2-4A5. Highest inhibition of CSF-1 induced growth was observed with the anti-CSF-IR antibodies according to the invention.

**Figure 3a** Biacore sensogram of binding of different anti-CSF-IR antibodies to immobilized human CSF-IR fragment delD4 (comprising the extracellular subdomains D1 -D3 and D5) (SEQ ID NO: 65) (y-axis: binding signal in Response Units (RU), baseline = 0 RU, x-axis: time in seconds (s)): While the antibodies Mab 3291 and sc 2-4A5 clearly show binding to this delD4 fragment, the antibodies according to the invention e.g. Mab 2F1 1, and Mab 2E10, did not bind to the CSF-IR fragment delD4. The control anti-CCR5 antibody m<CCR5>Pz03.1C5 did also not bind to the CSF-IR fragment delD4.

**Figure 3b** Biacore sensogram of binding of different anti-CSF-IR antibodies to immobilized human CSF-IR Extracellular Domain
All anti-CSF-1R antibodies show binding to CSF-1R-ECD. The control anti-CCR5 antibody m<CCR5>Pz03.1C5 did not bind to the CSF-1R-ECD.

**Figure 3c** Biacore sensogram of binding of different anti-CSF-1R antibodies to immobilized human CSF-1R fragment delD4 (comprising the extracellular subdomains D1-D5) (SEQ ID NO: 64) (y-axis: binding signal in Response Units (RU), baseline = 0 RU, x-axis: time in seconds (s)): Mab 1G10, Mab 2H7 and humanized hMab 2F1 l-e7 did not bind to the CSF-1R fragment delD4. The control anti-CCR5 antibody m<CCR5>Pz03.1C5 did also not bind to the CSF-1R fragment delD4.

**Figure 3d** Biacore sensogram of binding of different anti-CSF-1R antibodies to immobilized human CSF-1R Extracellular Domain (CSF-1R-ECD) (comprising the extracellular subdomains D1-D5) (SEQ ID NO: 64) (y-axis: binding signal in Response Units (RU), baseline = 0 RU, x-axis: time in seconds (s)): All anti-CSF-1R antibodies Mab 1G10, Mab 2H7 and humanized hMab 2F1 l-e7 showed binding to CSF-1R-ECD. The control anti-CCR5 antibody m<CCR5>Pz03.1C5 did not bind to the CSF-1R-ECD.

**Figure 3e** Biacore sensogram of binding of different anti-CSF-1R antibodies to immobilized human CSF-1R fragment delD4 (comprising the extracellular subdomains D1-D3 and D5) (SEQ ID NO: 65) (y-axis: binding signal in Response Units (RU), baseline = 0 RU, x-axis: time in seconds (s)): All anti-CSF-1R antibodies 1.2.SM, CXIIIG6, abl0676 and MAB3291 show binding to the CSF-1R fragment delD4. The control anti-CCR5 antibody m<CCR5>Pz03.1C5 did also not bind to the CSF-1R fragment delD4.

**Figure 3f** Biacore sensogram of binding of different anti-CSF-1R antibodies to immobilized human CSF-1R Extracellular Domain (CSF-1R-ECD) (comprising the extracellular subdomains D1-D5) (SEQ ID NO: 64) (y-axis: binding signal in Response Units (RU), baseline = 0 RU, x-axis: time in seconds (s)): All anti-CSF-1R antibodies show binding to CSF-1R-ECD. The control anti-CCR5 antibody m<CCR5>Pz03.1C5 did not bind to the CSF-1R-ECD.
All anti-CSF-1R antibodies 1.2.SM, CXIIG6, abl0676 and MAB3291 show binding to CSF-1R-ECD. The control anti-CCR5 antibody m<CCR5>Pz03.1C5 did not bind to the CSF-1R-ECD.

Figure 4a-d  CSF-1 levels in Cynomolgous monkey after application of different dosages of anti-CSF-1R antibody according to the invention.

Figure 5a-b  
5a: Human Monocytes differentiated into macrophages with coculture of GM-CSF or CSF-1 (100 ng/ml ligand). After 6 days differentiation addition of R07155. Cell viability was measured at day 7 of antibody treatment in a CTG Viability Assay (CellTiterGlo® Promega). Calculation of % cell viability: RLU signals from treated cells divided by RLU signal from untreated control without antibody, (n =4)

5b: Human Monocytes differentiated into macrophages with GM-CSF (M1) or M-CSF (M2) for 7 days. Phenotype analyzed by indirect fluorescence analysis - staining with anti CD163-PE, anti CD80-PE or anti HLA-DR/DQ/DP-Zenon-Alexa647 labeled. The number in each histogram corresponds to mean ratio fluorescence intensity (MRFI); calculated ratio between mean fluorescence intensity (MFI) of cells stained with the selected antibody (empty histogram) and of corresponding isotyp control (negative control; gray filled histogram) (mean ± SD; n ≥ 5).

Detailed Description of the Invention

Many tumors are characterized by a prominent immune cell infiltrate, including macrophages. Initially, the immune cells were thought to be part of a defense mechanism against the tumor, but recent data support the notion that several immune cell populations including macrophages may, in fact, promote tumor progression. Macrophages are characterized by their plasticity. Depending on the cytokine microenvironment, macrophages can exhibit so-called M1 or M2-subtypes. M2 macrophages are engaged in the suppression of tumor immunity. They also play an important role in tissue repair functions such as angiogenesis and tissue remodeling which are coopted by the tumor to support growth. In contrast to tumor promoting M2 macrophages, M1 macrophages exhibit antitumor activity via the secretion of inflammatory cytokines and their engagement in antigen

By secreting various cytokines such as colony stimulating factor 1 (CSF-1) and IL-10, tumor cells are able to recruit and shape macrophages into the M2 subtype, whereas cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF), IFN-gamma program macrophages towards the M1 subtype. Using immunohistochemistry, it is possible to distinguish between a macrophage subpopulation co-expressing CD68 and CD163, which is likely to be enriched for M2 Macrophages, and a subset showing the CD68+/MHC II+, or CD68+/CD80+ immunophenotype, likely to include M1 macrophages. Cell shape, size, and spatial distribution of CD68 and CD163 positive macrophages is consistent with published hypotheses on a tumor-promoting role of M2 macrophages, for example by their preferential location in tumor intersecting stroma, and vital tumor areas. In contrast, CD68+/MHC class II+ macrophages are ubiquitously found. Their hypothetical role in phagocytosis is reflected by clusters of the CD68+/MHC class II+, but CD163- immunophenotype near apoptotic cells and necrotic tumor areas.

The subtype and marker expression of different macrophage subpopulations is linked with their functional state. M2 macrophages can support tumorigenesis by:

a) enhancing angiogenesis via the secretion of angiogenic factors such as VEGF or bFGF,

b) supporting metastasis formation via secretion of matrix metalloproteinases(MMPs), growth factors and migratory factors guiding the tumor cells to the blood stream and setting up the metastatic niche (Wyckoff, J. et al, Cancer Res. 67 (2007) 2649-2656),

c) playing a role in building an immunosuppressive milieu by secreting immunosuppressive cytokines such as IL-4, 11-13, IL-1ra and IL-10, which in turn regulate T regulatory cell function. Conversely CD4 positive T cells have been shown to enhance the activity of tumor promoting macrophages in preclinical models (Mantovani, A. et al, Eur. J. Cancer 40 (2004) 1660-1667; DeNardo, D. et al, Cancer Cell 16 (2009) 91-102).

Accordingly, in several types of cancer (e.g. breast, ovarian, Hodgkin’s lymphoma) the prevalence of M2 subtype tumor associated macrophages (TAMs) has been associated with poor prognosis (Bingle, L. et al, J. Pathol. 3 (2002) 254-265; Orre,

Recently, a so-called immune signature comprising high numbers of macrophages and CD4 positive T cells, but low numbers of cytotoxic CD8 positive T cells was shown to correlate with reduced overall survival (OS) in breast cancer patients and to represent an independent prognostic factor (DeNardo, D. et al., Cancer Discovery 1 (2011) 54-67).

Consistent with a role for CSF-1 in driving the pro-tumorigenic function of M2 macrophages, high CSF-1 expression in rare sarcomas or locally aggressive connective tissue tumors, such as pigmented villonodular synovitis (PVNS) and tenosynovial giant cell tumor (TGCT) due in part to a translocation of the CSF-1 gene, leads to the accumulation of monocytes and macrophages expressing the receptor for CSF-1, the colony-stimulating factor 1 receptor (CSF-1R) forming the majority of the tumor mass (West, R.B. et al, Proc. Natl. Acad. Sci. USA 3 (2006) 690-695). These tumors were subsequently used to define a CSF-1 dependent macrophage signature by gene expression profiling. In breast cancer and leiomyosarcoma patient tumors this CSF-1 response gene signature predicts poor prognosis (Espinosa, I. et al, Am. J. Pathol. 6 (2009) 2347-2356; Beck, A. et al, Clin. Cancer Res. 3 (2009) 778-787).

CSF-1R belongs to the class III subfamily of receptor tyrosine kinases and is encoded by the c-fms proto-oncogene. Binding of CSF-1 or IL-34 induces receptor dimerization, followed by autophosphorylation and activation of downstream signaling cascades. Activation of CSF-1R regulates the survival, proliferation and differentiation of monocytes and macrophages (Xiong, Y. et al., J. Biol. Chem. 286 (2011) 952-960).

In addition to cells of the monocytic lineage and osteoclasts, which derive from the same hematopoietic precursor as the macrophage, CSF-1R/c-fms has also been
found to be expressed by several human epithelial cancers such as ovarian and breast cancer and in leiomyosarcoma and TGCT/PVNS, albeit at lower expression levels compared to macrophages. As with TGCT/PVNS, elevated levels of CSF-1, the ligand for CSF-1R, in serum as well as ascites of ovarian cancer patients have been correlated with poor prognosis (Scholl, S. et al., Br. J. Cancer 62 (1994) 342-346; Price, F. et al, Am. J. Obstet. Gynecol. 168 (1993) 520-527). Furthermore, a constitutively active mutant form of CSF-1R is able to transform NIH3T3 cells, one of the properties of an oncogene (Chambers, S., Future Oncol 5 (2009) 1429-1440).

Preclinical models provide validation of CSF-IR as an oncology target. Blockade of CSF-1 as well as CSF-IR activity results in reduced recruitment of TAMs. Chemotherapy resulted in elevated CSF-1 expression in tumor cells leading to enhanced TAM recruitment. Blockade of CSF-IR in combination with paclitaxel resulted in activation of CD8 positive cytotoxic T cells leading to reduced tumor growth and metastatic burden in a spontaneous transgenic breast cancer model (DeNardo, D. et al, Cancer Discovery 1 (2011) 54-67).

In one embodiment the invention comprises the combination therapy of an antibody binding to human CSF-IR, characterized in that the antibody binds to human CSF-IR Extracellular Domain (SEQ ID NO: 64) in combination with a TLR9 agonist for use in the treatment of cancer.

In one embodiment the invention comprises the combination therapy of an antibody binding to human CSF-IR, characterized in that the antibody binds to human CSF-IR Extracellular Domain (SEQ ID NO: 64) (comprising domains D1 to D5) and does not bind to domains D1 to D3 (SEQ ID NO: 66) of the extracellular domain of human CSF-IR in combination with a TLR9 agonist for use in the treatment of cancer.

In one embodiment the antibody binding to human CSF-IR used in the combination therapy is characterized in that

a) the heavy chain variable domain is SEQ ID NO:7 and the light chain variable domain is SEQ ID NO:8,

b) the heavy chain variable domain is SEQ ID NO:15 and the light chain variable domain is SEQ ID NO:16;

c) the heavy chain variable domain is SEQ ID NO:75 and the light chain variable domain is SEQ ID NO:76.
d) the heavy chain variable domain is SEQ ID NO: 83 and the light chain variable domain is SEQ ID NO: 84;

or a humanized version thereof.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain is SEQ ID NO: 23 and the light chain variable domain is SEQ ID NO: 24, or

b) the heavy chain variable domain is SEQ ID NO: 31 and the light chain variable domain is SEQ ID NO: 32, or

c) the heavy chain variable domain is SEQ ID NO: 39 and the light chain variable domain is SEQ ID NO: 40, or

d) the heavy chain variable domain is SEQ ID NO: 47 and the light chain variable domain is SEQ ID NO: 48, or

e) the heavy chain variable domain is SEQ ID NO: 55 and the light chain variable domain is SEQ ID NO: 56.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain is SEQ ID NO: 23 and the light chain variable domain is SEQ ID NO: 24, or

b) the heavy chain variable domain is SEQ ID NO: 31 and the light chain variable domain is SEQ ID NO: 32, or

c) the heavy chain variable domain is SEQ ID NO: 39 and the light chain variable domain is SEQ ID NO: 40, or

d) the heavy chain variable domain is SEQ ID NO: 47 and the light chain variable domain is SEQ ID NO: 48.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO: 23 and the light chain variable domain is SEQ ID NO: 24.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that
the heavy chain variable domain is SEQ ID NO:31 and the light chain variable domain is SEQ ID NO:32.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:39 and the light chain variable domain is SEQ ID NO:40.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:47 and the light chain variable domain is SEQ ID NO:48.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:15 and the light chain variable domain is SEQ ID NO:16, or a humanized version thereof.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:75 and the light chain variable domain is SEQ ID NO:76;

or a humanized version thereof.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:83 and the light chain variable domain is SEQ ID NO:84;

or a humanized version thereof.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:1, a CDR2 region of SEQ ID NO:2, and a CDR1 region of SEQ ID
NO:3, and the light chain variable domain comprises a CDR3 region of
SEQ ID NO: 4, a CDR2 region of SEQ ID NO: 5, and a CDR1 region of
SEQ ID NO: 6, or,

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID
NO: 9, a CDR2 region of SEQ ID NO: 10, and a CDR1 region of SEQ ID
NO: 11, and the light chain variable domain comprises a CDR3 region of
SEQ ID NO: 12, a CDR2 region of SEQ ID NO: 13, and a CDR1 region
of SEQ ID NO: 14, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID
NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ
ID NO: 19, and the light chain variable domain comprises a CDR3 region
of SEQ ID NO: 20, a CDR2 region of SEQ ID NO: 21, and a CDR1
region of SEQ ID NO: 22, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID
NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ
ID NO: 27, and the light chain variable domain comprises a CDR3 region
of SEQ ID NO: 28, a CDR2 region of SEQ ID NO: 29, and a CDR1
region of SEQ ID NO: 30, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID
NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ
ID NO: 35, and the light chain variable domain comprises a CDR3 region
of SEQ ID NO: 36, a CDR2 region of SEQ ID NO: 37, and a CDR1
region of SEQ ID NO: 38, or

f) the heavy chain variable domain comprises a CDR3 region of SEQ ID
NO: 41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ
ID NO: 43, and the light chain variable domain comprises a CDR3 region
of SEQ ID NO: 44, a CDR2 region of SEQ ID NO: 45, and a CDR1
region of SEQ ID NO: 46, or

g) the heavy chain variable domain comprises a CDR3 region of SEQ ID
NO: 49, a CDR2 region of SEQ ID NO: 50, and a CDR1 region of SEQ
ID NO: 51, and the light chain variable domain comprises a CDR3 region
of SEQ ID NO: 52, a CDR2 region of SEQ ID NO: 53, and a CDR1
region of SEQ ID NO: 54.
In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 2, and a CDR1 region of SEQ ID NO:3, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5, and a CDR1 region of SEQ ID NO:6, or

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 9, a CDR2 region of SEQ ID NO: 10, and a CDR1 region of SEQ ID NO: 11, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 12, a CDR2 region of SEQ ID NO: 13, and a CDR1 region of SEQ ID NO: 14, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO: 21, and a CDR1 region of SEQ ID NO: 22, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

f) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO: 43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO: 45, and a CDR1 region of SEQ ID NO: 46, or
g) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 49, a CDR2 region of SEQ ID NO: 50, and a CDR1 region of SEQ ID NO: 51, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:52, a CDR2 region of SEQ ID NO: 53, and a CDR1 region of SEQ ID NO: 54; or

h) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:69, a CDR2 region of SEQ ID NO: 70, and a CDR1 region of SEQ ID NO:71, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 72, a CDR2 region of SEQ ID NO:73, and a CDR1 region of SEQ ID NO:74, or

i) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 77, a CDR2 region of SEQ ID NO: 78, and a CDR1 region of SEQ ID NO: 79, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:80, a CDR2 region of SEQ ID NO: 81, and a CDR1 region of SEQ ID NO: 82.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:69, a CDR2 region of SEQ ID NO: 70, and a CDR1 region of SEQ ID NO:71, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 72, a CDR2 region of SEQ ID NO:73, and a CDR1 region of SEQ ID NO:74, or

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 77, a CDR2 region of SEQ ID NO: 78, and a CDR1 region of SEQ ID NO: 79, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:80, a CDR2 region of SEQ ID NO: 81, and a CDR1 region of SEQ ID NO: 82.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region...
of SEQ ID NO: 20, a CDR2 region of SEQ ID NO:21, and a CDR1 region of SEQ ID NO:22, or

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO:43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO: 45, and a CDR1 region of SEQ ID NO:46, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 49, a CDR2 region of SEQ ID NO: 50, and a CDR1 region of SEQ ID NO: 51, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:52, a CDR2 region of SEQ ID NO: 53, and a CDR1 region of SEQ ID NO: 54.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO:21, and a CDR1 region of SEQ ID NO:22, or

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region...
of SEQ ID NO:28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO:43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO:45, and a CDR1 region of SEQ ID NO:46.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO:21, and a CDR1 region of SEQ ID NO:22.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of
SEQ ID NO:36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38.

In one embodiment the antibody binding to human CSF-IR used in the combination therapy is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO:43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO:45, and a CDR1 region of SEQ ID NO:46.

In one embodiment the antibody binding to human CSF-IR used in the combination therapy is characterized in that the antibody binds to human CSF-IR fragment delD4 (SEQ ID NO: 65) and to human CSF-IR-ECD (SEQ ID NO: 64) with a ratio of 1:50 or lower, is further characterized in not binding to human CSF-IR fragment D1-D3 (SEQ ID NO: 66).

The term "antibody" encompasses the various forms of antibodies including but not being limited to whole antibodies, antibody fragments, human antibodies, humanized antibodies, chimeric antibodies, T cell epitope depleted antibodies, and further genetically engineered antibodies as long as the characteristic properties according to the invention are retained. "Antibody fragments" comprise a portion of a full length antibody, preferably the variable domain thereof, or at least the antigen binding site thereof. Examples of antibody fragments include diabodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. scFv antibodies are, e.g., described in Houston, J.S., Methods in Enzymol. 203 (1991) 46-88. In addition, antibody fragments comprise single chain polypeptides having the characteristics of a \( V_H \) domain binding to CSF-IR, namely being able to assemble together with a \( V_L \) domain, or of a \( V_L \) domain binding to CSF-IR, namely being able to assemble together with a \( V_H \) domain to a functional antigen binding site and thereby providing the property.

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of a single amino acid composition.

The term "chimeric antibody" refers to a monoclonal antibody comprising a variable region, i.e., binding region, from mouse and at least a portion of a constant
region derived from a different source or species, usually prepared by recombinant DNA techniques. Chimeric antibodies comprising a mouse variable region and a human constant region are especially preferred. Such rat/human chimeric antibodies are the product of expressed immunoglobulin genes comprising DNA segments encoding rat immunoglobulin variable regions and DNA segments encoding human immunoglobulin constant regions. Other forms of "chimeric antibodies" encompassed by the present invention are those in which the class or subclass has been modified or changed from that of the original antibody. Such "chimeric" antibodies are also referred to as "class-switched antibodies." Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques now well known in the art. See, e.g., Morrison, S.L., et al, Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855; US 5,202,238 and US 5,204,244.

The term "humanized antibody" refers to antibodies in which the framework or "complementarity determining regions" (CDR) have been modified to comprise the CDR of an immunoglobulin of different specificity as compared to that of the parent immunoglobulin. In a preferred embodiment, a murine CDR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See e.g. Riechmann, L., et al, Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. Optionally the framework region can be modified by further mutations. Also the CDRs can be modified by one or more mutations to generate antibodies according to the invention e.g. by mutagenesis based upon molecular modeling as described by Riechmann, L., et al, Nature 332 (1988) 323-327 and Queen, C, et al, Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033, or others. Particularly preferred CDRs correspond to those representing sequences recognizing the antigens noted above for chimeric antibodies. A "humanized version of an antibody according to the invention" (which is e.g. of mouse origin) refers to an antibody, which is based on the mouse antibody sequences in which the \( V_H \) and \( V_L \) are humanized by standard techniques (including CDR grafting and optionally subsequent mutagenesis of certain amino acids in the framework region and the CDRs ). Preferably such humanized version is chimerized with a human constant region (see e.g. Sequences SEQ ID NO:57-61).

