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(54) **Title**: HMGB1 SPECIFIC MONOCLONAL ANTIBODIES

(57) **Abstract**: The present invention concerns monoclonal antibodies specifically binding the human HMGB1 protein and further HMGB1 proteins of mammalian origin and also concerns fragments thereof, nucleic acids encoding such antibodies or fragments thereof, as well as vectors, cells, and compositions comprising the antibodies or nucleic acids, and uses thereof.

HMGB1 SPECIFIC MONOCLONAL ANTIBODIES

Field of the invention

The present invention concerns monoclonal antibodies specifically binding the human HMGB1 protein and further HMGB1 proteins of mammalian origin and also concerns fragments thereof, nucleic acids encoding such antibodies or fragments thereof, as well as vectors, cells, and compositions comprising the antibodies or nucleic acids, and uses thereof.

State of the art

High-mobility group protein B1 (HMGB1), also known as high-mobility group protein 1 (HMG-1) and amphoterin, is a protein that in humans is encoded by the *HMGB1* gene (Ferrari et al., 1996).

Activated macrophages and monocytes secrete HMGB1 as a mediator of inflammation (Wang et al., 1999). Moreover, HMGB1 forms complexes with several other molecules or complex structures, including lipopolysaccharide (LPS), IL-1b, DNA, nucleosomes (Bianchi, 2009), and others. HMGB1, alone or in complex with partners, binds to TLR4 (Yang et al., 2010, Yang and Tracey, 2010) to RAGE (receptor for advanced glycation end-products), TLR2 and other receptors (Bianchi, 2009). This signaling results in recruitment of inflammatory cells to sites of tissue damage, dendritic cell activation, cytokine secretion (Bianchi and Manfredi, 2009). This positions HMGB1 at the intersection of sterile and infectious inflammatory responses. Moreover, HMGB1 also promotes the reconstruction of damaged tissue after trauma or infection (Bianchi and Manfredi, 2009). Individual monoclonal antibodies that neutralize HMGB1 can confer protection against damage and tissue injury during arthritis, colitis, ischemia, sepsis, endotoxemia, and systemic lupus erythematosus, but not necessarily against all thee conditions at the same time, and not necessarily with the same profile of safety and lack of unwanted effects. The need and importance is therefore increasingly felt for the identification of a monoclonal antibody not only specific for HMGB1, but actually specific to well-determined surfaces on the protein, giving it the ability to interfere with the binding of HMGB1 to a subset of its partners.

It is therefore object of the present invention the development of a novel monoclonal antibody specific for HMGB1, and in particular capable of interfering with its chemoattractant activities towards inflammatory cells.

Summary of the invention

The present invention concerns a hybridoma which was deposited on the 22nd of December 2010, under deposit accession number PD 10002, with the International Deposit Authority for cell lines and hybridomas "Interlab Cell Line Collection" (ICLC), which is a Core Facility of the Istituto Nazionale per la Ricerca sul Cancro in Genoa (Italy).

The hybridoma generated according to the present invention (HG-HMGB1) produces a mouse anti-HMGB1 monoclonal antibody (mAb) also defined as DPH1.1.

A further aspect of the present invention is a mouse (murine) anti-HMGB1 monoclonal antibody produced from the HG-HMGB1 hybridoma, or fragments and derivatives thereof.

The present invention also provides the sequence of light and heavy chains of the murine anti-HMGB1 monoclonal antibody.

A still further aspect of the present invention is a cell or vector comprising the murine anti-HMGB1 monoclonal antibody produced from the HG-HMGB1 hybridoma.

The variable region of the heavy chain of the murine anti-HMGB1 monoclonal antibody according to the present invention has a nucleotide sequence which corresponds to SEQ ID NO. 2 and an amino acid sequence which corresponds to SEQ ID NO.4.

The variable domain of the light chain of the murine anti-HMGB1 monoclonal antibody according to the present invention, has a nucleotide sequence which corresponds to SEQ ID NO. 3 and an amino acid sequence which corresponds to SEQ ID NO.5.

The present invention further relates to a murine anti-HMGB1 monoclonal antibody, for use as a diagnostic agent.

The present invention still further relates to a murine anti-HMGB1 monoclonal antibody, for use as a therapeutic agent.

A further aspect is a pharmaceutical composition comprising the murine anti-HMGB1 monoclonal antibody of the present invention as an active ingredient and a pharmaceutically bioactive excipient.

As will be further described in the detailed description of the invention, the pharmaceutical composition of the present invention has the advantages of being specific for the inhibition of the chemoattractant activities of HMGB1.

Brief description of the drawings

The characteristics and advantages of the present invention will be apparent from the detailed description reported below, from the Examples given for illustrative and non-limiting purposes, and from the annexed Figures 1-6, wherein:

Figure 1: shows representative western blots of 500 ng (lane 1) or 100 ng (lane 2) of the recombinant BoxA fragment of HMGB1 (negative control) and 500 ng (lane 3) or 100 ng (lane 4) of recombinant HMGB1 incubated with either the anti-HMGB1 mAb DPH1.1 (top panel) or a control anti-Box-A subunit Ab control (bottom panel), as described in Example 3.

Figure 2: shows representative micrographs of mouse embryonic fibroblasts (MEFs) derived from either wild type (wt) mice or *Hmgb1*-/- mice that were stained by immunofluorescence with the anti-HMGB1 mAb DPH1.1. Scale bar represents 20 μm, as described in Example 3.

Figure 3: shows migration of 3T3 cells towards HMGB1 in the presence of the indicated concentrations of the anti-HMGB1 mAb, examined using a modified Boyden chamber assay. Each bar represents the mean number of migrated cells ± standard deviation of triplicate samples, as described in Example 4.

Figure 4: shows the results of the *in vivo* experiments of inflammatory cell recruitment, as described in Example 5.

Figure 5A and Figure 5B: show the results of the representative immunohistochemical micrographs of control (Figure 5A) or Clo-L-treated (Figure 5B) livers from HBV transgenic mice, two days after intravenous injection of 107 HBV-specific CTLs as described in Example 5. In particular: HMGB1 staining in brown. Note the nuclear-to-cytoplasm translocation of

HMGB1 in hepatocytes surrounded by PMNs (arrowhead). Scale bar represents 150 µm. The experiment was replicated in triplicate. Error bars show standard deviation. * $p<0.05$ and ** $p<0.01$ between DPH1.1 and isotype-matched irrelevant antibody (ANOVA plus t-test with Bonferroni correction).

Figure 6A: and Figure 6B: are representative graphs of the results of the *in vivo* experiments as described in Example 5. In particular, sALT levels in the blood (Figure 6A) and absolute number of Gr-1^{high} $CD11b^{+}$ neutrophils (PMNs) recovered from the livers (Figure 6B) of 6 HBV transgenic mice that received NaCl (white squares), Clo-L and irrelevant (Irr) IgG1 mAb (black triangles) or Clo-L and the anti-HMGB1 mAb DPH1.1 (black circles) along with the intravenous injection of 10^7 HBV-specific CTLs. Error bars show standard deviation. * $p<0.05$ and ** $p<0.01$ between DPH1.1 and isotype-matched irrelevant antibody (ANOVA plus t-test with Bonferroni correction).

Detailed description of the invention

The present invention concerns a hybridoma HB-HMGB1 which was internationally deposited on the 22nd of December 2010, under deposit accession number PD 10002, with the International Deposit Authority for cell lines and hybridomas "Interlab Cell Line Collection" (ICLC), which is a Core Facility of the Istituto Nazionale per la Ricerca sul Cancro in Genoa (Italy).

The hybridoma generated according to the present invention (HG-HMGB1) produces a mouse (murine) anti-HMGB1 monoclonal antibody (mAb).

The type of the immunized animal used in preparing the hybridoma can include a mouse, rat, hamster, rabbit, goat and horse, among which a mouse is preferably used. When a mouse is used, its strain is not particularly limited, but BALB/c mouse is preferably used.