Other forms of "humanized antibodies" encompassed by the present invention are those in which the constant region has been additionally modified or changed from that of the original antibody to generate the properties according to the invention, especially in regard to Clq binding and/or Fc receptor (FcR) binding.
In the following examples the terms "Mab" or "muMab" refer to murine monoclonal antibodies such as Mab 2F1 1 or Mab 2E10, whereas the term "hMab" refers to humanized monoclonal versions of such murine antibodies such as hMab 2F1 1-cl 1, hMab 2F1 1-d8, hMab 2F1 1-e7, hMab 2F1 1-f12, etc.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germ line immunoglobulin sequences. Human antibodies are well-known in the state of the art (van Dijk, M.A., and van de Winkel, J.G., Curr. Opin. Chem. Biol. 5 (2001) 368-374). Human antibodies can also be produced in transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire or a selection of human antibodies in the absence of endogenous immunoglobulin production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge (see, e.g., Jakobovits, A., et al, Proc. Natl. Acad. Sci. USA 90 (1993) 2551-2555; Jakobovits, A., et al, Nature 362 (1993) 255-258; Brueggemann, M., et al., Year Immunol. 7 (1993) 33-40). Human antibodies can also be produced in phage display libraries (Hoogenboom, H.R., and Winter, G.J. Mol. Biol. 227 (1992) 381-388; Marks, J.D., et al, J. Mol. Biol. 222 (1991) 581-597). The techniques of Cole, et al., and Boerner, et al., are also available for the preparation of human monoclonal antibodies (Cole, S.P.C., et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); and Boerner, P., et al, J. Immunol. 147 (1991) 86-95). As already mentioned for chimeric and humanized antibodies according to the invention the term "human antibody" as used herein also comprises such antibodies which are modified in the constant region to generate the properties according to the invention, especially in regard to Clq binding and/or FcR binding, e.g. by "class switching" i.e. change or mutation of Fc parts (e.g. from IgGl to IgG4 and/or IgGl/IgG4 mutation).

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell such as a NS0 or CHO cell or from an animal (e.g. a mouse) that is transgenic for human immunoglobulin genes or antibodies expressed using a recombinant expression vector transfected into a host cell. Such recombinant human antibodies have variable and constant regions in a rearranged form. The recombinant human antibodies according to the invention have been subjected to in vivo somatic hypermutation. Thus, the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences
that, while derived from and related to human germ line VH and VL sequences, may not naturally exist within the human antibody germ line repertoire in vivo.

The antibodies according to the invention include, in addition, such antibodies having "conservative sequence modifications", nucleotide and amino acid sequence modifications which do not affect or alter the above-mentioned characteristics of the antibody according to the invention. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions include ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a human anti-CSF-1R antibody can be preferably replaced with another amino acid residue from the same side chain family.


CSF-1R is the receptor for the CSF-1R ligands CSF-1 (macrophage colony stimulating factor, also called M-CSF) (SEQ ID No.: 86) and IL-34 (SEQ ID No.: 87) and mediates the biological effects of these cytokines (Sherr, C.J., et al, Cell 41 (1985) 665-676; Lin, H., et al, Science 320 (2008) 807-811). The cloning of the colony stimulating factor-1 receptor (also called c-fms) was described for the first time in Roussel, M.F., et al., Nature 325 (1987) 549-552. In that publication, it was shown that CSF-1R had transforming potential dependent on changes in the

CSF-1R is a single chain, transmembrane receptor tyrosine kinase (RTK) and a member of the family of immunoglobulin (Ig) motif containing RTKs characterized by 5 repeated Ig-like subdomains D1-D5 in the extracellular domain (ECD) of the receptor (Wang, Z., et al Molecular and Cellular Biology 13 (1993) 5348-5359). The human CSF-1R Extracellular Domain (CSF-1R-ECD) (SEQ ID NO: 64) comprises all five extracellular Ig-like subdomains D1-D5. The human CSF-1R fragment delD4 (SEQ ID NO: 65) comprises the extracellular Ig-like subdomains D1-D3 and D5, but is missing the D4 subdomain. The human CSF-1R fragment D1-D3 (SEQ ID NO: 66) comprises the respective subdomains D1-D3. The sequences are listed without the signal peptide MGSGPGVLLE LLVATAWHGQ G (SEQ ID NO: 67). The human CSF-1R fragment D4-D3 (SEQ ID NO: 85) comprises the respective subdomains D4-D3.

Currently two CSF-1R ligands that bind to the extracellular domain of CSF-1R are known. The first one is CSF-1 (colony stimulating factor 1, also called M-CSF, macrophage; human CSF-1, SEQ ID NO: 86) and is found extracellularly as a disulfide-linked homodimer (Stanley, E.R. et al., Journal of Cellular Biochemistry 21 (1983) 151-159; Stanley, E.R. et al, Stem Cells 12 Suppl. 1 (1995) 15-24). The second one is IL-34 (human IL-34; SEQ ID NO: 87) (Hume, D. A., et al, Blood 119 (2012) 1810-1820). Thus in one embodiment the term "CSF-1R ligand" refers to human CSF-1 (SEQ ID NO: 86) and/or human IL-34 (SEQ ID NO: 87).

For experiments often the active 149 amino acid (aa) fragment of human CSF-1 (aa 33-181 of SEQ ID NO: 86) is used. This active 149 aa fragment of human CSF-1 (aa 33-181 of SEQ ID NO: 86) is contained in all 3 major forms of CSF-1 and is sufficient to mediate binding to CSF-1R (Hume, D. A., et al, Blood 119 (2012) 1810-1820).

The main biological effects of CSF-1R signaling are the differentiation, proliferation, migration, and survival of hematopoietic precursor cells to the macrophage lineage (including osteoclast). Activation of CSF-1R is mediated by its CSF-1R ligands, CSF-1 (M-CSF) and IL-34. Binding of CSF-1 (M-CSF) to CSF-1R induces the formation of homodimers and activation of the kinase by tyrosine

The intracellular protein tyrosine kinase domain is interrupted by a unique insert domain that is also present in the other related RTK class III family members that include the platelet derived growth factor receptors (PDGFR), stem cell growth factor receptor (c-Kit) and fins-like cytokine receptor (FLT3). In spite of the structural homology among this family of growth factor receptors, they have distinct tissue-specific functions.


As used herein, "binding to human CSF-IR" or "specifically binding to human CSF-IR" or "which binds to human CSF-IR" or "anti-CSF-IR antibody" refers to an antibody specifically binding to the human CSF-IR antigen with a binding affinity of KD-value of 1.0 x 10⁻⁸ mol/l or lower at 35°C, in one embodiment of a KD-value of 1.0 x 10⁻⁹ mol/l or lower at 35°C. The binding affinity is determined with a standard binding assay at 35°C, such as surface plasmon resonance technique (BIACore®, GE-Healthcare Uppsala, Sweden) A method for determining the KD-value of the binding affinity is described in Example 4. Thus an "antibody binding to human CSF-IR" as used herein refers to an antibody specifically binding to the human CSF-IR antigen with a binding affinity of KD 1.0 x 10⁻⁸ mol/l or lower (preferably 1.0 x 10⁻⁸ mol/l - 1.0 x 10⁻¹² mol/l) at 35°C, preferably of a KD 1.0 x 10⁻⁹ mol/l or lower at 35°C (preferably 1.0 x 10⁻⁹ mol/l - 1.0 x 10⁻¹² mol/l).

The "binding to human CSF-IR fragment delD4 (SEQ ID NO: 65) and to human CSF-IR Extracellular Domain (SEQ ID NO: 64)" as used herein is measured by a Surface Plasmon Resonance assay (Biacore assay) as described in Example 4. The human CSF-IR fragment delD4 (SEQ ID NO: 65) or human CSF-IR Extracellular Domain (SEQ ID NO: 64), respectively, are captured to the surface (each to a separate surface) and the test antibodies were added (each in a separate
measurement) and the respective binding signals (Response Units (RU)) were determined. Reference signals (blank surface) were subtracted. If signals of nonbinding test antibodies were slightly below 0 the values were set as 0. Then the ratio of the respective binding signals (binding signal (RU) to human CSF-1R fragment delD4 /binding signal (RU) to human CSF-1R Extracellular Domain (CSF-1R-ECD)) is determined. The antibodies according to the invention have a ratio of the binding signals (RU(delD4) / RU(CSF-IR-ECD)) of 1:50 or lower, preferably of 1:100 or lower (the lower included end is 0 (e.g. if the RU is 0, then the ratio is 0:50 or 0:100)).

This means that such anti-CSF-1R antibodies according to the invention do not bind to the human CSF-1R fragment delD4 (like the anti-CCR5 antibody m<CCR5>Pz03.1C5 (deposited as DSM ACC 2683 on 18.08.2004 at DSMZ) and have binding signals for binding to the human CSF-1R fragment delD4 in the range of the anti-CCR5 antibody m<CCR5>Pz03.1C5, which are below 20 RU (Response Units), preferably below 10 RU in a Surface Plasmon Resonance (BIAcore) assay as shown in Example 4.

The term "binding to human CSF-1R fragment D1-D3" refers to a binding affinity determination by a Surface Plasmon Resonance assay (Biacore assay). The test antibody is captured to the surface and the human CSF-1R fragment D1-D3 (SEQ ID NO: 66) was added and the respective binding affinities were determined. The terms "not binding to human CSF-1R fragment D1-D3" or "which do not bind to human CSF-1R fragment D1-D3" denotes that in such an assay the detected signal was in the area of no more than 1.2 fold of background signal and therefore no significant binding could be detected and no binding affinity could be determined (see Example 10).

The term "ligand dependent" as used herein refers to a ligand-independent signaling through the extracellular ECD (and does not include the ligand independent signaling mediated by activating point mutations in the intracellular kinase domain).

In one embodiment CSF-1R ligand in this context refers a CSF-1R ligand selected from human CSF-1 (SEQ ID No: 86) and human IL-34 (SEQ ID No: 87); in one embodiment the CSF-1R ligand is human CSF-1 (SEQ ID No: 86); in one embodiment the CSF-1 R ligand is human IL-34 (SEQ ID No: 87).
The invention comprises an antibody binding to human CSF-IR, antibody binding to human CSF-IR, for use in the treatment of a patient having a tumor expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-IR ligand (in one embodiment the CSF-IR ligand is selected from human CSF-1 (SEQ ID No: 86) and human IL-34 (SEQ ID No: 87); in one embodiment the CSF-IR ligand is human CSF-1 (SEQ ID No: 86); in one embodiment the CSF-IR ligand is human IL-34 (SEQ ID No: 87)) (detectable in serum, urine or tumor biopsies), wherein the anti-CSF-IR antibody is administered in combination with TLR9 agonist. (In one embodiment the CSF-IR antibody is characterized in binding to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-IR.

The term "increase of CSF-IR ligand" refers to the overexpression of human CSF-IR ligand (in one embodiment the CSF-IR ligand is selected from human CSF-1 (SEQ ID No: 86) and human IL-34 (SEQ ID No: 87); in one embodiment the CSF-IR ligand is human CSF-1 (SEQ ID No: 86); in one embodiment the CSF-IR ligand is human IL-34 (SEQ ID No: 87)) (compared to normal tissue) before treatment or overexpression of human CSF-IR ligand induced by treatment with anti-CSF-IR antibody (and compared to the expression levels before treatment). In certain embodiments, the term "increase" or "above" refers to a level above the reference level or to an overall increase of 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 100% or greater, in CSF-IR ligand level detected by the methods described herein, as compared to the CSF-IR ligand level from a reference sample. In certain embodiments, the term increase refers to the increase in CSF-IR ligand level wherein, the increase is at least about 1.5-, 1.75-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 15-, 20-, 25-, 30-, 40-, 50-, 60-, 70-, 75-, 80-, 90-, or 100- fold higher as compared to the CSF-IR ligand level e.g. predetermined from a reference sample. In one preferred embodiment the term increased level relates to a value at or above a reference level.

In one embodiment of the invention the anti-CSF-IR antibody is characterized in that the antibody binds to human CSF-IR Extracellular Domain (SEQ ID NO: 64) (comprising domains D1 to D5) and binds to domains D1 to D3 (SEQ ID NO: 66) of the extracellular domain of human CSF-IR.

In one embodiment of the invention the anti-CSF-IR antibody is characterized in that the antibody binds to human CSF-IR Extracellular Domain (SEQ ID NO: 64)
(comprising domains D1 to D5) and does not bind to domains D1 to D3 (SEQ ID NO: 66) of the extracellular domain of human CSF-1R.

The term "Toll-like receptor 9" (TLR9, CD289; SEQ ID NO: 88) refers to a protein of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. They recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression. This gene is preferentially expressed in immune cell rich tissues, such as spleen, lymph node, bone marrow and peripheral blood leukocytes. Studies in mice and human indicate that this receptor mediates cellular response to unmethylated CpG dinucleotides in bacterial DNA to mount an innate immune response.

TLR9 is mainly found in the endosomal compartment of B cells, monocytes, macrophages and plasmacytoid Dendritic Cells (Galluzzi et al, OncolImmunology, 1:5, (2012) 699-716). The main ligand of TLR9 is bacterial/viral DNA, differing from its mammalian counterpart for the elevated frequency of unmethylated CpG oligodeoxynucleotides. Indeed, whereas mammalian DNA has no immunostimulatory activity, the administration of bacterial/viral DNA induces a potent Th1 immune response in vivo, which is entirely abrogated in TLR9<sup>-/-</sup> mice. CpG oligodeoxynucleotides (or CpG ODN) are short single-stranded synthetic DNA molecules that contain a cytidine triphosphate deoxynucleotide ("C") followed by a guanidine triphosphate deoxynucleotide ("G"). The "p" refers to the phosphodiester link between consecutive nucleotides, although some ODN have a modified phosphorothioate (PS) backbone instead. When these CpG motifs are unmethylated, they act as immunostimulants (Weiner, GJ; et al, PNAS 94 (1997) 10833-7). Thus "Toll-like receptor 9 agonists" (TLR9 agonist) are characterized in binding to Toll-like receptor 9 and in stimulating TLR9 immune response. E.g. in one embodiment a Toll-like receptor 9 agonist (TLR9 agonist) is characterized by binding to Toll-like receptor 9 on human plasmacytoid dendritic cells (pDCs) and by induction of IFN-alpha, IL-6, and/or IL-12 (elevating the levels of IFN-alpha, IL-6, and/or IL-12) in these plasmacytoid dendritic cells (pDCs).
CpG motifs are considered pathogen-associated molecular patterns (PAMPs) due to their abundance in microbial genomes but their rarity in vertebrate genomes (Bauer, S.; Current Topics in Microbiology and Immunology 270 (2002) 145-54). The CpG PAMP is recognized by the pattern recognition receptor (PRR) Toll-Like Receptor 9 (TLR9), which is constitutively expressed only in B cells and plasmacytoid dendritic cells (pDCs) in humans and other higher primates (Rothenfusser, S.; et al, Human immunology 63 (2002) 1111-9).

Synthetic CpG ODN differ from microbial DNA in that they have a partially or completely phosphorothioated (PS) backbone instead of the typical phosphodiester backbone and a poly G tail at the 3’ end, 5’ end, or both. PS modification protects the ODN from being degraded by nucleases such as DNase in the body and poly G tail enhances cellular uptake (Dalpke, AH et al, Immunology 106 (2002) 102—12).The poly G tails form intermolecular tetrads that result in high molecular weight aggregates. These aggregates are responsible for the increased activity the poly G sequence impart; not the sequence itself.

These synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs), such as ODN 1826, have been extensively studied as adjuvants (Steinhagen F. et al, 2011; Vaccine 29(17):3341-55). These CpG motifs are present at a 20-fold greater frequency in bacterial DNA compared to mammalian DNA (Hemmi H. et al, 2000. Nature 408: 740-5). CpG ODNs agonize TLR9, which is expressed on human B cells and plasmacytoid dendritic cells (pDCs), thereby inducing Th1-dominated immune responses (Coffman et al., 2010. Immunity 33(4):492-503). Pre-clinical studies, conducted in rodents and non-human primates, and human clinical trials have demonstrated that CpG ODNs can significantly improve vaccine-specific antibody responses (Steinhagen F. et al, 2011; Vaccine 29(17):3341-55).

Numerous sequences have been shown to stimulate TLR9 with variations in the number and location of CpG dimers, as well as the precise base sequences flanking the CpG dimers. This led to the creation of classes or categories of CpG ODN, which are all TLR9 agonist based on their sequence, secondary structures, and effect on human peripheral blood mononuclear cells (PBMCs). The three main classes of CpG ODNs are class A, B and C, which differ in their immune-stimulatory activities (Krug A. et al., 2001, Eur J Immunol, 31(7): 2154-63). Furthermore, CpG ODNs activate TLR9 in a species-specific manner (Bauer, S. et al, 2001, PNAS, 98(16):9237-42). One of the first Class A ODN, ODN 2216, was
described in 2001 by Krug et al (see above) This class of ODN was distinctly different from the previously described Class B ODN (i.e., ODN 2006) in that it stimulated the production of large amounts of Type I interferons, the most important one being IFNA, and induced the maturation of pDCs.

Class A ODN are also strong activators of NK cells through indirect cytokine signaling. Class A ODN typically contain 7 to 10 PS-modified bases at one or both ends that resist degradation by nucleases and increase the longevity of the ODN. The above rules strictly define the class, but variability of the sequence within these "rules" is possible. It should also be noted that changes to the sequence will affect the magnitude of the response. For example, the internal palindrome sequence can be 4 to 8 base pairs in length and vary in the order of bases, however the pattern, 5'-Pu Pu CG Pu Py CG Py Py-3', was found to be the most active when compared to several other sequences. The poly G tail found at either end of the DNA strand can vary in length and even number, but its presence is critical to the activity of the molecule.

Class B ODN (i.e. ODN 2007) are strong stimulators of human B cell and monocyte maturation. They also stimulate the maturation of pDC but to a lesser extent than Class A ODN and very small amounts of IFNA. The strongest ODN in this class have three 6mer sequences. Class B ODNs have been studied extensively as therapeutic agents because of their ability to induce a strong humoral immune response, making them ideal as a vaccine adjuvant.


Moreover, the administration of type B CpG oligonucleotides (alone or combined with chemotherapeutics or peptide vaccines) to tumor-bearing rodents reportedly exerts potent anticancer effects. Initial Phase I/II clinical trials to test the safety and efficacy of CpG-7909 for oncological indications were launched in April 2000.
Approximately in the same period, CpG-7909 begun to be extensively investigated as an adjuvant for cancer-unrelated indications (mainly antiviral vaccines), showing no severe side effects and encouraging efficacy.

During the last decade, the safety and anticancer potential of CpG-7909 (as a standalone agent or in combination with chemotherapy and/or vaccination approaches) have been investigated in a large number of Phase I/II clinical trials, including studies with leukemia, lymphoma, basal cell carcinoma, melanoma, esophageal squamous cell carcinoma, NSCLC, renal cell carcinoma, and prostate cancer patients. Several TLR9 agonist are known and currently developed in clinical testing Agatolimod (tricosasodium salt of a synthetic 24-mer oligonucleotide containing 3 CpG motifs; Pfizer) GNKG168 (CpG ODN; SBI Biotech), IMO-2055 (synthetic oligonucleotide containing unmethylated CpG dinucleotides; Idera Pharmaceuticals), MGN-1703 (Mologen). Typically these TLR9 agonist are used in the treatment of different cancers:

Bacterial and synthetic DNA containing unmethylated CpG motifs act as agonists of TLR9 and induce Th1-type immune response profiles. The immune-stimulatory effects of TLR9 agonists are multifactorial and depend on the nucleotide sequence, the nature of the backbone and the presence of specific structural motifs. Based on the cytokine profiles induced, three distinct types of TLR9 agonists, class A, B and C, have been described. Each class of TLR9 agonist is composed of a different nucleotide sequence that allows formation of structures (or no structures) that generate different immune profiles.