A further aspect of the present invention is a mouse anti-HMGB1 monoclonal antibody produced from the HG-HMGB1 hybridoma, also defined as DPH1.1, or fragments and derivatives thereof.

The definition monoclonal antibody used herein includes fragments and derivatives of the monoclonal antibody, or any humanized anti-HMGB1

whose sequence is derived from DPH1.1 or from any antibody produced from the HG-HMGB1 hybridoma. Specifically, the fragments and derivatives of the monoclonal antibody are exemplified by Fab, Fab', F(ab)2 and sFv fragment, and of sequences in the present antibody replacing sequences of antibodies of human origin, or from other mammalian species, not limited to IgG subclasses, but comprising IgM, igE, IgD, IgE or the like.

The monoclonal antibody in the present embodiment can be obtained by immunizing an animal with HMGB1Ag as antigen by a known immunological method and then using cells of the immunized animal to prepare a hybridoma. DPH1.1 monoclonal antibodies of the present invention are produced by the hybridoma HB-HMGB1.

The present invention also provides the sequence of light and heavy chains of the murine anti-HMGB1 monoclonal antibody.

Each heavy chain has two regions, the constant region and the variable region. The constant region of the heavy chain corresponds to the Mus musculus immunoglobulin heavy chain complex (Igh) on chromosome 12 NCBI Reference Sequence: NG_005838.1

The variable region of the heavy chain of the murine anti-HMGB1 monoclonal antibody according to the present invention has a nucleotide sequence which corresponds to SEQ ID NO. 2 and an amino acid sequence which corresponds to SEQ ID NO.4.

The light chain has two successive domains: one constant domain and one variable domain. The constant domain corresponds to the Mus musculus immunoglobulin kappa chain complex (Igk) on chromosome 6 NCBI Reference Sequence: NG_005612.

The variable domain of the light chain of the murine anti-HMGB1 monoclonal antibody according to the present invention has a nucleotide sequence which corresponds to SEQ ID NO. 3 and an amino acid sequence which corresponds to SEQ ID NO.5.

For the purposes of the present invention, the HMGB1 monoclonal antibody (mAb) has a corresponding SEQ ID NO. as follows:

SEQ ID NO. 1 corresponds to the amino acid sequence of the 17-mer

peptide P1 (KGKPDAAKKGVVKAEKS) derived from HMGB1;

SEQ ID NO. 2 corresponds to the nucleotide sequence of the cDNAs of the variable regions of the heavy chain of DPH1.1, [mus musculus];

CTGCTCGAGGAGTCTGGGGCAGAGCTTGTGAAGCCAGGGGCCTCAGTCAA GTTGTCCTGCACAGCTTCTGGCTTCAACATTGAAGACACCTATGTGCACT GGGTGAAGCAGAGGCCTGAACAGGGCCTGGAGTGGATTGGAAGGATTGAT CCTGCGAATGGTAATAGTAAATATGACCCGAAGTTCCAGGGCAAGGCCAC TCTAACAGCAGACACATTCTCCAACACAGCCTACCTGCAGCTCAGCAGCC TGACATCTGAGGACACTGCCGTCTATTACTGTG*CT* *GTAACTGGGACGGGG GCTTTG*GACTACTGGGGTCAAGGAACC

wherein: the "**bold**" sequence corresponds to the V heavy chain exon in the VHSM7 mouse germline genomic locus, the sequence in "*italic*" corresponds to the diversity region and the "underlined" sequence corresponds to the mouse germline IGHJ4 genomic locus.

The correspondence between the cDNA and the genomic loci is approximate, as expected, due to mutations introduced during B cell maturation.