The structure-activity relationships of oligonucleotides that act as agonists of TLR9 was systematically studied (Kandimalla, E.R. and Agrawal, S. (2005) in Toll and Toll Receptors: An Immunologic Perspective (Rich, T., ed.), pp. 181-212, Kluwer Academic/Plenum Publishers, New York). The presence of a CpG motif in oligonucleotides is required for TLR9 stimulation. Oligonucleotides with phosphodiester and phosphorothioate backbone stimulate TLR9-mediated immune responses. Phosphorothioate backbone oligonucleotides are commonly used because they are less susceptible to degradation by ubiquitous nucleases than are phosphodiester oligonucleotides. Introduction of a sulfur atom on the internucleotide phosphodiester bond results in the formation of Rp and Sp diastereoisomers; the Rp diastereomer of phosphorothioate linkage stimulates a stronger TLR9-mediated immune response than does the Sp diastereomer. The negative charges on phosphates between and adjacent to cytosine (C) and guanine...
(G) are also required for TLR9-mediated activity. Neutralization of charges by incorporation of methylphosphonate linkages at these positions results in the loss of immune-stimulatory activity. Moreover, TLR9 activation is also dependent on the sequences flanking the CpG dinucleotide, the nature of the nucleotide backbone and the secondary structures.

Flanking sequences play a significant role in TLR9 stimulation

Chemical modifications introduced at the 2'-position of the sugar ring of a C or G nucleotide in the CpG motif result in the loss of immune-stimulatory activity of TLR9 agonists. In addition, studies of TLR9 agonists containing chemical modifications such as methylphosphonate linkages, 2'-alkyl or 3'-deoxy or -alkyl ribonucleosides, non-nucleotide linkers or abasic nucleotides in the flanking sequences indicate that substitutions incorporated at the fourth to sixth nucleotide positions 5' to the CpG dinucleotide significantly enhance immune-stimulatory activity. In general, modifications incorporated in the 3'-flanking sequence distal to the CpG dinucleotide have effects dependent on the nature of the modification.

TLR9 requires a free 5'-end of agonist for stimulation

Two CpG oligonucleotides linked through their 5'-ends do not activate immune cells despite the availability of two CpG motifs. When the same oligonucleotides are linked through their 3'-ends, they produce higher and distinct cytokine profiles than the parent CpG oligonucleotide with a single 5'-end. These are the first studies demonstrating the requirement of an accessible or free 5'-end for TLR9 activation and that the receptor reads the sequence from the 5'-end. The transcription factor NF-kB is rapidly activated by TLR9 agonists that contain two 5'-ends, but these compounds have the same activity as conventional TLR9 agonists on the MAPK (mitogen-activated protein kinase) pathway in J774 cells.

These studies suggest that agonists containing two 5'-ends facilitate dimerization of the receptor, leading to rapid activation of immune responses. Moreover, TLR9 activation can be modulated through appropriate presentation of the free 5'-ends and synthetic immune-stimulatory motifs, leading to changes in the downstream cytokine induction profiles. Consistent with these results, recent studies have shown that TLR9 exists in dimer form and binds to single-stranded oligonucleotides. However, only oligonucleotides containing the CpG motif cause conformational changes in the receptor, leading to the activation of immune signalling pathways.
The attachment of oligonucleotides through their 3'-ends not only provides two 5'-ends for optimal activation of TLR9, but also increases the stability against 3'-exonucleases. Oligonucleotides with a phosphodiester backbone and as short as 5 and 6 nt linked through their 3'-ends act as potent TLR9 agonists and produce immune responses. Moreover, oral administration of the novel structure containing TLR9 agonists induces potent mucosal immune responses, acts as an adjuvant with antigens, and prevents and reverses peanut allergy in mouse models because of their greater stability in the gastrointestinal tract.

Functional groups of cytosine and guanine required for TLR9 stimulation

As described above, certain chemical modifications introduced within the CpG dinucleotide that alter structure and conformation lead to the loss of immune-stimulatory activity of agonists. One such modification is a replacement of the methyl group at the 5-position of cytosine in the CpG motif of TLR9 agonists. Vertebrates use this feature to distinguish self-DNA from that of bacterial DNA, which contains more unmethylated CpG motifs.


Systematic studies of several TLR9 agonists that have two 5'-ends and contain synthetic CpR dinucleotides in different nucleotide compositions in mouse, human and monkey systems suggest that nucleotide sequence and secondary structures play a role in modulating the immune response. Based on these studies, we have broadly identified two different groups of synthetic agonists of TLR9.

In one embodiment the TLR9 agonist is characterized by induction of IFN-alpha, IL-6, and/or IL-12 (elevating the levels of IFN-alpha, IL-6, and/or IL-12) in plasmacytoid dendritic cells (pDCs). In one embodiment the TLR9 agonist is characterized by elevating the level of IFN-alpha in human plasmacytoid dendritic cells (pDCs) (as measured by sandwich ELISA as described below or e.g in in WO2010/088395)

Assay for measuring IFN-alpha induction (elevating the levels of IFN-alpha, IL-6, and/or IL-12) by TLR9 agonist of the invention in human pDCs:

Human PBMC isolation: Peripheral blood mononuclear cells (PBMCs) from freshly drawn healthy volunteer blood (CBR Laboratories, Boston, MA) are isolated by Ficoll density gradient centrifugation method (Histopaque-1077, Sigma).

Human pDC isolation: Human plasmacytoid dendritic cells (pDCs) are isolated from freshly obtained healthy human volunteer’s blood PBMCs by positive selection using the BDC A4 cell isolation kits (Miltenyi Biotec) according to the manufacturer's instructions.

Human pDCs are plated in 96-well dishes using 1 x 10⁶ cells/ml. Individual immune modulatory compounds from Table I were dissolved in DPBS (pH 7.4; Mediatech) are added to the cell cultures at doses of 0, 0.1, 0.3, 1.0, 3.0, or 10.0 .micro.g/ml. The cells were then incubated at 37 (0)C for 24 hours and the supernatants were collected for luminex multiplex or ELISA assays.

In the levels of IFN-alpha, IL-6, and/or IL-12 are measured by sandwich ELISA. The required reagents, including cytokine antibodies and standards, can be purchased from PharMingen.

IFN-alpha has been known as an antiviral cytokine for many years. It stimulates Th1 cell development, therefore promoting the effects of CG-containing DNA molecules. IFN-alpha also exhibits antitumour activity in mouse and human
malignancies and is capable of decreasing the tumourigenicity of transplanted
tumour cells, partially by activating cytotoxic T cells and thereby increasing the
likelihood of tumour-cell cytolysis. NK cell and macrophage activity, both also
important for antitumour cytotoxicity, are also increased by IFN-alpha (Brassard et
al., J. Leukoc. Biol. 2002 71: 565-81). Therefore, increasing the amount of IFN-
alpha upon stimulation with the DNA constructs of the present disclosure is
expected to be beneficial for the treatment of cancer.

In one embodiment of the invention the TLR9 agonist of the invention is an
oligodeoxynucleotides containing a) cytosine-phosphate-guanosine (CpG) motifs
(CpG ODNs) b) pyrimidine -phosphate-guanosine (YpG) motifs (YpG ODNs) or
c) cytosine-phosphate-purine (CpR) motifs (CpR ODNs).

In one embodiment of the invention the TLR9 agonist of the invention is an
oligodeoxynucleotides containing a) cytosine-phosphate-guanosine (CpG) motifs
(CpG ODNs) b) pyrimidine -phosphate-guanosine (YpG) motifs (YpG ODNs) or
c) Purine-phosphate-guanosine (RpG) motifs (RpG ODNs) wherein the TLR9
agonist stimulates TLR9 (in one embodiment the TLR9 agonist induces the
maturation of plasmacytoid dendritic cells (pDCs); in one embodiment the TLR9
agonist is characterized by human B cell maturation; in one embodiment)

In one embodiment of the invention the TLR9 agonist of the invention is an
oligodeoxynucleotides containing cytosine-phosphate-guanosine (CpG) motifs
(CpG ODNs).

In one embodiment the TLR9 agonist of the invention is a Class A
CpG ODN.

In one embodiment the TLR9 agonist of the invention is an oligodeoxynucleotides
comprising

a) a poly G sequence at the 5’ end, or the 3’ end, or at both ends

b) an internal palindrome sequence;

c) GC dinucleotides contained within the internal palindrome, and

d) a partially PS-modified backbone
Class A CpG ODN typically contain 7 to 10 PS-modified bases at one or both ends that resist degradation by nucleases and increase the longevity of the ODN. The above rules strictly define the class, but variability of the sequence within these rules is possible. The internal palindrome sequence can be 4 to 8 base pairs in length and vary in the order of bases, however the pattern, 5'-Pu Pu CG Pu Py CG Py Py-3', was found to be the most active when compared to several other sequences. The poly G tail found at either end of the DNA strand can vary in length and number.

In one embodiment the Class A CpG ODN (Xueqing Liang, et al, Blood. 2010 June 17; 115(24): 5041-5052) is selected from the group consisting of CpG ODN 2216 (5'-ggGGGACGATCGTCgggggG-3') (SEQ ID NO: 89) CpG ODN PB4 (5'-tcgGACGATCGTCggggG-3') (SEQ ID NO: 90); or CpG ODN 1002 (5'-ggGGTCGTTCCGTGTTggggG-3') (SEQ ID NO: 91).

In one embodiment of the invention the TLR9 agonist of the invention is a Class B CpG ODN.

In one embodiment the TLR9 agonist of the invention is a oligodeoxynucleotides comprising

a) one or more 6mer unmethylated cytosine-phosphate-guanosine (CpG) motifs 5'-Pu Py C G Py Pu-3' (one or more 6mer 5'-RYCGYR-3' 6-mers (R = A or G; Y = T or C))

b) a fully phosphorothioated (PS-modified) backbone; and

c) 18 to 28 nucleotides in length

In one embodiment the Class B CPG ODN is selected from the group consisting of CpG-28, CpG-685 (GNKG168; CpG ODN; SBI Biotech), CpG-684 and CpG-7909 (CPG-ODN 2006, PF-35 12676, Agatolimod).

CpG-7909 (CpG 2006, PF-35 12676, Agatolimod) is a Synthetic, 24-mer phosphothioate oligodeoxynucleotide (d(P-Thio)(T-C-G-T-C-G-T-T-T-G-T-C-G-T-T-G-T-C-G-T-C-G-T-C-G-T))DNA) (5'-tcgctgttggctttgctttctg-3') (SEQ ID NO: 92) containing multiple cytosine-phosphate-guanosine (CpG) motifs or one of its derivatives like tricosasodium salt. The preparation is described e.g. in WO 9818810 or US 7223741)
CpG-685 (GNKG168; CpG ODN; SBI Biotech) is synthetic, 21-mer, unmethylated CpG motif-based oligodeoxynucleotide (ODN) (685, 5'-tcgacgtcgtcgtcgtcgtc-3') (SEQ ID NO: 93), with immunostimulatory activity. CpG685 (GNKG168), a 21-mer fully phosphorothioated oligonucleotides designed to directly target Toll-like receptor 9 that mediates cellular responses in B cells, showed antitumor effects in SCID mouse and is under clinical development for the treatment of human chronic lymphocytic leukemia (B-CLL) by SBI Biotech Co. Herein, a sensitive and specific assay was developed in plasma and cell lysate to support its preclinical pharmacology studies. CpG oligodeoxynucleotide GNKG168 binds to and activates Toll-like receptor 9 (TLR9) and is taken up into cells by endocytosis; once internalized, it may activate numerous signaling transduction pathways resulting in the release of multiple cytokines, such as immunoglobulins (Igs), interferons (IFNs), interleukins (ILs) and tumor necrosis factor (TNF).

CpG-684 is synthetic, 23-mer, unmethylated CpG motif-based oligodeoxynucleotide (ODN) 684, 5'-tcgacgtcgtcgtcgtcgtc-3' (SEQ ID NO: 94);

CpG-28 synthetic unmethylated CpG motif-based oligodeoxynucleotide (ODN), containing multiple repeats of unmethylated CpG motifs (CpG ODN) with immunostimulatory activity (5'-TAAACGTATAACGTATGACGTAC-3') (SEQ ID NO: 95) with a wholly phosphorothioate backbone (Carpentier AF, et al Front Biosci. 2003;8:el 15-el27; Meng Y, et al, Int J Cancer. 2005;1 16:992-997; or Carpentier A, et al. Neuro-Oncology 2006;8:60-66). Upon entering the cell via endocytosis, CpG-28 activates numerous signaling transduction pathways resulting in the release of multiple cytokines. CpG-28 has immunomodulatory properties with direct activation of B-lymphocytes, dendritic and NK cells resulting in the stimulation of innate immunity and antibody-dependant cell cytotoxicity (ADCC). Additionally, this agent indirectly modulates T-cell responses though the release of cytokines (IL-12 and IFN gamma) to induce a preferential shift to the Th1 (helper) phenotype resulting in enhanced CD8+ cellular cytotoxicity.

In one embodiment of the invention the TLR9 agonist of the invention is a oligodeoxynucleotides containing pyrimidine-phosphate-guanosine (YpG) motifs (YpG ODNs).

In one embodiment of the invention the TLR9 agonist of the invention is a oligodeoxynucleotides containing cytosine-phosphate-purine (CpR) motifs (CpR ODNs).
In one embodiment of the invention the TLR9 agonist of the invention is IMO-2055 (Idera) (ODN consisting of 3'-3' linked structure and synthetic CpR(R = 2'-deoxy-7-deazaguanosine) motif)

In one embodiment of the invention the TLR9 agonist of the invention is a oligodeoxynucleotides containing a) cytosine-phosphate-guanosine (CpG) motifs (CpG ODNs) b) pyrimidine-phosphate-guanosine (YpG) motifs (YpG ODNs) or c) cytosine-phosphate-purine (CpR) motifs (CpR ODNs).

In one embodiment of the invention the TLR9 agonist of the invention is a oligodeoxynucleotides based CpG motif-containing circular ODN (e.g. MGN-1703 from Mologen as described in WO2012/085291) based on the dSLIM® technology (this technology is described in WO2001/07055).

In one embodiment of the invention the TLR9 agonist is selected from the group consisting of CpG ODN 2216 CpG ODN 1002 CpG-28, CpG-685, CpG-684, CpG-7909, IMO-2055 or MGN-1703. In one embodiment of the invention the TLR9 agonist is selected from the group consisting of CpG-685, CpG-7909, IMO-2055 or MGN-1703. In one embodiment the TLR9 agonist is selected from the group consisting of CpG-7909, IMO-2055 or MGN-1703.

In one embodiment of the invention the CSF-1R antibody is selected from antibodies described in WO 2009/026303, WO 2009/112245, WO2011/123381(A1) or WO2011/070024; and the TLR9 agonist is selected from the group consisting of CpG-685, CpG-7909, IMO-2055 or MGN-1703.

In one embodiment of the invention the CSF-1R antibody is selected from antibodies binding to human CSF-1R, characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:1, a CDR2 region of SEQ ID NO: 2, and a CDR1 region of SEQ ID NO:3, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5, and a CDR1 region of SEQ ID NO:6, or,

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 9, a CDR2 region of SEQ ID NO: 10, and a CDR1 region of SEQ ID NO: 11, and the light chain variable domain comprises a CDR3 region of
SEQ ID NO: 12, a CDR2 region of SEQ ID NO: 13, and a CDR1 region of SEQ ID NO: 14, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO: 21, and a CDR1 region of SEQ ID NO: 22, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

f) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO: 43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO: 45, and a CDR1 region of SEQ ID NO: 46, or

g) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 49, a CDR2 region of SEQ ID NO: 50, and a CDR1 region of SEQ ID NO: 51, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 52, a CDR2 region of SEQ ID NO: 53, and a CDR1 region of SEQ ID NO: 54;

and the TLR9 agonist is selected from the group consisting of CpG-685, CpG-7909, IMO-2055 or MGN-1703

In one embodiment of the invention the CSF-1R antibody is selected from antibodies binding to human CSF-1R, is characterized in that
the heavy chain variable domain is SEQ ID NO:39 and the light chain variable domain is SEQ ID NO:40.

and the TLR9 agonist is selected from the group consisting of CpG-685, CpG-7909, IMO-2055 or MGN-1703

In general, many suitable TLR9 agonists are known in the art. These TLR9 agonists are contemplated to be used for the present combination therapy of the invention. TLR9 specifically recognizes CpG DNA that is unmethylated, and initiates a signalling cascade leading to the production of proinflammatory cytokines. Methylation of the cytosine within the CpG motif strongly reduces the affinity of TLR9. Double stranded (ds) CpG DNA is a weaker stimulator of TLR9 compared to its single stranded (ss) counterpart.


CPG 7909 is an immunostimulatory TLR9 agonist oligodeoxynucleotide that was found to be well tolerated in a phase 1/1 I clinical study (Cooper et al. (2004) J. Clin. Immunol, 24(6): 693-701). The CpG enriched, synthetic oligodeoxynucleotide TLR9 agonist PF-3512676 was found to have antilymphoma activity in a phase 1/1 I clinical study (Brody et al (2010) J. Clin. Oncol., 28(28): 4324-32).

Nucleic Acids Res. 30:4460-4469; Yu, D. et al. (2002) J. Med. Chem. 45:4540-

US 2009/0053206 describes a number of TLR9 agonists, in particular compounds
1-169 listed in Table 1; US 2008/0292648 describes a number of TLR9 agonists,
in particular compounds 1-92 listed in Table 1; and US 2007/0105800 describes
oligonucleotidebased compounds that are TLR9 agonists (Idera Pharmaceuticals).
Suitable TLR9 agonists may also include the selective TLR9 agonists IMO-2055,
IMO-2125 and IMO- 2134 that are undergoing phase 1 /phase 2 clinical trials
(Idera Pharmaceuticals). US 2010/0016250 describes a number of TLR9 agonists,
in particular compounds of Formula I (Kyowa Hakko Kirin Co). As mentioned
above, US 2009/0041809 describes compositions that are TLR9 agonists or both
TLR3 and TLR9 agonists (Nventa Pharmaceuticals).

Different TLR9 agonists to be used for the present combination therapy of the
invention are described in detail in WO2007/7047396, WO2007/7047396,

The term "epitope" denotes a protein determinant of human CSF-IR capable of
specifically binding to an antibody. Epitopes usually consist of chemically active
surface groupings of molecules such as amino acids or sugar side chains and
usually epitopes have specific three dimensional structural characteristics, as well
as specific charge characteristics. Conformational and nonconformational epitopes
are distinguished in that the binding to the former but not the latter is lost in the
presence of denaturing solvents. Preferably an antibody according to the invention
binds specifically to native and to denatured CSF-IR.

The "variable domain" (variable domain of a light chain (V_L), variable domain of a
heavy chain (V_H)) as used herein denotes each of the pair of light and heavy chain
domains which are involved directly in binding the antibody to the antigen. The
variable light and heavy chain domains have the same general structure and each
domain comprises four framework (FR) regions whose sequences are widely
conserved, connected by three "hypervariable regions" (or complementary determining regions, CDRs). The framework regions adopt a β-sheet conformation and the CDRs may form loops connecting the β-sheet structure. The CDRs in each chain are held in their three-dimensional structure by the framework regions and form together with the CDRs from the other chain the antigen binding site. The antibody's heavy and light chain CDR3 regions play a particularly important role in the binding specificity/affinity of the antibodies according to the invention and therefore provide a further object of the invention.

The term "antigen-binding portion of an antibody" when used herein refer to the amino acid residues of an antibody which are responsible for antigen-binding. The antigen-binding portion of an antibody comprises amino acid residues from the "complementary determining regions" or "CDRs". "Framework" or "FR" regions are those variable domain regions other than the hypervariable region residues as herein defined. Therefore, the light and heavy chain variable domains of an antibody comprise from N- to C-terminus the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Especially, CDR3 of the heavy chain is the region which contributes most to antigen binding and defines the antibody's properties. CDR and FR regions are determined according to the standard definition of Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) and/or those residues from a "hypervariable loop".

The terms "nucleic acid" or "nucleic acid molecule", as used herein, are intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "amino acid" as used within this application denotes the group of naturally occurring carboxy a-amino acids comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gin, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

The antibodies according to the invention are preferably produced by recombinant means. Therefore the antibody is preferably an isolated monoclonal antibody. Such recombinant methods are widely known in the state of the art and comprise protein
expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody polypeptide and usually purification to a pharmaceutically acceptable purity. For the protein expression, nucleic acids encoding light and heavy chains or fragments thereof are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells like CHO cells, NSO cells, SP2/0 cells, HEK293 cells, COS cells, yeast, or E.coli cells, and the antibody is recovered from the cells (supernatant or cells after lysis).


The antibodies may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. Purification is performed in order to eliminate other cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al, ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).


The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, enhancers and polyadenylation signals.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a
preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The monoclonal antibodies are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. DNA and RNA encoding the monoclonal antibodies are readily isolated and sequenced using conventional procedures. The hybridoma cells can serve as a source of such DNA and RNA. Once isolated, the DNA may be inserted into expression vectors, which are then transfected into host cells such as HEK 293 cells, CHO cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of recombinant monoclonal antibodies in the host cells.

As used herein, the expressions "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

The "Fc part" of an antibody is not involved directly in binding of an antibody to an antigen, but exhibit various effector functions. A "Fc part of an antibody" is a term well known to the skilled artisan and defined on the basis of papain cleavage of antibodies. Depending on the amino acid sequence of the constant region of their heavy chains, antibodies or immunoglobulins are divided in the classes: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgGl, IgG2, IgG3, and IgG4, IgAl, and IgA2. According to the heavy chain constant regions the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The Fc part of an antibody is directly involved in

In one embodiment the antibody according to the invention comprises a Fc part derived from human origin and preferably all other parts of the human constant regions. As used herein the term "Fc part derived from human origin" denotes a Fc part which is either a Fc part of a human antibody of the subclass IgG1, IgG2, IgG3 or IgG4, preferably a Fc part from human IgG1 subclass, a mutated Fc part from human IgG1 subclass (preferably with a mutation on L234A + L235A), a Fc part from human IgG4 subclass or a mutated Fc part from human IgG4 subclass (preferably with a mutation on S228P). Mostly preferred are the human heavy chain constant regions of SEQ ID NO: 58 (human IgG1 subclass), SEQ ID NO: 59 (human IgG1 subclass with mutations L234A and L235A), SEQ ID NO: 60 human IgG4 subclass), or SEQ ID NO: 61 (human IgG4 subclass with mutation S228P).

Preferably the antibody according to the invention is of human IgG1 subclass or of human IgG4 subclass. In one embodiment the antibody according to the invention is of human IgG1 subclass. In one embodiment the antibody according to the invention is of human IgG4 subclass.

In one embodiment the antibody according to the invention is characterized in that the constant chains are of human origin. Such constant chains are well known in the state of the art and e.g. described by Kabat, E.A., (see e.g. Johnson, G. and Wu,
T.T., Nucleic Acids Res. 28 (2000) 214-218). For example, a useful human heavy chain constant region comprises an amino acid sequence of SEQ ID NO: 58. For example, a useful human light chain constant region comprises an amino acid sequence of a kappa-light chain constant region of SEQ ID NO: 57.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain is SEQ ID NO:23 and the light chain variable domain is SEQ ID NO:24, or
b) the heavy chain variable domain is SEQ ID NO:31 and the light chain variable domain is SEQ ID NO:32, or
c) the heavy chain variable domain is SEQ ID NO:39 and the light chain variable domain is SEQ ID NO:40, or
d) the heavy chain variable domain is SEQ ID NO:47 and the light chain variable domain is SEQ ID NO:48, or
e) the heavy chain variable domain is SEQ ID NO:55 and the light chain variable domain is SEQ ID NO:56.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain is SEQ ID NO:23 and the light chain variable domain is SEQ ID NO:24, or
b) the heavy chain variable domain is SEQ ID NO:31 and the light chain variable domain is SEQ ID NO:32, or
c) the heavy chain variable domain is SEQ ID NO:39 and the light chain variable domain is SEQ ID NO:40, or
d) the heavy chain variable domain is SEQ ID NO:47 and the light chain variable domain is SEQ ID NO:48.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:23 and the light chain variable domain is SEQ ID NO:24.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

...
the heavy chain variable domain is SEQ ID NO:31 and the light chain variable domain is SEQ ID NO:32.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:39 and the light chain variable domain is SEQ ID NO:40.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:47 and the light chain variable domain is SEQ ID NO:48.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:15 and the light chain variable domain is SEQ ID NO:16, or a humanized version thereof.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:75 and the light chain variable domain is SEQ ID NO:76;
or a humanized version thereof.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

the heavy chain variable domain is SEQ ID NO:83 and the light chain variable domain is SEQ ID NO:84;
or a humanized version thereof.

In one embodiment the antibody binding to human CSF-1R used in the combination therapy is characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:1, a CDR2 region of SEQ ID NO:2, and a CDR1 region of SEQ ID
NO:3, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5, and a CDR1 region of SEQ ID NO:6, or,

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 9, a CDR2 region of SEQ ID NO: 10, and a CDR1 region of SEQ ID NO: 11, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 12, a CDR2 region of SEQ ID NO: 13, and a CDR1 region of SEQ ID NO: 14, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO: 21, and a CDR1 region of SEQ ID NO: 22, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

f) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO: 43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO: 45, and a CDR1 region of SEQ ID NO: 46, or

g) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 49, a CDR2 region of SEQ ID NO: 50, and a CDR1 region of SEQ ID NO: 51, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 52, a CDR2 region of SEQ ID NO: 53, and a CDR1 region of SEQ ID NO: 54; or
h) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:69, a CDR2 region of SEQ ID NO: 70, and a CDR1 region of SEQ ID NO:71, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 72, a CDR2 region of SEQ ID NO:73, and a CDR1 region of SEQ ID NO:74, or

i) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 77, a CDR2 region of SEQ ID NO: 78, and a CDR1 region of SEQ ID NO: 79, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:80, a CDR2 region of SEQ ID NO: 81, and a CDR1 region of SEQ ID NO: 82.

In one embodiment the combination therapy with an antibody binding to human CSF-IR,i is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO: 21, and a CDR1 region of SEQ ID NO: 22.

In one embodiment the combination therapy with an antibody binding to human CSF-IR,i is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30.

In one embodiment the combination therapy with an antibody binding to human CSF-IR,i is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38.
In one embodiment the combination therapy with an antibody binding to human CSF-1R, is characterized in that

the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO:43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO:45, and a CDR1 region of SEQ ID NO:46.

The invention comprises a method for the treatment of a patient in need of therapy, characterized by administering to the patient a therapeutically effective amount of an antibody according to the invention.

The invention comprises the use of an antibody according to the invention for the described therapy.

One preferred embodiment of the invention are the CSF-1R antibodies of the present invention for use in the treatment of "CSF-1R mediated diseases" or the CSF-1R antibodies of the present invention for use for the manufacture of a medicament in the treatment of "CSF-1R mediated diseases", which can be described as follows:


Two point mutations were seen in CSF-1R in about 10-20% of acute myelocytic leukemia, chronic myelocytic leukemia and myelodysplasia patients tested in one study, and one of the mutations was found to disrupt receptor turnover (Ridge, S.A., et al, Proc. Natl. Acad. Sci USA 87 (1990) 1377-1380). However the incidence of the mutations could not be confirmed in later studies (Abu-Duhier, F.M., et al, Br. J. Haematol. 120 (2003) 464-470). Mutations were also found in some cases of hepatocellular cancer (Yang, D.H., et al, Hepatobiliary Pancreat. Dis. Int. 3 (2004) 86-89) and idiopathic myelofibrosis (Abu-Duhier, F.M., et al, Br. J. Haematol. 120 (2003) 464-470). Recently, in the GDM-1 cell line derived
from a patient with myelomonoblastic leukemia the Y571D mutation in CSF-1R was identified (Chase, A., et al, Leukemia 23 (2009) 358-364).

Pigmented villonodular synovitis (PVNS) and Tenosynovial Giant cell tumors (TGCT) can occur as a result of a translocation that fuses the M-CSF gene to a collagen gene COL6A3 and results in overexpression of M-CSF (West, R.B., et al, Proc. Natl. Acad. Sci. USA 103 (2006) 690-695). A landscape effect is proposed to be responsible for the resulting tumor mass that consists of monocytic cells attracted by cells that express M-CSF. TGCTs are smaller tumors that can be relatively easily removed from fingers where they mostly occur. PVNS is more aggressive as it can recur in large joints and is not as easily controlled surgically.

The second mechanism is based on blocking signaling through M-CSF/CSF-IR at metastatic sites in bone which induces osteoclastogenesis, bone resorption and osteolytic bone lesions. Breast, multiple myeloma and lung cancers are examples of cancers that have been found to metastasize to the bone and cause osteolytic bone disease resulting in skeletal complications. M-CSF released by tumor cells and stroma induces the differentiation of hematopoietic myeloid monocyte progenitors to mature osteoclasts in collaboration with the receptor activator of nuclear factor kappa-B ligand-RANKL. During this process, M-CSF acts as a permissive factor by giving the survival signal to osteoclasts (Tanaka, S., et al, J. Clin. Invest. 91 (1993) 257-263). Inhibition of CSF-1R activity during osteoclast differentiation and maturation with an anti-CSF-1R antibody is likely to prevent unbalanced activity of osteoclasts that cause osteolytic disease and the associated skeletal related events in metastatic disease. Whereas breast, lung cancer and multiple myeloma typically result in osteolytic lesions, metastasis to the bone in prostate cancer initially has an osteoblastic appearance in which increased bone forming activity results in 'woven bone' which is different from typical lamellar structure of normal bone. During disease progression bone lesions display a significant osteolytic component as well as high serum levels of bone resorption and suggests that anti-resorptive therapy may be useful. Bisphosphonates have been shown to inhibit the formation of osteolytic lesions and reduced the number of skeletal-related events only in men with hormone-refractory metastatic prostate cancer but at this point their effect on osteoblastic lesions is controversial and bisphosphonates have not been beneficial in preventing bone metastasis or hormone responsive prostate cancer to date. The effect of anti-resorptive agents in mixed osteolytic/osteoblastic prostate cancer is still being studied in the clinic (Choueiri,

The third mechanism is based on the recent observation that tumor associated macrophages (TAM) found in solid tumors of the breast, prostate, ovarian and cervical cancers correlated with poor prognosis (Bingle, L., et al, J. Pathol. 196 (2002) 254-265; Pollard, J.W., Nat. Rev. Cancer 4 (2004) 71-78). Macrophages are recruited to the tumor by M-CSF and other chemokines. The macrophages can then contribute to tumor progression through the secretion of angiogenic factors, proteases and other growth factors and cytokines and may be blocked by inhibition of CSF-1R signaling. Recently it was shown by Zins et al (Zins, K., et al., Cancer Res. 67 (2007) 1038-1045) that expression of siRNA of Tumor necrosis factor alpha (TNF alpha), M-CSF or the combination of both would reduce tumor growth in a mouse xenograft model between 34% and 50% after intratumoral injection of the respective siRNA. SiRNA targeting the TNF alpha secreted by the human SW620 cells reduced mouse M-CSF levels and led to reduction of macrophages in the tumor. In addition treatment of MCF7 tumor xenografts with an antigen binding fragment directed against M-CSF did result in 40% tumor growth inhibition, reversed the resistance to chemotherapeutics and improved survival of the mice when given in combination with chemotherapeutics (Paulus, P., et al., Cancer Res. 66 (2006) 4349-4356).

TAMs are only one example of an emerging link between chronic inflammation and cancer. There is additional evidence for a link between inflammation and cancer as many chronic diseases are associated with an increased risk of cancer, cancers arise at sites of chronic inflammation, chemical mediators of inflammation are found in many cancers; deletion of the cellular or chemical mediators of inflammation inhibits development of experimental cancers and long-term use of anti-inflammatory agents reduce the risk of some cancers. A link to cancer exists for a number of inflammatory conditions among- those H.pylori induced gastritis for gastric cancer, Schistosomiasis for bladder cancer, HHVX for Kaposi's sarcoma, endometriosis for ovarian cancer and prostatitis for prostate cancer (Balkwill, F., et al, Cancer Cell 7 (2005) 211-217). Macrophages are key cells in chronic inflammation and respond differentially to their microenvironment. There are two types of macrophages that are considered extremes in a continuum of functional states: M1 macrophages are involved in Type 1 reactions. These reactions involve the activation by microbial products and consequent killing of pathogenic microorganisms that result in reactive oxygen intermediates. On the
other end of the extreme are M2 macrophages involved in Type 2 reactions that promote cell proliferation, tune inflammation and adaptive immunity and promote tissue remodeling, angiogenesis and repair (Mantovani, A., et al, Trends Immunol. 25 (2004) 677-686). Chronic inflammation resulting in established neoplasia is usually associated with M2 macrophages. A pivotal cytokine that mediates inflammatory reactions is TNF alpha that true to its name can stimulate anti-tumor immunity and hemorrhagic necrosis at high doses but has also recently been found to be expressed by tumor cells and acting as a tumor promoter (Zins, K., et al, Cancer Res. 67 (2007) 1038-1045; Balkwill, F., Cancer Metastasis Rev. 25 (2006) 409-416). The specific role of macrophages with respect to the tumor still needs to be better understood including the potential spatial and temporal dependence on their function and the relevance to specific tumor types.

Thus one embodiment of the invention are the CSF-1R antibodies of the present invention for use in the treatment of cancer. The term "cancer" as used herein may be, for example, lung cancer, non small cell lung (NSCL) cancer, bronchioloalviolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwanomas, ependymomas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma, lymphoma, lymphocytic leukemia, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers. In one preferred embodiment such cancer is a breast cancer, colorectal cancer, melanoma, head and neck cancer, lung cancer or prostate cancer. In one preferred embodiment such cancer is a breast cancer, ovarian cancer, cervical cancer, lung cancer or prostate cancer. In one preferred embodiment such cancers are further characterized by CSF-1 or CSF-IR expression or overexpression. One further embodiment the invention are the CSF-
IR antibodies of the present invention for use in the simultaneous treatment of primary tumors and new metastases.

Thus another embodiment of the invention are the CSF-IR antibodies of the present invention for use in the treatment of periodontitis, histiocytosis X, osteoporosis, Paget's disease of bone (PDB), bone loss due to cancer therapy, periprosthetic osteolysis, glucocorticoid-induced osteoporosis, rheumatoid arthritis, psiratic arthritis, osteoarthritis, inflammatory arthridities, and inflammation.


Histiocytosis X (also called Langerhans cell histiocytosis, LCH) is a proliferative disease of Langerhans dendritic cells that appear to differentiate into osteoclasts in bone and extra osseous LCH lesions. Langerhans cells are derived from circulating monocytes. Increased levels of M-CSF that have been measured in sera and lesions where found to correlate with disease severity (da Costa, C.E., et al, J. Exp. Med. 201 (2005) 687-693). The disease occurs primarily in a pediatric patient population and has to be treated with chemotherapy when the disease becomes systemic or is recurrent.

The pathophysiology of osteoporosis is mediated by loss of bone forming osteoblasts and increased osteoclast dependent bone resorption. Supporting data has been described by Cenci et al showing that an anti-M-CSF antibody injection preserves bone density and inhibits bone resorption in ovariectomized mice (Cenci, S., et al, J. Clin. Invest. 105 (2000) 1279-1287). Recently a potential link between postmenopausal bone loss due to estrogen deficiency was identified and found that the presence of TNF alpha producing T-cell affected bone metabolism (Roggia, C., et al., Minerva Med. 95 (2004) 125-132). A possible mechanism could be the induction of M-CSF by TNF alpha in vivo. An important role for M-CSF in TNF-alpha-induced osteoclastogenesis was confirmed by the effect of an antibody directed against M-CSF that blocked the TNF alpha induced osteolysis in mice and thereby making inhibitors of CSF-IR signaling potential targets for inflammatory arthritis (Kitaura, H., et al, J. Clin. Invest. 115 (2005) 3418-3427).

Paget's disease of bone (PDB) is the second most common bone metabolism disorder after osteoporosis in which focal abnormalities of increased bone turnover.
lead to complications such as bone pain, deformity, pathological fractures and deafness. Mutations in four genes have been identified that regulate normal osteoclast function and predispose individuals to PDB and related disorders: insertion mutations in TNFRSF1 IA, which encodes receptor activator of nuclear factor (NF) kappaB (RANK)-a critical regulator of osteoclast function, inactivating mutations of TNFRSF1 IB which encodes osteoprotegerin (a decoy receptor for RANK ligand), mutations of the sequestosome 1 gene (SQSTM1), which encodes an important scaffold protein in the NFkappaB pathway and mutations in the valosin-containing protein (VCP) gene. This gene encodes VCP, which has a role in targeting the inhibitor of NFkappaB for degradation by the proteasome (Daroszewska, A. and Ralston, S.H., Nat. Clin. Pract. Rheumatol. 2 (2006) 270-277). Targeted CSF-1R inhibitors provide an opportunity to block the deregulation of the RANKL signaling indirectly and add an additional treatment option to the currently used bisphosphonates.


Targeted inhibition of CSF-1R signaling is likely to be beneficial in other indications as well when targeted cell types include osteoclasts and macrophages e.g. treatment of specific complications in response to joint replacement as a consequence of rheumatoid arthritis. Implant failure due to periprosthetic bone loss and consequent loosing of prostheses is a major complication of joint replacement and requires repeated surgery with high socioeconomic burdens for the individual patient and the health-care system. To date, there is no approved drug therapy to prevent or inhibit periprosthetic osteolysis (Drees, P., et al., Nat. Clin. Pract. Rheumatol. 3 (2007) 165-171).
Glucocorticoid-induced osteoporosis (GIOP) is another indication in which a CSF-1R inhibitor could prevent bone loss after long-term glucocorticosteroid use that is given as a result of various conditions among those chronic obstructive pulmonary disease, asthma and rheumatoid arthritis (Guzman-Clark, J.R., et al., Arthritis Rheum. 57 (2007) 140-146; Feldstein, A.C., et al, Osteoporos. Int. 16 (2005) 2168-2174).

Rheumatoid arthritis, psoriatic arthritis and inflammatory arthritides are in itself potential indications for CSF-1R signaling inhibitors in that they consist of a macrophage component and to a varying degree bone destruction (Ritchlin, C.T., et al., J. Clin. Invest. 111 (2003) 821-831). Osteoarthritis and rheumatoid arthritis are inflammatory autoimmune disease caused by the accumulation of macrophages in the connective tissue and infiltration of macrophages into the synovial fluid, which is at least partially mediated by M-CSF. Campbell, I., K., et al., J. Leukoc. Biol. 68 (2000) 144-150, demonstrated that M-CSF is produced by human-joint tissue cells (chondrocytes, synovial fibroblasts) in vitro and is found in synovial fluid of patients with rheumatoid arthritis, suggesting that it contributes to the synovial tissue proliferation and macrophage infiltration which is associated with the pathogenesis of the disease. Inhibition of CSF-1R signaling is likely to control the number of macrophages in the joint and alleviate the pain from the associated bone destruction. In order to minimize adverse effects and to further understand the impact of the CSF-1R signaling in these indications, one method is to specifically inhibit CSF-1R without targeting a myriad other kinases, such as Raf kinase.


Expression and signaling of M-CSF and CSF-1R is found in activated microglia. Microglia, which are resident macrophages of the central nervous system, can be activated by various insults, including infection and traumatic injury. M-CSF is considered a key regulator of inflammatory responses in the brain and M-CSF levels increase in HIV-1, encephalitis, Alzheimer's disease (AD) and brain tumors. Microgliosis as a consequence of autocrine signaling by M-CSF/CSF-1R results in
induction of inflammatory cytokines and nitric oxides being released as demonstrated by e.g. using an experimental neuronal damage model (Hao, A.J., et al, Neuroscience 112 (2002) 889-900; Murphy, G.M., Jr., et al, J. Biol. Chem. 273 (1998) 20967-20971). Microglia that have increased expression of CSF-IR are found to surround plaques in AD and in the amyloid precursor protein V717F transgenic mouse model of AD (Murphy, G.M., Jr., et al, Am. J. Pathol. 157 (2000) 895-904). On the other hand op/op mice with fewer microglia in the brain resulted in fibrilar deposition of A-beta and neuronal loss compared to normal control suggesting that microglia do have a neuroprotective function in the development of AD lacking in the op/op mice (Kaku, M., et al., Brain Res. Brain Res. Protoc. 12 (2003) 104-108).

Expression and signaling of M-CSF and CSF-IR is associated with inflammatory bowel disease (IBD) (WO 2005/046657). The term "inflammatory bowel disease" refers to serious, chronic disorders of the intestinal tract characterized by chronic inflammation at various sites in the gastrointestinal tract, and specifically includes ulcerative colitis (UC) and Crohn's disease.

Thus another embodiment of the invention are the CSF-IR antibodies of the present invention for use in the treatment of periodontitis, histiocytosis X, osteoporosis, Paget's disease of bone (PDB), bone loss due to cancer therapy, periprosthetic osteolysis, glucocorticoid-induced osteoporosis, rheumatoid arthritis, psiratic arthritis, osteoarthritis, inflammatory arthridities, and inflammation.

The invention comprises the combination therapy with an antibody binding to human CSF-IR being characterized by the above mentioned epitope binding properties or alternatively by the above mentioned amino acid sequences and amino acid sequence fragments with an TLR9 agonist for the treatment of cancer.

The invention comprises the combination therapy with an antibody binding to human CSF-IR being characterized by the above mentioned epitope binding properties or alternatively by the above mentioned amino acid sequences and amino acid sequence fragments with an TLR9 agonist for the treatment of bone loss.

The invention comprises the combination therapy with an antibody binding to human CSF-IR being characterized by the above mentioned epitope binding properties or alternatively by the above mentioned amino acid sequences and
amino acid sequence fragments with an TLR9 agonist for the prevention or
treatment of metastasis.

The invention comprises the combination therapy with an antibody binding to
human CSF-1R being characterized by the above mentioned epitope binding
properties or alternatively by the above mentioned amino acid sequences and
amino acid sequence fragments with an TLR9 agonist for treatment of
inflammatory diseases.

The invention comprises the use of an antibody characterized in comprising the
antibody binding to human CSF-1R being characterized by the above
mentioned epitope binding properties or alternatively by the above mentioned
amino acid sequences and amino acid sequence fragments for the
combination treatment of cancer as described herein or alternatively for the
manufacture of a medicament for the combination treatment of cancer with
an TLR9 agonist as described herein.

The invention comprises the use of an antibody characterized in comprising the
antibody binding to human CSF-1R being characterized by the above
mentioned epitope binding properties or alternatively by the above mentioned
amino acid sequences and amino acid sequence fragments for the
combination treatment as described herein of bone loss or alternatively for
the manufacture of a medicament for the combination treatment of bone loss
with an TLR9 agonist as described herein.

The invention comprises the use of an antibody characterized in comprising the
antibody binding to human CSF-1R being characterized by the above
mentioned epitope binding properties or alternatively by the above mentioned
amino acid sequences and amino acid sequence fragments for the prevention
or treatment of metastasis with the combination as described herein or
alternatively for the manufacture of a medicament for the prevention or
treatment of metastasis with the combination with an TLR9 agonist as
described herein.

The invention comprises the use of an antibody characterized in comprising the
antibody binding to human CSF-1R being characterized by the above
mentioned epitope binding properties or alternatively by the above mentioned
amino acid sequences and amino acid sequence fragments for combination
treatment of inflammatory diseases as described herein or alternatively for
the manufacture of a medicament for the combination treatment of inflammatory diseases with an TLR9 agonist as described herein.

The antibodies according to the invention are preferably produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in prokaryotic and eukaryotic cells with subsequent isolation of the antibody polypeptide and usually purification to a pharmaceutically acceptable purity. For the protein expression nucleic acids encoding light and heavy chains or fragments thereof are inserted into expression vectors by standard methods. Expression is performed in appropriate prokaryotic or eukaryotic host cells, such as CHO cells, NSO cells, SP2/0 cells, HEK293 cells, COS cells, yeast, or E. coli cells, and the antibody is recovered from the cells (from the supernatant or after cells lysis).


The antibodies may be present in whole cells, in a cell lysate, or in a partially purified, or substantially pure form. Purification is performed in order to eliminate other cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. See Ausubel, F., et al, ed. Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987).

Nucleic acid molecules encoding amino acid sequence variants of anti-CSF-IR antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of humanized anti-CSF-IR antibody.

The heavy and light chain variable domains according to the invention are combined with sequences of promoter, translation initiation, constant region, 3′ untranslated region, polyadenylation, and transcription termination to form expression vector constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a single host cell expressing both chains.

In another aspect, the present invention provides a composition, e.g. a pharmaceutical composition, containing one or a combination of monoclonal antibodies, or the antigen-binding portion thereof, of the present invention, formulated together with a pharmaceutically acceptable carrier.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption/resorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for injection or infusion.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. In addition to water, the carrier can be, for example, an isotonic buffered saline solution.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into
pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient (effective amount). The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

The term "a method of treating" or its equivalent, when applied to, for example, cancer refers to a procedure or course of action that is designed to reduce or eliminate the number of cancer cells in a patient, or to alleviate the symptoms of a cancer. "A method of treating" cancer or another proliferative disorder does not necessarily mean that the cancer cells or other disorder will, in fact, be eliminated, that the number of cells or disorder will, in fact, be reduced, or that the symptoms of a cancer or other disorder will, in fact, be alleviated. Often, a method of treating cancer will be performed even with a low likelihood of success, but which, given the medical history and estimated survival expectancy of a patient, is nevertheless deemed to induce an overall beneficial course of action.

The terms "administered in combination with" or "co-administration", "co-administering" or "a combination" refer to the administration of the anti-CSF-IR, and the TLR 9 agonist e.g. as separate formulations/applications (or as one single formulation/application). The co-administration can be simultaneous or sequential in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Said antibody and said TLR9 agonist are co-administered either simultaneously or sequentially (e.g. intravenous (i.v.) through a continuous infusion. When both therapeutic agents are co-administered sequentially the dose is administered either on the same day in two separate administrations, or one of the agents is administered on day 1 and the second is co-administered on day 2 to day 7, preferably on day 2 to 4. Thus in one
embodiment the term "sequentially" means within 7 days after the dose of the first component, preferably within 4 days after the dose of the first component; and the term "simultaneously" means at the same time. The terms "co-administration" with respect to the maintenance doses of anti-CSF-IR antibody mean that the maintenance doses can be either co-administered simultaneously, if the treatment cycle is appropriate for both drugs, e.g. every week. Or the further agent is e.g. administered e.g. every first to third day and said antibody is administered every week. Or the maintenance doses are co-administered sequentially, either within one or within several days.

It is self-evident that the antibodies are administered to the patient in a "therapeutically effective amount" (or simply "effective amount") which is the amount of the respective compound or combination that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician.

The amount of co-administration and the timing of co-administration will depend on the type (species, gender, age, weight, etc.) and condition of the patient being treated and the severity of the disease or condition being treated. Said anti-CSF-IR antibody and further agent are suitably co-administered to the patient at one time or over a series of treatments e.g. on the same day or on the day after.

Depending on the type and severity of the disease, about 0.1 mg/kg to 50 mg/kg (e.g. 0.1-20 mg/kg) of said anti-CSF-IR antibody; is an initial candidate dosage for co-administration of both drugs to the patient The invention comprises the use of the antibodies according to the invention for the treatment of a patient suffering from cancer, especially from colon, lung or pancreas cancer.

In addition to the anti-CSF-IR antibody in combination with the TLR9 agonist also a chemotherapeutic agent can be administered.

In one embodiment such additional chemotherapeutic agents, which may be administered with anti-CSF-IR antibody and the TLR9 agonist, include, but are
not limited to, anti-neoplastic agents including alkylating agents including: nitrogen mustards, such as mechloremethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil; nitrosoureas, such as carmustine (BCNU), lomustine (CCNU), and semustine (methyl-CCNU); Temodal(TM) (temozolamide), ethylenimines/methylmelamine such as thriethylenemelamine (TEM), triethylene, thiophosphoramid (tiotepa), hexamethylmelamine (HMM, altretamine); alkyl sulfonates such as busulfan; triazines such as dacarbazine (DTIC); antimetabolites including folic acid analogs such as methotrexate and trimetrexate, pyrimidine analogs such as 5-fluorouracil (5FU), fluorodeoxyuridine, gemcitabine, cytosine arabinoside (AraC, cytarabine), 5-azacytidine, 2,2'- difluorodeoxycytidine, purine analogs such as 6-merca.rho.topurine, 6-thioguanamne, azathioprine, T-deoxycoformycin (pentostatin), erythrophoxynonyladenine (EHNA), fludarabine phosphate, and 2- chlorodeoxyadenosine (cladribine, 2-CdA); natural products including antimitotic drugs such as paclitaxel, vinca alkaloids including vinblastine (VLB), vincristine, and vinorelbine, taxotere, estramustine, and estramustine phosphate; pipodophytoxins such as etoposide and teniposide; antibiotics such as actimomycin D, daunomycin (rubidomycin), doxorubicin, mitoxantrone, idarubicin, bleomycins, plicamycin (mithramycin), mitomycinC, and actinomycin; enzymes such as L-asparaginase; biological response modifiers such as interferon-alpha, IL-2, G-CSF and GM-CSF; miscellaneous agents including platinum coordination complexes such as oxaliplatin, cisplatin and carboplatin, anthracenediones such as mitoxantrone, substituted urea such as hydroxyurea, methylhydrazine derivatives including N- methylhydrazine (MIH) and procarbazine, adrenocortical suppressants such as mitotane (o, p-DDD) and aminoglutethimide; hormones and antagonists including adrenocorticosteroid antagonists such as prednisone and equivalents, dexamethasone and aminoglutethimide; Gemzar(TM) (gemcitabine), progestin such as hydroxyprogesterone caproate, medroxyprogesterone acetate and megestrol acetate; estrogen such as diethylstilbestrol and ethinyl estradiol equivalents; antiestrogen such as tamoxifen; androgens including testosterone propionate and fluoxymesterone/equivalents; antiandrogens such as flutamide, gonadotropin-releasing hormone analogs and leuprolide; and non-steroidal antiandrogens such as flutamide. Therapies targeting epigenetic mechanism including, but not limited to, histone deacetylase inhibitors, demethylating agents (e.g., Vidaza) and release of transcriptional repression (ATRA) therapies can also be combined with the antigen binding proteins. In one embodiment the chemotherapeutic agent is selected from the group consisting of taxanes (like e.g. paclitaxel (Taxol), docetaxel (Taxotere),
modified paclitaxel (e.g., Abraxane and Opaxio), doxorubicin, sunitinib (Sutent), sorafenib (Nexavar), and other multikinase inhibitors, oxaliplatin, cisplatin and carboplatin, etoposide, gemcitabine, and vinblastine. In one embodiment the chemotherapeutic agent is selected from the group consisting of taxanes (like e.g. taxol (paclitaxel), docetaxel (Taxotere), modified paclitaxel (e.g. Abraxane and Opaxio). In one embodiment, the additional chemotherapeutic agent is selected from 5-fluorouracil (5-FU), leucovorin, irinotecan, or oxaliplatin. In one embodiment the chemotherapeutic agent is 5-flourouracil, leucovorin and irinotecan (FOLFIRI). In one embodiment the chemotherapeutic agent is 5-fluorouracil, and oxaliplatin (FOLFOX).

Specific examples of combination therapies with additional chemotherapeutic agents include, for instance, therapies taxanes (e.g., docetaxel or paclitaxel) or a modified paclitaxel (e.g., Abraxane or Opaxio), doxorubicin), capecitabine and/or bevacizumab (Avastin) for the treatment of breast cancer; therapies with carboplatin, oxaliplatin, cisplatin, paclitaxel, doxorubicin (or modified doxorubicin (Caelyx or Doxil)), or topotecan (Hycamtin) for ovarian cancer, the therapies with a multi-kinase inhibitor, MKI, (Sutent, Nexavar, or 706) and/or doxorubicin for treatment of kidney cancer; therapies with oxaliplatin, cisplatin and/or radiation for the treatment of squamous cell carcinoma; therapies with taxol and/or carboplatin for the treatment of lung cancer.

Therefore, in one embodiment the additional chemotherapeutic agent is selected from the group of taxanes (docetaxel or paclitaxel or a modified paclitaxel (Abraxane or Opaxio), doxorubicin, capecitabine and/or bevacizumab for the treatment of breast cancer.

In one embodiment the CSF-1R antibody/TLR9 agonist combination therapy is no chemotherapeutic agents are administered.

The invention comprises also a method for the treatment of a patient suffering from such disease.

The invention further provides a method for the manufacture of a pharmaceutical composition comprising an effective amount of an antibody according to the invention together with a pharmaceutically acceptable carrier and the use of the antibody according to the invention for such a method.
The invention further provides the use of an antibody according to the invention in an effective amount for the manufacture of a pharmaceutical agent, preferably together with a pharmaceutically acceptable carrier, for the treatment of a patient suffering from cancer.

The invention also provides the use of an antibody according to the invention in an effective amount for the manufacture of a pharmaceutical agent, preferably together with a pharmaceutically acceptable carrier, for the treatment of a patient suffering from cancer.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

**Description of the Sequences**

SEQ ID NO: 1 heavy chain CDR3, Mab 2F1 1
SEQ ID NO: 2 heavy chain CDR2, Mab 2F1 1
SEQ ID NO: 3 heavy chain CDR1, Mab 2F1 1
SEQ ID NO: 4 light chain CDR3, Mab 2F1 1
SEQ ID NO: 5 light chain CDR2, Mab 2F1 1
SEQ ID NO: 6 light chain CDR1, Mab 2F1 1
SEQ ID NO: 7 heavy chain variable domain, Mab 2F1 1
SEQ ID NO: 8 light chain variable domain, Mab 2F1 1
SEQ ID NO: 9 heavy chain CDR3, Mab 2E10
SEQ ID NO: 10 heavy chain CDR2, Mab 2E10
SEQ ID NO: 11 heavy chain CDR1, Mab 2E10
SEQ ID NO: 12 light chain CDR3, Mab 2E10
SEQ ID NO: 13 light chain CDR2, Mab 2E10
SEQ ID NO: 14 light chain CDR1, Mab 2E10
SEQ ID NO: 15 heavy chain variable domain, Mab 2E10
SEQ ID NO: 16 light chain variable domain, Mab 2E10
SEQ ID NO: 17 heavy chain CDR3, hMab 2F1 1-cl 1
SEQ ID NO: 18 heavy chain CDR2, hMab 2F1 1-cl 1
SEQ ID NO: 19 heavy chain CDR1, hMab 2F1 1-cl 1
SEQ ID NO: 20 light chain CDR3, hMab 2F1 1-cl 1
SEQ ID NO: 21 light chain CDR2, hMab 2F1 1-cl 1
SEQ ID NO: 22  light chain CDR1, hMab 2F1 1-cl 1
SEQ ID NO: 23  heavy chain variable domain, hMab 2F1 1-cl 1
SEQ ID NO: 24  light chain variable domain, hMab 2F1 1-cl 1
SEQ ID NO: 25  heavy chain CDR3, hMab 2F1 l-d8
SEQ ID NO: 26  heavy chain CDR2, hMab 2F1 l-d8
SEQ ID NO: 27  heavy chain CDR1, hMab 2F1 l-d8
SEQ ID NO: 28  light chain CDR3, hMab 2F1 l-d8
SEQ ID NO: 29  light chain CDR2, hMab 2F1 l-d8
SEQ ID NO: 30  light chain CDR1, hMab 2F1 l-d8
SEQ ID NO: 31  heavy chain variable domain, hMab 2F1 l-d8
SEQ ID NO: 32  light chain variable domain, hMab 2F1 l-d8
SEQ ID NO: 33  heavy chain CDR3, hMab 2F1 l-e7
SEQ ID NO: 34  heavy chain CDR2, hMab 2F1 l-e7
SEQ ID NO: 35  heavy chain CDR1, hMab 2F1 l-e7
SEQ ID NO: 36  light chain CDR3, hMab 2F1 l-e7
SEQ ID NO: 37  light chain CDR2, hMab 2F1 l-e7
SEQ ID NO: 38  light chain CDR1, hMab 2F1 l-e7
SEQ ID NO: 39  heavy chain variable domain, hMab 2F1 l-e7
SEQ ID NO: 40  light chain variable domain, hMab 2F1 l-e7
SEQ ID NO: 41  heavy chain CDR3, hMab 2F1 l-f12
SEQ ID NO: 42  heavy chain CDR2, hMab 2F1 l-f12
SEQ ID NO: 43  heavy chain CDR1, hMab 2F1 l-f12
SEQ ID NO: 44  light chain CDR3, hMab 2F1 l-f12
SEQ ID NO: 45  light chain CDR2, hMab 2F1 l-f12
SEQ ID NO: 46  light chain CDR1, hMab 2F1 l-f12
SEQ ID NO: 47  heavy chain variable domain, hMab 2F1 l-f12
SEQ ID NO: 48  light chain variable domain, hMab 2F1 l-f12
SEQ ID NO: 49  heavy chain CDR3, hMab 2F1 l-gl
SEQ ID NO: 50  heavy chain CDR2, hMab 2F1 l-gl
SEQ ID NO: 51  heavy chain CDR1, hMab 2F1 l-gl
SEQ ID NO: 52  light chain CDR3, hMab 2F1 l-gl
SEQ ID NO: 53  light chain CDR2, hMab 2F1 l-gl
SEQ ID NO: 54  light chain CDR1, hMab 2F1 l-gl
SEQ ID NO: 55  heavy chain variable domain, hMab 2F1 l-gl
SEQ ID NO: 56  light chain variable domain, hMab 2F1 l-gl
SEQ ID NO: 57  human kappa light chain constant region
SEQ ID NO: 58  human heavy chain constant region derived from IgGl
SEQ ID NO: 59  human heavy chain constant region derived from IgGl
   mutated on L234A and L235A
SEQ ID NO: 60  human heavy chain constant region derived from IgG4
SEQ ID NO: 61  human heavy chain constant region derived from IgG4
   mutated on S228P
SEQ ID NO: 62  human wildtype CSF-IR (wt CSF-IR)
SEQ ID NO: 63  human mutant CSF-IR L301S Y969F
SEQ ID NO: 64  human CSF-IR Extracellular Domain (domains D1-D5)
SEQ ID NO: 65  human CSF-IR fragment delD4
SEQ ID NO: 66  human CSF-IR fragment domains D1-D3
SEQ ID NO: 67  signal peptide
SEQ ID NO: 68  Primer
SEQ ID NO: 69  heavy chain CDR3, Mab 1G10
SEQ ID NO: 70  heavy chain CDR2, Mab 1G10
SEQ ID NO: 71  heavy chain CDR1, Mab 1G10
SEQ ID NO: 72  light chain CDR3, Mab 1G10
SEQ ID NO: 73  light chain CDR2, Mab 1G10
SEQ ID NO: 74  light chain CDR1, Mab 1G10
SEQ ID NO: 75  heavy chain variable domain, Mab 1G10
SEQ ID NO: 76  light chain variable domain, Mab 1G10
SEQ ID NO: 77  heavy chain CDR3, Mab 2H7
SEQ ID NO: 78  heavy chain CDR2, Mab 2H7
SEQ ID NO: 79  heavy chain CDR1, Mab 2H7
SEQ ID NO: 80  light chain CDR3, Mab 2H7
SEQ ID NO: 81  light chain CDR2, Mab 2H7
SEQ ID NO: 82  light chain CDR1, Mab 2H7
SEQ ID NO: 83  heavy chain variable domain, Mab 2H7
SEQ ID NO: 84  light chain variable domain, Mab 2H7
SEQ ID NO: 85  human CSF-IR fragment domains D4-D5
SEQ ID NO: 86  human CSF-1
SEQ ID NO: 87  human IL-34
SEQ ID NO: 88  human toll-like receptor 9 (TLR9)
SEQ ID NO: 89  TLR9 agonist CpG ODN 2216
SEQ ID NO: 90  TLR9 agonist CpG ODN PB4
SEQ ID NO: 91  TLR9 agonist CpG ODN 1002
SEQ ID NO: 92  TLR9 agonist CpG-7909 (CpG 2006, PF-35 12676, Agatolimod)
In the following embodiment of the invention are described:

1. An antibody which binds to human CSF-IR wherein the antibody is administered in combination with a Toll-like receptor 9 (TLR9) agonist for use in the treatment of cancer.

2. Use of a combination of
   i) an antibody which binds to human CSF-IR, and
   ii) a Toll-like receptor 9 (TLR9) agonist

   for the manufacture of a medicament for use in the treatment of cancer.

3. The antibody or use according to embodiments 1 or 2, wherein the cancer is further characterized by CSF-IR expression or overexpression.

4. The antibody or use according to any one of embodiments 1 or 2, wherein the cancer is a breast cancer, colorectal cancer, melanoma, head and neck cancer, lung cancer or prostate cancer.

5. An antibody which binds to human CSF-IR characterized in binding to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-IR for use in
   a) the inhibition of cell proliferation in CSF-IR ligand-dependent and/or CSF-1 ligand-independent CSF-IR expressing tumor cells;
   b) the inhibition of cell proliferation of tumors with CSF-IR ligand-dependent and/or CSF-IR ligand-independent CSF-IR expressing macrophage infiltrate;
   c) the inhibition of cell survival (in CSF-IR ligand-dependant and/or CSF-IR ligand-independent) CSF-IR expressing monocytes and macrophages; and/or
d) the inhibition of cell differentiation (in CSF-IR ligand-dependent and/or CSF-IR ligand-independent) CSF-IR expressing monocytes into macrophages,

wherein the antibody is administered in combination with a TLR9 agonist.

6. Use of a combination of

i) an antibody which binds to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-IR, and

ii) a Toll-like receptor 9 (TLR9) agonist

for the manufacture of a medicament for use in

a) the inhibition of cell proliferation in CSF-IR ligand-dependent and/or CSF-1 ligand-independent CSF-IR expressing tumor cells;

b) the inhibition of cell proliferation of tumors with CSF-IR ligand-dependent and/or CSF-IR ligand-independent CSF-IR expressing macrophage infiltrate;

c) the inhibition of cell survival (in CSF-IR ligand-dependant and/or CSF-IR ligand-independent) CSF-IR expressing monocytes and macrophages; and/or

d) the inhibition of cell differentiation (in CSF-IR ligand-dependent and/or CSF-IR ligand-independent) CSF-IR expressing monocytes into macrophages.

7. An antibody which binds to human CSF-IR, for use in the treatment of a patient having a CSF-IR expressing tumor or having a tumor with CSF-IR expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-IR ligand and wherein the anti-CSF-IR antibody is administered in combination with a TLR9 agonist.

8. Use of a combination of

i) an antibody which binds to human CSF-IR, and

ii) a Toll-like receptor 9 (TLR9) agonist
for the manufacture of a medicament for use in the treatment of a patient having a CSF-1R expressing tumor or having a tumor with CSF-1R expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-1R ligand.

9. The antibody or use according to any one of embodiments 1 or 8 wherein the TLR9 agonist is characterized by induction of IFN-alpha, IL-6, and/or IL-12 in plasmacytoid dendritic cells (pDCs).

10. The antibody or use according to any one of embodiments 1 to 8, wherein the TLR9 agonist is a oligodeoxynucleotides containing cytosine-phosphate-guanosine (CpG) motifs (CpG ODNs).

11. The antibody or use according to any one of embodiments 1 to 10 wherein the antibody is characterized in binding to the domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-1R.

12. The antibody according any one of the preceding embodiments, wherein the antibody is characterized in that the antibody does not bind to human CSF-1R fragment delD4 (SEQ ID NO: 65).

13. The antibody according any one of the preceding embodiments, characterized in that

a) the heavy chain variable domain is SEQ ID NO: 7 and the light chain variable domain is SEQ ID NO: 8,

b) the heavy chain variable domain is SEQ ID NO: 15 and the light chain variable domain is SEQ ID NO: 16;

c) the heavy chain variable domain is SEQ ID NO: 75 and the light chain variable domain is SEQ ID NO: 76;

d) the heavy chain variable domain is SEQ ID NO: 83 and the light chain variable domain is SEQ ID NO: 84;

e) the heavy chain variable domain is SEQ ID NO: 23 and the light chain variable domain is SEQ ID NO: 24, or

f) the heavy chain variable domain is SEQ ID NO: 31 and the light chain variable domain is SEQ ID NO: 32, or
g) the heavy chain variable domain is SEQ ID NO:39 and the light chain variable domain is SEQ ID NO:40, or

h) the heavy chain variable domain is SEQ ID NO:47 and the light chain variable domain is SEQ ID NO:48, or

i) the heavy chain variable domain is SEQ ID NO:55 and the light chain variable domain is SEQ ID NO:56.

14. The antibody according any one of the preceding embodiments, characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO: 2, and a CDR1 region of SEQ ID NO:3, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5, and a CDR1 region of SEQ ID NO:6, or

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 9, a CDR2 region of SEQ ID NO: 10, and a CDR1 region of SEQ ID NO: 11, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 12, a CDR2 region of SEQ ID NO: 13, and a CDR1 region of SEQ ID NO: 14, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO: 19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO: 21, and a CDR1 region of SEQ ID NO: 22, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region
of SEQ ID NO:36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

f) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO:43, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 44, a CDR2 region of SEQ ID NO:45, and a CDR1 region of SEQ ID NO:46, or

g) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 49, a CDR2 region of SEQ ID NO: 50, and a CDR1 region of SEQ ID NO: 51, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:52, a CDR2 region of SEQ ID NO: 53, and a CDR1 region of SEQ ID NO: 54; or

h) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:69, a CDR2 region of SEQ ID NO: 70, and a CDR1 region of SEQ ID NO: 71, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:72, a CDR2 region of SEQ ID NO: 73, and a CDR1 region of SEQ ID NO: 74, or

i) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 77, a CDR2 region of SEQ ID NO: 78, and a CDR1 region of SEQ ID NO: 79, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:80, a CDR2 region of SEQ ID NO: 81, and a CDR1 region of SEQ ID NO: 82.

15. The antibody according any one of the preceding embodiments, characterized in that said antibody is of human IgG1 subclass or is of human IgG4 subclass.

16. A method of treatment comprising administering to a patient suffering from cancer an effective amount of an antibody which binds to human CSF-IR wherein the antibody is administered in combination with a Toll-like receptor 9 (TLR9) agonist.

17. The method according to embodiment 16, wherein the cancer is further characterized by CSF-IR expression or overexpression.
18. The method according to embodiment 16, wherein the cancer is a breast cancer, colorectal cancer, melanoma, head and neck cancer, lung cancer or prostate cancer.

19. A method comprising administering an effective amount of an antibody which binds to human CSF-1R and is characterized in binding to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-1R for use in

a) the inhibition of cell proliferation in CSF-1R ligand-dependent and/or CSF-1 ligand-independent CSF-1R expressing tumor cells;

b) the inhibition of cell proliferation of tumors with CSF-1R ligand-dependent and/or CSF-1R ligand-independent CSF-1R expressing macrophage infiltrate;

c) the inhibition of cell survival (in CSF-1R ligand-dependant and/or CSF-1R ligand-independent) CSF-1R expressing monocytes and macrophages; and/or

d) the inhibition of cell differentiation (in CSF-1R ligand-dependent and/or CSF-1R ligand-independent) CSF-1R expressing monocytes into macrophages,

wherein the antibody is administered in combination with an effective amount of a TLR9 agonist.

20. A method of treatment comprising administering an effective amount of an antibody which binds to human CSF-1R, for use in the treatment of a patient having a CSF-1R expressing tumor or having a tumor with CSF-1R expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-1R ligand and wherein the anti-CSF-1R antibody is administered in combination with an effective amount of a TLR9 agonist.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.
Examples

Example 1
Generation of a hybridoma cell line producing anti-CSF-IR antibodies

Immunization procedure of NMRI mice

NMRI mice were immunized with an expression vector pDisplay™ (Invitrogen, USA) encoding the extracellular domain of huCSF-IR by utilizing electroporation. Every mouse was 4 times immunized with 100 µg DNA. When serum titers of anti-huCSF-IR were found to be sufficient, mice were additionally boosted once with 50 µg of a 1:1 mixture huCSF-IR ECD/huCSF-IR ECDhuFc chimera in 200 µl PBS intravenously (i.v.) 4 and 3 days before fusion.

Antigen specific ELISA

Anti-CSF-IR titers in sera of immunized mice were determined by antigen specific ELISA.

0.3 µg/ml huCSF-IR-huFc chimera (soluble extracellular domain) was captured on a streptavidin plate (MaxiSorb; MicroCoat, DE, Cat.No. 11974998/MC1099) with 0.1 mg/ml biotinylated anti Fey (Jackson ImmunoResearch., Cat.No. 109-066-098) and horse radish peroxidase (HRP)-conjugated F(ab′)₂ anti-mouse IgG (GE Healthcare, UK, Cat.No.NA9310V) diluted 1/800 in PBS/0.05% Tween20/0.5% BSA was added. Sera from all taps were diluted 1/40 in PBS/0.05% Tween20/0.5% BSA and serially diluted up to 1/1638400. Diluted sera were added to the wells. Pre-tap serum was used as negative control. A dilution series of mouse anti-human CSF-1R Mab3291 (R&D Systems, UK) from 500 ng/ml to 0.25 ng/ml was used as positive control. All components were incubated together for 1.5 hours, Wells were washed 6 times with PBST (PBS/0.2% Tween20) and assays were developed with freshly prepared ABTS® solution (1 mg/ml) (ABTS: 2,2’-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) for 10 minutes at RT. Absorbance was measured at 405 nm.

Hybridoma generation

The mouse lymphocytes can be isolated and fused with a mouse myeloma cell line using PEG based standard protocols to generate hybridomas. The resulting hybridomas are then screened for the production of antigen-specific antibodies. For
example, single cell suspensions of splenic derived lymphocytes from immunized mice are fused to Ag8 non-secreting mouse myeloma cells P3X63Ag8.653 (ATCC, CRL-1580) with 50% PEG. Cells are plated at approximately $10^4$ in flat bottom 96 well micro titer plate, followed by about two weeks incubation in selective medium. Individual wells are then screened by ELISA for human anti-CSF-IR monoclonal IgM and IgG antibodies. Once extensive hybridoma growth occurs, the antibody secreting hybridomas are replated, screened again, and if still positive for human IgG, anti-CSF-IR monoclonal antibodies, can be subcloned by FACS. The stable subclones are then cultured in vitro to produce antibody in tissue culture medium for characterization. Antibodies according to the invention could be selected using the determination of the binding of anti-CSF-IR antibodies to human CSF-IR fragment delD4 and to human CSF-IR Extracellular Domain (CSF-1R-ECD) as described in Example 4, as well as the determination of growth inhibition of NIH3T3 cells transfected with wildtype CSF-IR (ligand dependent signalling) or mutant CSF-IR L301S Y969F (ligand independent signalling) under treatment with anti-CSF-IR monoclonal antibodies as described in Example 5.

**Culture of hybridomas**

Generated mMuMAb hybridomas were cultured in RPMI 1640 (PAN - Catalogue No. (Cat. No.) P04- 17500) supplemented with 2 mM L-glutamine (GIBCO - Cat. No.35050-038), 1 mM Na-Pyruvat (GIBCO - Cat. No.1 360-039), 1x NEAA (GIBCO - Cat. No.1 360-035), 10% FCS (PAA - Cat. No.A15-649), 1x Pen Strep (Roche - Cat. No.1074440), 1x Nutridoma CS (Roche - Cat. No.1363743), 50 µM Mercaptoethanol (GIBCO - Cat. No.3 1350-010) and 50 U/ml IL 6 mouse (Roche - Cat. No.1 444 581) at 37°C and 5% CO$_2$. Some of the resulting mouse antibodies have been humanized (e.g. Mab 2F1) and been expressed recombinantly.

**Example 2**

**Inhibition of CSF-1 binding to CSF-IR (ELISA)**

By setting-up this assay to first allow for anti-CSF-IR antibody binding to the CSF-1R-ECD followed by detection of ligand not bound to the receptor both-ligand displacing antibodies and dimerization inhibitor anti-CSF-IR antibodies - can be tested. The test was performed on 384 well microtiter plates (MicroCoat, DE, Cat.No. 464718) at RT. After each incubation step plates were washed 3 times with PBST.
At the beginning, plates were coated with 0.5 mg/ml goat F(ab')2 biotinylated anti Fey (Jackson ImmunoResearch., Cat.No. 109-006-170) for 1 hour (h).

Thereafter the wells were blocked with PBS supplemented with 0.2% Tween®-20 and 2% BSA (Roche Diagnostics GmbH, DE) for 0.5 h. 75 ng/ml of huCSF-1R-huFc chimera (which forms the dimeric soluble extracellular domain of huCSF-1R) was immobilized to plate for 1 h. Then dilutions of purified antibodies in PBS/0.05% Tween20/0.5% BSA were incubated for 1 h. After adding a mixture of 3 ng/ml hu CSF-1 (active 149 aa fragment of human CSF-1 (aa 33-181 of SEQ ID NO: 86) ;Biomol, DE, Cat.No.60530), 50ng/ml biotinylated anti CSF-1 clone BAF216 (R&D Systems,UK) and 1:5000 diluted streptavidin HRP (Roche Diagnostics GmbH, DE, Cat.No.l 1089153001) for 1 h the plates were washed 6 times with PBST. Anti CSF-1R SC 2-4A5 (Santa Cruz Biotechnology, US), which inhibits the ligand- receptor interaction, was used as positive control. Plates were developed with freshly prepared BM blue® POD substrate solution (BM blue®: 3,3’-5,5’-Tetramethylbenzidine, Roche Diagnostics GmbH, DE, Cat.No. 11484281001) for 30 minutes at RT. Absorbance was measured at 370 nm. A decrease of absorbance is found, if the anti-CSF-1R antibody causes a release of CSF-1 from the dimeric complex. All anti-CSF-1R antibodies showed significant inhibition of the CSF-1 interaction with CSF-1R (see Table 1). Anti CSF-1R SC 2-4A5 (Santa Cruz Biotechnology, US see also Sherr, C.J. et al, Blood 73 (1989) 1786-1793), which inhibits the ligand- receptor interaction, was used as reference control.

Table 1:
Calculated IC50 values for the inhibition of the CSF-1/CSF-1R interaction

<table>
<thead>
<tr>
<th>CSF-1R Mab</th>
<th>IC50 CSF-1 /CSF-1R Inhibition [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab 2Fl 1</td>
<td>19.3</td>
</tr>
<tr>
<td>Mab 2E10</td>
<td>20.6</td>
</tr>
<tr>
<td>Mab 2H7</td>
<td>18.2</td>
</tr>
<tr>
<td>Mab 1G10</td>
<td>11.8</td>
</tr>
<tr>
<td>SC-2-4A5</td>
<td>35.2</td>
</tr>
</tbody>
</table>
Example 3
Inhibition of CSF-1-induced CSF-IR phosphorylation in NIH3T3-CSF-1R recombinant cells

4.5x10^3 NIH 3T3 cells, retrovirally infected with an expression vector for full-length CSF-IR, were cultured in DMEM (PAA Cat. No.E15-01 1), 2mM L-glutamine (Sigma, Cat.No.G7513), 2mM Sodium pyruvate, 1x nonessential aminoacids, 10% FKS (PAA, Cat.No.AI 5-649) and 100µg/ml PenStrep (Sigma, Cat.No. P4333 [10mg/ml]) until they reached confluency. Thereafter cells were washed with serum-free DMEM media (PAA Cat.No.E15-01 1) supplemented with sodium selenite [5ng/ml] (Sigma, Cat.No. S9133), transferrin [10µg/ml] (Sigma, Cat.No. T8158), BSA [400µg/ml] (Roche Diagnostics GmbH, Cat.No. 10735078), 4mM L-glutamine (Sigma, Cat.No.G7513), 2mM sodium pyruvate (Gibco, Cat.No. 11360), 1x nonessential aminoacids (Gibco, Cat: 11140-035), 2-mercaptoethanol [0.05mM] (Merck, Cat.No. M7522), 100µg/ml and PenStrep (Sigma, Cat. No. P4333) and incubated in 30 µl of the same medium for 16 hours to allow for receptor up-regulation. 10 µl of diluted anti-CSF-IR antibodies were added to the cells for 1.5 h. Then cells were stimulated with 10 µl of 100 ng/ml hu CSF-1 (active 149 aa fragment of human CSF-1 (aa 33-181 of SEQ ID NO: 86) ;Biomol, DE, Cat.No.60530) for 5 min. After the incubation, supernatant was removed, cells were washed twice with 80 µl of ice-cold PBS and 50 µl of freshly prepared ice-cold lysis buffer (150mM NaCl/ 20mM Tris pH 7.5 / 1mM EDTA/ 1mM EGTA/ 1% Triton X-100 \ protease inhibitor tablet (Roche Diagnostics GmbH Cat.No. 1836 170) per 10 ml buffer,l0µl/ml phosphatase inhibitor cocktail 1 (Sigma Cat.No. P-2850, 10Ox Stock), lOµl/ml protease inhibitor 1 (Sigma Cat.No.P-5726, 10Ox Stock) /10µH2O 1 M NaF ) was added. After 30 minutes on ice the plates were shaken vigourously on a plateshaker for 3 minutes and then centrifuged 10 minutes at 2200 rpm (Heraeus Megafuge 10).

The presence of phosphorylated and total CSF-1 receptor in the cell lysate was analyzed with Elisa. For detection of the phosphorylated receptor the kit from R&D Systems (Cat. No. DYC3268-2) was used according to the instructions of the supplier. For detection of total CSF-IR 10 µl of the lysate was immobilized on plate by use of the capture antibody contained in the kit. Thereafter 1:750 diluted biotinylated anti CSF-IR antibody BAF329 (R&D Systems) and 1:1000 diluted streptavidin-HRP conjugate was added. After 60 minutes plates were developed with freshly prepared ABTS® solution and the absorbance was detected. Data were calculated as % of positive control without antibody and the ratio value
phospho/total receptor expressed. The negative control was defined without addition of M-CSF-1. Anti CSF-IR SC 2-4A5 (Santa Cruz Biotechnology, US, see also Sherr, C.J. et al, Blood 73 (1989) 1786-1793), which inhibits the ligand-receptor interaction, was used as reference control.

Table 2:
Calculated IC50 values for the inhibition of CSF-1 receptor phosphorylation.

<table>
<thead>
<tr>
<th>CSF-1R Mab</th>
<th>IC50 CSF-1R Phosphorylation [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab 2F11</td>
<td>219.4</td>
</tr>
<tr>
<td>Mab 2E10</td>
<td>752.0</td>
</tr>
<tr>
<td>Mab 2H7</td>
<td>703.4</td>
</tr>
<tr>
<td>Mab 1G10</td>
<td>56.6</td>
</tr>
<tr>
<td>SC-2-4A5</td>
<td>1006.6</td>
</tr>
</tbody>
</table>

Example 4
Determination of the binding of anti-CSF-IR antibodies to human CSF-IR fragment delD4 and to human CSF-IR Extracellular Domain (CSF-1R-ECD)

Preparation of human CSF-IR Extracellular Domain (CSF-1R-ECD) (comprising the extracellular subdomains D1-D5, hCSF-1R-ECD) of SEQ ID NO: 64:

pCMV-preS-Fc-hCSF-IR-ECD (7836bp) encodes the complete ECD of human CSF-IR (SEQ ID NO: 64) C-terminally fused to a PreScission protease cleavage site, followed by aal00-330 of human IgG1 and a 6xHis-Tag, under the control of CMV promoter. The natural signal peptide has been varied by insertion of amino acids G and S after the first M, in order to create a BamHI restriction site.

Preparation of human CSF-IR fragment delD4 (comprising the extracellular subdomains D1-D3 and D5, hCSF-IR-delD4) of SEQ ID NO: 65:

hCSFIR-delD4-V1-PreSc-hFc-His was cloned from pCMV-preS-Fc-hCSF-IR-ECD by means of the Stratagene QuikChange XL site-directed mutagenesis protocol, using delD4-for with sequence CACCTCCATGTTCCTCGGTACCCCCGAGGTAAG (SEQ ID NO: 68) as the forward primer and delD4-rev with the reverse complement sequence as the reverse primer. A protocol variation published in BioTechniques 26 (1999) 680
was used to extend both primers in separate reactions in three cycles preceeding the regular Stratagene protocol:

Two separate 50 μl reaction mixtures were set up according to the manufacturer's manual, each containing 10 ng plasmid pCMV-preS-Fc-hCSF1R-ECD as the template and 10 pM of one of the primers delD4-for or delD4-rev, and 0.5 μl Pfu DNA polymerase as provided with the kit. Three PCR cycles 95 °C 30 sec / 55 °C 60 sec / 68 °C 8 min were run, then 25 μl each of both reaction mixtures were combined in a new tube and 0.5 μl fresh Pfu DNA polymerase were added. The regular PCR protocol with 18 temperature cycles as specified by Stratagene in the kit manual was carried out, followed by 2 hrs final digestion with the DpnI restriction enzyme provided with the kit. Clones bearing the deletion were detected by digestion with Cel II and Not I and verified by sequencing.

Protein was prepared by transient transfection in the Hek293 FreeStyle suspension cell system (Invitrogen) according to the manufacturer's specifications. After 1 week 500 ml supernatant was filtered and loaded onto a 1ml HiTrap MabSelect Xtra (GE healthcare) protein A column (0.2 ml/min). The column was washed first with PBS, then with 50 mM Tris/ 150 mM NaCl/ 1 mM EDTA/ pH 7.3. 75 μl PreScission Protease (GE #27-0843-01) diluted in 375 μl of the same buffer were loaded onto the column and the closed column was incubated overnight at 4 °C with rolling. The column was mounted on top of a 1 ml GSTrap FF column (GE Healthcare) and the desired protein was eluted (0.2 ml/min, 0.2 ml fractions). Pooled fractions were concentrated from 1.8 ml to 0.4 ml by centrifugal ultrafiltration via a 3k Nanosep and chromatographed over an S200 HR SEC in PBS (0.5 ml/min).

Human CSF-1R fragment delD4 was obtained in two fractions as a dimeric molecule (pool1, V=1.5 ml; c=0.30 mg/ml; apparent mass on SDS page 83 kDa, reduced 62 kDa) and as the monomer (pool 2, V=1.4 ml; c=0.25 mg/ml apparent mass on SDS page 62 kDa). The dimeric form was used for all experiments.

**Determination of the binding of anti-CSF-1R antibodies to human CSF-1R fragment delD4 and to human CSF-1R Extracellular Domain (CSF-1R-ECD)** (binding signals as Response Units (RU)):

**Instrument:** Biacore T100 (GE Healthcare)

**Software:** T100 Control, Version 2.0.1
T100 Evaluation, Version 2.0.2

**Assayformat** Chip: CM5
Temperature: 25°C

CSF-IR fragments were immobilized via amine coupling. To compare the binding of different anti-CSF-IR antibodies according to the invention one concentration of the test antibody was injected. Anti CSF-IR Mab3291 (R&D-Systems) and SC 2-4A5 (Santa Cruz Biotechnology, US- see also Sherr, C.J. et al, Blood 73 (1989) 1786-1793), was used as reference control, anti-CCR5 m<CCR5>Pz03.1C5 (deposited as DSM ACC 2683 on 18.08.2004 at DSMZ) as negative control, all under the same conditions as the anti-CSF-IR antibodies according to the invention.

Amine coupling of CSF-IR fragments

Standard amine coupling according to the manufacturer's instructions: running buffer: PBS-T (Roche: 11 666 789 + 0.05% Tween20: 11 332 465), activation by mixture of EDC/NHS, injection of human CSF-IR fragment delD4 (comprising the extracellular subdomains D1 -D3 and D5) (SEQ ID NO: 65) and human CSF-IR Extracellular Domain (CSF-1R-ECD) (comprising the extracellular subdomains D1 -D5) (SEQ ID NO: 64) for 600 seconds at flow rate 10µL/min; diluted in coupling buffer NaAc, pH 5.0, c = 10 µg/mL; finally remaining activated carboxyl groups were blocked by injection of 1M Ethanolamin.

Binding of <CSF-1R> Mab 2F11, Mab 2E10, Mab 3291 and sc2-4A5 and other anti-CSF-IR antibodies to human CSF-IR fragment delD4 and human CSF-IR Extracellular Domain (CSF-1R-ECD) at 25°C

Running buffer: PBS-T (Roche: 11 666 789 + 0.05% Tween20: 11 332 465)

Analyte sample:
Binding was measured at a flow rate of 30 µL/ηη by one injection of the analyte with concentration c = 10 nM. (for Mab 1G10, Mab 2H7 and humanized hMab 2F11-e7 in second experiment) Each injection was 700 seconds long, followed by a dissociation phase of 180 seconds. Final regeneration was performed after each cycle using 50 mM NaOH, contact time 60 seconds, flow rate 30 µL/ηη.

Signals were measured by a report point 10 seconds after end of injection. Reference signals (signals from a blank reference flow cell (treated with EDC/NHS and ethanolamine, only) were subtracted to give the binding signals (as RU). If binding signals of nonbinding antibodies were slightly below 0 (Mab 2F11 =-3;
Mab 2E10 = -2; Mab 1G10 = -6, Mab 2H7 = -9; and humanized hMab 2F1 1-e7 = -7) the values were set as 0.

Table 3a:

<table>
<thead>
<tr>
<th></th>
<th>Binding to delD4 [RU]</th>
<th>Binding to CSF-1R-ECD [RU]</th>
<th>Ratio of binding of anti-CSF1R antibodies to CSF1R fragment delD4 / to CSF-1R-ECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab 3291</td>
<td>1015</td>
<td>627</td>
<td>1015/627= 1.61</td>
</tr>
<tr>
<td>sc2-4A5</td>
<td>374</td>
<td>249</td>
<td>374/249= 1.50</td>
</tr>
<tr>
<td>Mab 2F11</td>
<td>0</td>
<td>176</td>
<td>0/176 = 0</td>
</tr>
<tr>
<td>hMab 2F11-e7</td>
<td>0</td>
<td>237</td>
<td>0/237 = 0</td>
</tr>
<tr>
<td>Mab 2E10</td>
<td>0</td>
<td>120</td>
<td>0/120 = 0</td>
</tr>
<tr>
<td>Mab 1G10</td>
<td>0</td>
<td>2708</td>
<td>0/2708 = 0</td>
</tr>
<tr>
<td>Mab 2H7</td>
<td>0</td>
<td>147</td>
<td>0/147 = 0</td>
</tr>
<tr>
<td>m&lt;CCR5&gt;Pz03.1C5</td>
<td>2</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

Mab 2F11 and Mab 2E10 showed binding to the human CSF-IR Extracellular Domain (CSF-IR-ECD) (see Fig. 3b); however no binding was detected to CSF-IR fragment delD4. (see Fig. 3a).

Sc2-4A5 and MAB3291 showed binding to CSF-IR-ECD and to del D4 (see Fig. 3b and 3a).

Thus the ratio of binding of anti-CSFIR antibodies Mab 2F11 and Mab 2E10 to CSF1R fragment delD4 / to CSF-IR-ECD was clearly below 1:50 (= 0.02), while the binding ratio of MAB3291 and Sc2-4A5 were 1.61 and 1.50, respectively and were highly above 1:50 (= 0.02). Negative control antibody m<CCR5>Pz03.1C5 did not show any binding (as expected).

Mab 1G10, Mab 2H7 and humanized hMab 2F1 1-e7 showed binding to the human CSF-IR Extracellular Domain (CSF-IR-ECD) (see Fig. 3d); however no binding was detected to CSF-IR fragment delD4. (see Fig.3). Thus the ratio of binding of anti-CSFIR antibodies Mab 1G10, Mab 2H7 and humanized hMab 2F1 1-e7 to CSF1R fragment delD4 / to CSF-IR-ECD was clearly below 1:50 (= 0.02).
In a further experiment anti-CSF-IR antibodies 1.2.SM (ligand displacing CSF-IR antibody described in WO2009026303), CXIIG6 (ligand displacing CSF-IR antibody described in WO 2009/1 12245), the goat polyclonal anti-CSF-IR antibody ab10676 (abeam) were investigated. Anti-CSF-IR antibody Mab3291 (R&D-Systems) was used as reference control. Anti-CCR5 m<CCR5>Pz03.1C5 (deposited as DSM ACC 2683 on 18.08.2004 at DSMZ) was used as negative control.

**Table 3b:**

<table>
<thead>
<tr>
<th></th>
<th>Binding to delD4 [RU]</th>
<th>Binding to CSF-1R-ECD [RU]</th>
<th>Ratio of binding of anti-CSF1R antibodies to CSF1R fragment delD4 / to CSF-1R-ECD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAB3291</td>
<td>1790</td>
<td>1222</td>
<td>1790/1222 = 1.47</td>
</tr>
<tr>
<td>1.2.SM</td>
<td>469</td>
<td>704</td>
<td>469/704 = 0.67</td>
</tr>
<tr>
<td>CXIIG6</td>
<td>1983</td>
<td>1356</td>
<td>1983/1356 = 1.46</td>
</tr>
<tr>
<td>ab10676</td>
<td>787</td>
<td>547</td>
<td>787/547 = 1.44</td>
</tr>
<tr>
<td>m&lt;CCR5&gt;Pz03.1C5</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

1.2.SM, CXIIG6, abl0676 and MAB3291 showed binding to CSF-1R-ECD and to del D4 (see Fig. 3f and 3e).

The binding ratio of 1.2.SM, CXIIG6, abl0676 and MAB3291 was highly above 1:50 (= 0.02). Negative control antibody m<CCR5>Pz03.1C5 did not show any binding (as expected).

**Example 5**

**Growth inhibition of NIH3T3-CSF-1R recombinant cells in 3D culture under treatment with anti-CSF-IR monoclonal antibodies (CellTiterGlo-assay)**

NIH 3T3 cells, retrovirally infected with either an expression vector for full-length wildtype CSF-IR (SEQ ID NO: 62) or mutant CSF-IR L301S Y969F (SEQ ID NO: 63), were cultured in DMEM high glucose media (PAA, Pasching, Austria) supplemented with 2mM L-glutamine, 2mM sodium pyruvate and non-essential amino acids and 10% fetal bovine serum (Sigma, Taufkirchen, Germany) on poly-
HEMA (poly(2-hydroxyethylmethacrylate)) (Polysciences, Warrington, PA, USA)) coated dishes to prevent adherence to the plastic surface. Cells are seeded in medium replacing serum with 5ng/ml sodium selenite, 10mg/ml transferrin, 40(^g/ml BSA and 0.05 mM 2-mercaptoethanol. When treated with 100ng/ml hu CSF-1 (active 149 aa fragment of human CSF-1 (aa 33-181 of SEQ ID NO: 86); Biomol, DE, Cat.No.60530) wtCSF-1R (expressing cells form dense spheroids that grow three dimensionally, a property that is called anchorage independence. These spheroids resemble closely the three dimensional architecture and organization of solid tumors in situ. Mutant CSF-1R recombinant cells are able to form spheroids independent of the CSF-1 ligand. Spheroid cultures were incubated for 3 days in the presence of different concentrations of antibody in order to determine an IC50 (concentration with 50 percent inhibition of cell viability). The CellTiterGlo assay was used to detect cell viability by measuring the ATP-content of the cells.

<table>
<thead>
<tr>
<th>CSF-1R Mab</th>
<th>wtCSF-1R IC₅₀ [µg/ml]</th>
<th>Mutant CSF-1R IC₅₀ [µg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab 2F11</td>
<td>1.1</td>
<td>8.0</td>
</tr>
<tr>
<td>Mab 2E10</td>
<td>0.49</td>
<td>4.9</td>
</tr>
<tr>
<td>Mab 2H7</td>
<td>0.31</td>
<td>5.3</td>
</tr>
<tr>
<td>Mab 1G10</td>
<td>0.29</td>
<td>14.2</td>
</tr>
<tr>
<td>SC 2-4A5</td>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 4:

Reference control Mab R&D-Systems 3291 did not show inhibition of mutant CSF-1R recombinant cell proliferation.

In a further experiment the anti-CSF-1R antibody according to the invention hMab 2F11c7 and the anti-CSF-1R antibodies 1.2.SM (ligand displacing CSF-1R antibody described in WO 2009/026303), CXIIIG6 (ligand displacing CSF-1R antibody described in WO 2009/112245), the goat polyclonal anti-CSF-1R antibody ab10676 (abeam), and SC 2-4A5 (Santa Cruz Biotechnology, US- see also Sherry, C.J. et al, Blood 73 (1989) 1786-1793) were investigated.
Spheroid cultures were incubated for 3 days in the presence of different concentrations of antibody in order to determine an IC30 (concentration with 30 percent inhibition of cell viability). Maximum concentration was 20 µg/ml. The CellTiterGlo assay was used to detect cell viability by measuring the ATP-content of the cells.

Table S:

<table>
<thead>
<tr>
<th>CSF-1R Mab</th>
<th>wtCSF-1R IC₃₀ [µg/ml]</th>
<th>Mutant CSF-1R IC₃₀ [µg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMab 2F11-e7</td>
<td>4.91</td>
<td>0.54</td>
</tr>
<tr>
<td>1.2.SM</td>
<td>1.19</td>
<td>&gt; 20 µg/ml (-19% inhibition at 20 µg/ml = 19% stimulation)</td>
</tr>
<tr>
<td>CXIIG6</td>
<td>&gt; 20 µg/ml (21% inhibition at 20 µg/ml)</td>
<td>&gt; 20 µg/ml (-36% inhibition at 20 µg/ml = 36% stimulation)</td>
</tr>
<tr>
<td>ab10676</td>
<td>14.15</td>
<td>&gt; 20 µg/ml (0% inhibition at 20 µg/ml)</td>
</tr>
<tr>
<td>SC 2-4A5</td>
<td>16.62</td>
<td>2.56</td>
</tr>
</tbody>
</table>

Example 6

Growth inhibition of BeWo tumor cells in 3D culture under treatment with anti-CSF-1R monoclonal antibodies (CellTiterGlo-assay)

BeWo choriocarcinoma cells (ATCC CCL-98) were cultured in F12K media (Sigma, Steinheim, Germany) supplemented with 10% FBS (Sigma) and 2mM L-glutamine. 5x10⁴ cells/well were seeded in 96-well poly-HEMA (poly(2-hydroxyethylmethacrylate)) coated plates containing F12K medium supplemented with 0.5% FBS and 5% BSA. Concomitantly, 200 ng/ml huCSF-1 (active 149 aa fragment of human CSF-1 (aa 33-181 of SEQ ID NO: 86)) and 10 µg/ml of different anti-CSF-1R monoclonal antibodies were added and incubated for 6 days. The CellTiterGlo assay was used to detect cell viability by measuring the ATP-content of the cells in relative light units (RLU). When BeWo spheroid cultures were treated with different anti-CSF-1R antibodies (10 µg/ml) inhibition of CSF-1 induced growth was observed. To calculate antibody-mediated inhibition the mean RLU value of unstimulated BeWo cells was subtracted from all samples. Mean RLU value of CSF-1 stimulated cells was set arbitrarily to 100%. Mean RLU
values of cells stimulated with CSF-1 and treated with anti-CSF-IR antibodies were calculated in % of CSF-1 stimulated RLUs. The Table 6 shows the calculated data of growth inhibition of BeWo tumor cells in 3D culture under treatment with anti-CSF-IR monoclonal antibodies; Fig.2a and b depicts normalized mean RLU values.

Table 6:

<table>
<thead>
<tr>
<th>CSF-1R Mab</th>
<th>% inhibition 10μg/ml antibody concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF-1 only</td>
<td>0</td>
</tr>
<tr>
<td>Mab 2F11</td>
<td>70</td>
</tr>
<tr>
<td>Mab 2E10</td>
<td>102</td>
</tr>
<tr>
<td>Mab 2H7</td>
<td>103</td>
</tr>
<tr>
<td>Mab 1G10</td>
<td>99</td>
</tr>
<tr>
<td>SC 2-4A5</td>
<td>39</td>
</tr>
</tbody>
</table>

Example 7

Inhibition of human macrophage differentiation under treatment with anti-CSF-1R monoclonal antibodies (CellTiterGlo-assay)

Human monocytes were isolated from peripheral blood using the RosetteSep™ Human Monocyte Enrichment Cocktail (StemCell Tech. - Cat. No. 15028). Enriched monocyte populations were seeded into 96 well microtiterplates (2.5x10^4 cells/well) in 100 μl RPMI 1640 (Gibco - Cat. No. 31870) supplemented with 10% FCS (GIBCO - Cat. No. 011-090014M), 4 mM L-glutamine (GIBCO - Cat. No. 25030) and 1x PenStrep (Roche Cat. No. 1 074 440) at 37°C and 5% CO_2 in a humidified atmosphere. When 150 ng/ml huCSF-1 was added to the medium, a clear differentiation into adherent macrophages could be observed. This differentiation could be inhibited by addition of anti-CSF-IR antibodies. Furthermore, the monocyte survival is affected and could be analyzed by CellTiterGlo (CTG) analysis. From the concentration dependent inhibition of the survival of monocytes by antibody treatment, an IC_{50} was calculated (see Table 7).
In a separate test series humanized versions of Mab 2 F1l, e.g. hMab 2F1-l-cl 1, hMab 2F1-l-d8, hMab 2F1-l-e7, hMab 2F1-l-fl2, showed IC50 values of 0.07 µg/ml (hMab 2F1-l-cl 1), 0.07 µg/ml (hMab 2F1-l-d8), 0.04 µg/ml (hMab 2F1-l-e7) and 0.09 µg/ml (hMab 2F1-l-fl2).

**Example 8**

Inhibition of cynomolgous macrophage differentiation under treatment with anti-CSF-IR monoclonal antibodies (CellTiterGlo-assay)

Cynomolgous monocytes were isolated from peripheral blood using the CD14 MicroBeads non-human primate kit (Miltenyi Biotec - Cat.No. 130-091-097) according to the manufacturers description. Enriched monocyte populations were seeded into 96 well microtiterplates (1-3x10^4 cells/well) in 100 μl RPMI 1640 (Gibco - Cat. No.31870) supplemented with 10% FCS (GIBCO - Cat. No.01-090014M), 4 mM L-glutamine (GIBCO - Cat. No.25030) and 1x PenStrep (Roche Cat. No.1 074 440) at 37°C and 5% CO₂ in a humidified atmosphere. When 150 ng/ml huCSF-1 was added to the medium, a clear differentiation into adherent macrophages could be observed. This differentiation could be inhibited by addition of anti-CSF-IR antibodies. Furthermore, the monocyte survival is affected and could be analyzed by CellTiterGlo (CTG) analysis. The viability was analyzed at a concentration of 5 µg/ml antibody treatment (see Table 8).
Table 8:

<table>
<thead>
<tr>
<th>CSF-1R Mab</th>
<th>% survival</th>
<th>% inhibition (of survival) = (100% - %survival)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab 2F11</td>
<td>4 *</td>
<td>96</td>
</tr>
<tr>
<td>Mab 2E10</td>
<td>17 **</td>
<td>83</td>
</tr>
<tr>
<td>Mab 2H7</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>Mab 1G10</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>SC 2-4A5</td>
<td>31</td>
<td>69</td>
</tr>
</tbody>
</table>

* mean of four experiments (3 expts. using the murine, 1 expt. using the chimeric mAb)

** mean of two experiments using the murine mAb only

Example 9

Inhibition of human M1 and M2 macrophage differentiation under treatment with anti-CSF-1R monoclonal antibodies (CellTiterGlo-assay)

Human monocytes were isolated from peripheral blood using the RosetteSep™ Human Monocyte Enrichment Cocktail (StemCell Tech. - Cat. No.15028). Enriched monocyte populations were seeded into 96 well microtiterplates (2.5x10⁴ cells/well) in 100 μι RPMI 1640 (Gibco - Cat. No.31870) supplemented with 10% FCS (GIBCO - Cat. No.01 1-090014M), 4 mM L-glutamine (GIBCO - Cat. No.25030) and 1x PenStrep (Roche Cat. No.1 074 440) at 37°C and 5% CO₂ in a humidified atmosphere. When 100 ng/ml huCSF-1 was added for 6 days to the medium, a clear differentiation into adherent, M2 macrophages with elongated morphology could be observed. When 100 ng/ml huGM-CSF was added to the medium for 6 days, a clear differentiation into adherent, M1 macrophages with round morphology could be observed. This differentiation was associated with the expression of certain markers such as CD163 for M2 macrophages and CD80 or high MHC class II for M1 macrophages as assessed by flow cytometry. Cells were washed with PBS and, if adherent, detached using a 5mM EDTA solution in PBS (20min at 37°C). Cells were then well resuspended, washed with staining buffer (5% FCS in PBS) and centrifuged at 300xg for 5min. Pellets were resuspended in 1ml staining buffer and cells counted in a Neubauer chamber. Approximately 1x10⁵ cells were transferred in each FACS tube, centrifuged at 300xg for 5min and resuspended in staining buffer. Fey receptors were blocked by incubation with
1 µg human IgG/2.5x10^4 cells (JIR Cat.No.009-000-003) in staining buffer for 20 min on ice. Cells were then mixed with 1.5 µl antibody/2.5x10^4 cells for CD80 and CD163 detection whereas 5 µl antibody/2.5x10^4 cells for MHC class II detection was used: PE labeled mouse anti human CD163 (BD Bioscience Cat.No.556018), PE labeled mouse anti human CD80 (BD Bioscience Cat.No. 557227) and Alexa 647 labeled mouse anti human MHC class II (Dako-Cat.No. M0775). The Alexa 647 label was conjugated to the antibody by using the Zenon Alexa 647 mouse IgG labeling kit (Invitrogen Cat.No. Z25008) After a 1-hour incubation on ice cells were washed twice with staining buffer, resuspended and measured at a FACS Canto II.

Exclusively M2 macrophage differentiation which is characterized by the expression of CD163, absence of CD80 and low MHC class II expression could be inhibited by addition of humanized anti-CSF-1R antibody hMab 2Fl 1-e7. Furthermore, the M2 but not M1 macrophage survival is affected and could be analyzed by CellTiterGlo (CTG) analysis. Concentration dependent inhibition of the survival of macrophages by antibody treatment for 7 days is depicted in Figure 5a. Expression of M1 and M2 macrophage markers assessed by flow cytometry is shown in Figure 5b.

**Example 10**

**Determination of the binding affinity of anti-CSF-1R antibodies to human CSF-1R**

Instrument: BIACORE® A100  
Chip: CM5 (Biacore BR-1006-68)  
Coupling: amine coupling  
Buffer: PBS (Biacore BR-1006-72), pH 7.4, 35°C

For affinity measurements 36 µg/ml anti mouse Fey antibodies (from goat, Jackson Immuno Research JIRl 15-005-071) have been coupled to the chip surface for capturing the antibodies against CSF-1R. Human CSF-1R Extracellular Domain (CSF-1R-ECD) (comprising the extracellular subdomains D1 -D5) (SEQ ID NO: 64) (R&D-Systems 329-MR or subcloned pCMV-presS-HisAvitag-hCSF-IR-ECD) was added in various concentrations in solution. Association was measured by an CSF-1R-injection of 1.5 minutes at 35 °C; dissociation was measured by washing the chip surface with buffer for 10 minutes at 35 °C. For calculation of kinetic parameters the Langmuir 1:1 model was used.
In a separate biacore binding assay using the CSF-IR ECD (data not shown) some competition of the antibodies Mab 2F11 and Mab 2E10 with the antibody Ab SC-2-4A5 was shown. However Mab 2F11/Mab 2E10 do not bind to the human CSF-IR fragment delD4, whereas Ab SC-2-4A5 binds to this delD4 fragment (see Example 4 and Fig 3a). Thus the binding region of Mab 2F11/Mab 2E10 is clearly distinct from the binding region of Ab SC-2-4A5, but probably located in a vicinity area. In such competition assay both antibodies Mab 2F11 and Mab 2E10 did not compete with Mab3291 from R&D-Systems (data not shown).

**Example 11**

**Determination of the binding of anti-CSF-IR antibodies to human CSF-IR fragment D1-D3**

Instrument: Biacore T100 (GE Healthcare)  
Software: T100 Control, Version 1.1.1 1  
B3000 Evaluation, Version 4.01  
Scrubber, Version 2.0a  
Assay format: Chip:CM5-Chip

Antibodies against CSF-IR were captured via amine coupled capture molecules. Using the single cycle kinetics five increasing concentrations of human CSF-IR fragment D1-D3 (SEQ ID NO: 66) were injected. Human CSF-IR fragment D1-D3 was subcloned into pCMV-presS-HisAvitag expression vector.

Anti CSF-IR SC 2-4A5 (Santa Cruz Biotechnology, US; Sherr, C.J. et al, Blood 73 (1989) 1786-1793) which inhibits the ligand-receptor interaction, and Mab 3291 (R&D-Systems) were used as reference controls.
Capture molecules: Anti mouse Fey antibodies (from goat, Jackson Immuno Research JIRl 15-005-071) for antibodies according to the invention and the R&D-Systems control Mab 3291 and Anti rat Fey antibodies (from goat, Jackson Immuno Research JIRl 12-005-071) for the reference control anti CSF-1R SC 2-4A5.

**Amine coupling of capture molecules**

Standard amine coupling according to the manufacturer's instructions: running buffer: HBS-N buffer, activation by mixture of EDC/NHS, aim for ligand density of 2000 RU; the capture-Abs were diluted in coupling buffer NaAc, pH 4.5, c = 10 µg/mL; finally remaining activated carboxyl groups were blocked by injection of 1 M Ethanolamin.

**Kinetic characterization of human CSF-1R fragments D1-D3 binding to MAbs <CSF-1R> at 37°C**

Running buffer: PBS (Biacore BR-1006-72)

Capturing of Mabs <CSF-1R> on flow cells 2 to 4: Flow 20 µL/ηιη, contact time 90 seconds, c(Abs<CSF-1R>) = 50 nM, diluted with running buffer + 1 mg/mL BSA;

Analyte sample:

Single Cycle Kinetics was measured at a flow rate of 30 µL/ηιη by five consecutive injections of the analyte with concentrations, c = 7.8 , 31.25, 125 500 and 2000 nM, without regeneration. Each injection was 30 seconds long and followed by a dissociation phase of 120 Seconds for the first four injections, and finally 1200 seconds for the highest concentration (=last injection).

Final regeneration was performed after each cycle using 10 mM Glycin pH 1.5 (Biacore BR-1003-54), contact time 60 seconds, flow rate 30 µL/ηιη.

Kinetic parameters were calculated by using the usual double referencing (control reference: binding of analyte to capture molecule; Flow Cell: subdomain CSF-1R concentration "0" as Blank) and calculation with model 'titration kinetics 1:1 binding with draft'.
Table 10:
Affinity data for binding of human CSF-1R fragment D1-D3 measured by SPR

<table>
<thead>
<tr>
<th>CSF-1R Mab</th>
<th>Sub domain</th>
<th>K_D (nM)</th>
<th>k_a (1/Ms)</th>
<th>k_d (1/s)</th>
<th>t_{1/2} (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mab 2F11</td>
<td>D1-D3</td>
<td>no binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mab 2E10</td>
<td>D1-D3</td>
<td>no binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mab 2H7</td>
<td>D1-D3</td>
<td>not determined</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mab 1G10</td>
<td>D1-D3</td>
<td>no binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC-2-4A5</td>
<td>D1-D3</td>
<td>no binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R&amp;D-Systems 3291</td>
<td>D1-D3</td>
<td>5.4</td>
<td>2.2E^{-5}</td>
<td>1.2E^{-3}</td>
<td>9.6</td>
</tr>
</tbody>
</table>

The antibodies Mab 2F1, Mab 2E10 and Mab 1G10 showed no binding to human CSF-1R fragment D1-D3.

Also reference control-Ab SC-2-4A5 did not bind to human CSF-1R fragment D1-D3.

The reference control Mab R&D-Systems 3291 showed binding to the human CSF-1R fragment D1-D3.

**Example 12**

**CSF-1 level increase during CSF-1R inhibition in Cynomolgus monkey**

Serum CSF-1 levels provide a pharmacodynamic marker of CSF-1R neutralizing activity of anti-human CSF-1R dimerization inhibitor hMab 2Fl l-e7. One male and one female cynomolgus monkey per dosage group (1 and 10 mg/kg) were intravenously administered anti-CSFIR antibody hMab 2Fl l-e7. Blood samples for analysis of CSF-1 levels were collected 1 week before treatment (pre-dose), 2, 24, 48, 72, 96, 168 hours post-dose and weekly for two additional weeks. CSF-1 levels were determined using a commercially available ELISA kit (Quantikine® human M-CSF) according to the manufacturer's instructions (R&D Systems, UK). Monkey CSF-1 level were determined by comparison with CSF-1 standard curve samples provided in the kit.
Administration of hMab 2F1 l-e7 induced a dramatic increase in CSF-1 by ~ 1000-fold, which depending on the dose administered lasted for 48 hr (1mg/kg) or 15 days (10mg/kg). Hence, a dimerization inhibitor for CSF-1R offers the advantage to not directly compete with the dramatically upregulated ligand for binding to the receptor in contrast to a ligand displacing antibody. (Results are shown in Figure 4)

**Example 13**

**Inhibition of tumor growth under treatment with anti-CSF-1R monoclonal antibody in combination with chemotherapy or cancer immunotherapy in subcutaneous syngeneic MC38 colon carcinoma models**

Cells of the murine colorectal adenocarcinoma cell line MC-38 (obtained from Beckman Research Institute of the City of Hope, California, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM, PAN Biotech) supplemented with 10% FCS and 2mM L-glutamine at 37°C in a water saturated atmosphere at 5% CO2. At the day of inoculation, MC38 tumor cells were harvested with PBS from culture flasks and transferred into culture medium, centrifuged, washed once and re-suspended in PBS. For injection of cells, the final titer was adjusted to 1x10^7 cells/ml. Subsequently 100 µl of this suspension (1x10^6 cells) were inoculated subcutaneously into 7-9 weeks old female C57BL/6N mice (obtained from Charles River, Sulzfeld, Germany). Treatment with control antibody (MOPC-21; Bio X Cell, West Lebanon), anti-murine CSF-1R mAb <mouse CSF1R> antibody at a weekly dose of 30 mg/kg i.p. alone or in combination the TLR9 agonist CpG ODN 1826 (ODN 1826, class B CpG ODN, VacciGrade, InvivoGen, 100 µg peritumoral, lx). Tumor volume was measured twice a week and animal weights were monitored in parallel.

In a separate study with comparable set-up, primary tumors from indicated treatment groups were excised, weighed and subjected to FACS analysis. Primary tumor material was collected between study day 20-25 as indicated. To obtain single cell suspensions amenable for flow cytometry analysis the tumors were minced by using the Mcllwain tissue chopper. Subsequently, the tumor pieces were resuspended in RPMI media supplemented with collagenase I, dispase II and DNase I, incubated at 37°C and cell suspension were passed through a mash. CD45 positive cells were enriched by magnetic cell separation according to the manufacturer's instructions (Miltenyi). Briefly cells were labeled with anti-mouse CD45 conjugated with APC (BD, Cat.No 559864) and separated with anti APC microbeads. To analyse CD8+ T cells these CD45 positive cells were stained with
Monotherapy with <mouse CSF1R> antibody inhibited primary tumor growth when compared to control antibody treatment (TGI: 67%, TCR: 0.38 CI: 0.15-0.64). Also TLR9 agonist (CpG ODN 1826) monotherapy had an effect on MC38 primary tumor growth (TGI: 74%, TCR: 0.28 CI: 0.10-0.50). Addition of <mouse CSF1R> antibody to TLR9 agonist therapy led to a clearly superior anti-tumor efficacy compared to TLR9 agonist treatment alone (TGI: 95%, TCR: 0.08 CI: -0.1 1-0.28). (see table 11).

**Table 11:**

<table>
<thead>
<tr>
<th>Group</th>
<th>TGI (day 24)</th>
<th>TCR (day 24)</th>
<th>Median time to progression TV &gt; 700 mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Mouse IgG1)</td>
<td>--</td>
<td>--</td>
<td>21</td>
</tr>
<tr>
<td>&lt;mouse CSF1R&gt; antibody</td>
<td>67%</td>
<td>0.38</td>
<td>24</td>
</tr>
<tr>
<td>TLR9 agonist (CpG)</td>
<td>73%</td>
<td>0.28</td>
<td>24</td>
</tr>
<tr>
<td>&lt;mouse CSF1R&gt; antibody/TLR9</td>
<td>95%</td>
<td>0.08</td>
<td>46</td>
</tr>
<tr>
<td>agonist (CpG)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Evaluation on Tumor Progression**

Additionally to the assessment of median tumor volume after 2 weeks of treatment the progression of individual tumors in the study was followed until progression > 700 mm³ (Figure 1 (CpG is TLR9 agonist CpG ODN 1826) and Table 11)

Median time to progression > 700 mm³ was 21 days for IgG control treatment group. Slight improvement of median progression time was achieved by treatment with <CSF1R> antibody monotherapy (24 days).
Monotherapy with TLR9 agonist resulted in a median time to progression of 24 days. Addition of TLR9 agonist (CpG) to anti-CSF-IR antibody therapy resulted in a statistically significant more than additive improvement of median time to progression (46 days) compared to anti-CSF-IR antibody monotherapy or TLR9 agonist monotherapy.
Patent Claims

1. An antibody which binds to human CSF-IR wherein the antibody is administered in combination with a Toll-like receptor 9 (TLR9) agonist for use in the treatment of cancer.

2. Use of a combination of
   i) an antibody which binds to human CSF-IR, and
   ii) a Toll-like receptor 9 (TLR9) agonist

   for the manufacture of a medicament for use in the treatment of cancer.

3. The antibody or use according to claims 1 or 2, wherein the cancer is further characterized by CSF-IR expression or overexpression

4. The antibody or use according to any one of claims 1 or 2, wherein the cancer is a breast cancer, colorectal cancer, melanoma, head and neck cancer, lung cancer or prostate cancer.

5. An antibody which binds to human CSF-IR characterized in binding to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-IR for use in
   a) the inhibition of cell proliferation in CSF-IR ligand-dependent and/or CSF-1 ligand-independent CSF-IR expressing tumor cells;
   b) the inhibition of cell proliferation of tumors with CSF-IR ligand-dependent and/or CSF-IR ligand-independent CSF-IR expressing macrophage infiltrate;
   c) the inhibition of cell survival (in CSF-IR ligand-dependant and/or CSF-IR ligand-independent) CSF-IR expressing monocytes and macrophages; and/or
   d) the inhibition of cell differentiation (in CSF-IR ligand-dependent and/or CSF-IR ligand-independent) CSF-IR expressing monocytes into macrophages,

   wherein the antibody is administered in combination with a TLR9 agonist.
6. Use of a combination of

i) an antibody which binds to the (dimerization) domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-IR, and

ii) a Toll-like receptor 9 (TLR9) agonist

for the manufacture of a medicament for use in

a) the inhibition of cell proliferation in CSF-IR ligand-dependent and/or CSF-1 ligand-independent CSF-IR expressing tumor cells;

b) the inhibition of cell proliferation of tumors with CSF-IR ligand-dependent and/or CSF-IR ligand-independent CSF-IR expressing macrophage infiltrate;

c) the inhibition of cell survival (in CSF-IR ligand-dependant and/or CSF-IR ligand-independent) CSF-IR expressing monocytes and macrophages; and/or

d) the inhibition of cell differentiation (in CSF-IR ligand-dependent and/or CSF-IR ligand-independent) CSF-IR expressing monocytes into macrophages.

7. An antibody which binds to human CSF-IR, for use in the treatment of a patient having a CSF-IR expressing tumor or having a tumor with CSF-IR expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-IR ligand and wherein the anti-CSF-IR antibody is administered in combination with a TLR9 agonist.

8. Use of a combination of

i) an antibody which binds to human CSF-IR, and

ii) a Toll-like receptor 9 (TLR9) agonist

for the manufacture of a medicament for use in for use in the treatment of a patient having a CSF-IR expressing tumor or having a tumor with CSF-IR expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-IR ligand.
9. The antibody or use according to any one of claims 1 or 8 wherein the TLR9 agonist is characterized by induction of IFN-alpha, IL-6, and/or IL-12 in plasmacytoid dendritic cells (pDCs).

10. The antibody or use according to any one of claims 1 to 8, wherein the TLR9 agonist is is a oligodeoxynucleotides containing cytosine-phosphate-guanosine (CpG) motifs (CpG ODNs).

11. The antibody or use according to any one of claims 1 to 10 wherein the antibody is characterized in binding to the domains D4 to D5 (SEQ ID No: 85) of the extracellular domain of human CSF-1R.

12. The antibody according any one of the preceding claims, wherein the antibody is characterized in that the antibody does not bind to human CSF-1R fragment delD4 (SEQ ID NO: 65).

13. The antibody according any one of the preceding claims, characterized in that
   a) the heavy chain variable domain is SEQ ID NO: 7 and the light chain variable domain is SEQ ID NO: 8;
   b) the heavy chain variable domain is SEQ ID NO: 15 and the light chain variable domain is SEQ ID NO: 16;
   c) the heavy chain variable domain is SEQ ID NO: 75 and the light chain variable domain is SEQ ID NO: 76;
   d) the heavy chain variable domain is SEQ ID NO: 83 and the light chain variable domain is SEQ ID NO: 84;
   e) the heavy chain variable domain is SEQ ID NO: 23 and the light chain variable domain is SEQ ID NO: 24, or
   f) the heavy chain variable domain is SEQ ID NO: 31 and the light chain variable domain is SEQ ID NO: 32, or
   g) the heavy chain variable domain is SEQ ID NO: 39 and the light chain variable domain is SEQ ID NO: 40, or
   h) the heavy chain variable domain is SEQ ID NO: 47 and the light chain variable domain is SEQ ID NO: 48, or
i) the heavy chain variable domain is SEQ ID NO:55 and the light chain variable domain is SEQ ID NO:56.

14. The antibody according any one of the preceding claims, characterized in that

a) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 1, a CDR2 region of SEQ ID NO: 2, and a CDR1 region of SEQ ID NO:3, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 4, a CDR2 region of SEQ ID NO:5, and a CDR1 region of SEQ ID NO:6, or

b) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 9, a CDR2 region of SEQ ID NO: 10, and a CDR1 region of SEQ ID NO: 11, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:12, a CDR2 region of SEQ ID NO: 13, and a CDR1 region of SEQ ID NO: 14, or

c) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 17, a CDR2 region of SEQ ID NO: 18, and a CDR1 region of SEQ ID NO:19, and the light chain variable domain comprises a CDR3 region of SEQ ID NO: 20, a CDR2 region of SEQ ID NO:21, and a CDR1 region of SEQ ID NO: 22, or

d) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 25, a CDR2 region of SEQ ID NO: 26, and a CDR1 region of SEQ ID NO: 27, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:28, a CDR2 region of SEQ ID NO: 29, and a CDR1 region of SEQ ID NO: 30, or

e) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: 33, a CDR2 region of SEQ ID NO: 34, and a CDR1 region of SEQ ID NO: 35, and the light chain variable domain comprises a CDR3 region of SEQ ID NO:36, a CDR2 region of SEQ ID NO: 37, and a CDR1 region of SEQ ID NO: 38, or

f) the heavy chain variable domain comprises a CDR3 region of SEQ ID NO:41, a CDR2 region of SEQ ID NO: 42, and a CDR1 region of SEQ ID NO:43, and the light chain variable domain comprises a CDR3 region
of SEQ ID NO: 44, a CDR2 region of SEQ ID NO:45, and a CDR1 region of SEQ ID NO:46, or

\[ \text{g}) \text{ the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: } 49, \text{ a CDR2 region of SEQ ID NO: } 50, \text{ and a CDR1 region of SEQ ID NO: } 51, \text{ and the light chain variable domain comprises a CDR3 region of SEQ ID NO: } 52, \text{ a CDR2 region of SEQ ID NO: } 53, \text{ and a CDR1 region of SEQ ID NO: } 54; \text{ or} \]

\[ \text{h}) \text{ the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: } 69, \text{ a CDR2 region of SEQ ID NO: } 70, \text{ and a CDR1 region of SEQ ID NO: } 71, \text{ and the light chain variable domain comprises a CDR3 region of SEQ ID NO: } 72, \text{ a CDR2 region of SEQ ID NO: } 73, \text{ and a CDR1 region of SEQ ID NO: } 74; \text{ or} \]

\[ \text{i}) \text{ the heavy chain variable domain comprises a CDR3 region of SEQ ID NO: } 77, \text{ a CDR2 region of SEQ ID NO: } 78, \text{ and a CDR1 region of SEQ ID NO: } 79, \text{ and the light chain variable domain comprises a CDR3 region of SEQ ID NO: } 80, \text{ a CDR2 region of SEQ ID NO: } 81, \text{ and a CDR1 region of SEQ ID NO: } 82. \]

15. The antibody according any one of the preceding claims, characterized in that said antibody is of human IgGl subclass or is of human IgG4 subclass.

16. A method of treatment comprising administering an effective amount of an antibody which binds to human CSF-IR, for use in the treatment of a patient having a CSF-IR expressing tumor or having a tumor with CSF-IR expressing macrophage infiltrate, wherein the tumor is characterized by an increase of CSF-IR ligand and wherein the anti-CSF-IR antibody is administered in combination with an effective amount of a TLR9 agonist.
Fig. 2a

BeWo, 5x10E4 cells/well, 3D

Fig. 2b

BeWo, 5x10E4 cells/well, 3D
Fig. 4a

CSF-1 ELISA - Cyto Plasma Batch 1
(0.1 mg/kg anti-CSF1R/hMab 2F11-e7)-Animal 1

M-CSF (pg/mL)

16000 14000 12000 10000 8000 6000 4000 2000 0

Time Point

Pre-Dose
24 hr, 48 hr, 72 hr, 96 hr, 168 hr d15, d22
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/28 A61K39/395 A61K45/06

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>wo 2011/070024 AI (HOFFMANN LA ROCHE [CH]; DIMOUDIS NI KOLAOS [DE]; FERTIG GEORG [DE]; FID) 16 June 2011 (2011-06-16) cited in the application on page 9, line 17 - page 10, line 9 claims 15-19; examples 1-12</td>
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<td>wo 2013/132044 AI (HOFFMANN LA ROCHE [CH]; CANNARI LE MICHAEL [DE]; RUI ES CAR0LA [DE]; RUET) 12 September 2013 (2013-09-12) examples 1-17</td>
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* Special categories of cited documents:

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Date of the actual completion of the international search 4 August 2014

Date of mailing of the international search report 01/09/2014

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer

Domingues, Helena
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