SEQ ID NO. 3 corresponds to the nucleotide sequence of the cDNAs of the variable domains of the light chain of DPH1.1, [mus musculus];

TTGTTATTACTCGCTGCCCAACCAGCCATGGCC**GAGCTCGTGTTGACGCA GCCGCCATCGTCTCTGGCTGTGTCTGCAGGAGAAAAGGTCACTATGAGCT GTAAGTCCAGTCAAAGTGTTTTATACAGTTCAAATCAGAAGAACTACTTG GCCTGGTACCAGCAGAAACCAGGGCAGTCTCCTAAACTGCTGATCTACTG GGCATCCACTAGGGAGTCTGGTGTCCCTGATCGCTTCACAGGCAGTGGAT CTGGGACAGATTTTACTCTTAACATCACCAATGTACAACCTGACGACCTG GCAGTTTATTACTGTCATCAATACCTCTCCTCG***GTCACGTTCGGTGCTGG *GACCAAGCTGGAACTGAAACGGGCTGATGCTGCACC*

wherein: the "**bold**" sequence corresponds to the Igkv8-27 kappa light chain V exon in the mouse germline genomic locus, the sequence in "*italic*" corresponds to the mouse germline IGKJ5 genomic locus.

SEQ ID NO. 4 corresponds to the amino acid sequence of variable regions of the heavy chain of DPH1.1, [mus musculus];

LLEESGAELVKPGASVKLSCTAS**GFNIEDTY**VHWVKQRPEQGLEWIGR**ID PANGNS**KYDPKFQGKATLTADTFSNTAYLQLSSLTSEDTAVYYC**AVTGTG ALDY**WGQGT

where the "**bold**" sequences correspond to the CDR regions 1, 2 and 3 respectively, as identified through the VBASE2 website (http://www.vbase2.org/).

SEQ ID NO. 5 corresponds to the amino acid sequence of variable domains of the light chain of DPH1.1, [mus musculus];

ELVLTQPPSSLAVSAGEKVTMSCKSS**QSVLYSSNQKNY**LAWYQQKPGQ S

PKLLIY**WAS**TRESGVPDRFTGSGSGTDFTLNITNVQPDDLAVYYC**HQYLS SVT**FGAGTKLELKRADAA

where the "**bold**" sequences correspond to the CDR regions 1, 2 and 3 respectively, as identified through the VBASE2 website (http://www.vbase2.org/).

The murine anti-HMGB1 monoclonal antibody according to the present invention is specific for a known epitope of HMGB1, represented by the sequence KGKPDAAKKGVVKAEKS that is identical in mouse, human and most other mammals, and shows minimal cross-reactivity with other proteins, including HMGB2 which is 80% identical in sequence to HMGB1.

Specificity of the anti-HMGB1 mAb has been in fact seen with respect to, but is not limited to the following mammalian HMGB1 genes:

- Homo sapiens, where HMGB1 has the following Reference Sequences (Ref Seq): NM_002128.4 → NP_002119.1 ;
- Mus musculus HMGB1: NM_010439.3 → NP_034569.1;
- Rattus norvegicus HMGB1 NM_012963.2 → NP_037095.1;
- Bos taurus HMGB1: NM_176612.1 → NP_788785.1;
- Equus caballus HMGB1: NM_001081835.1 → NP_001075304.1

It is therefore an advantage of the murine anti-HMGB1 monoclonal antibody according to the present invention to be specific for a epitope of HMGB1 that is identical in many mammalian species.

A further advantage of the murine anti-HMGB1 monoclonal antibody

according to the present invention is that it shows minimal cross-reactivity with other proteins, including HMGB2 which is 80% identical in sequence to HMGB1.

A still further advantage of the murine anti-HMGB1 monoclonal antibody DPH1.1 is that of being able to block HMGB1-elicited cell migration. This unexpected advantage is important since the recruitment of inflammatory cells to the site of tissue damage is an important element in the causation and continuation of acute and chronic inflammation.

It is shown in the example that HMGB1 release from hepatocytes contributes to recruit PMNs into the liver, and that neutralization of HMGB1 with DPH1.1 mAb reduces the recruitment of PMNs.

The DPH1.1 mAb according to the present invention advantageously blocks cell migration, and in particular has been seen to reduce polymorphonuclear leukocytes recruitment *in vivo*, as well as *in vitro.* Accordingly, the severity of hepatitis (as measured by sALT levels in the blood) is reduced.

A still further aspect of the present invention is a cell or a vector comprising the murine anti-HMGB1 monoclonal antibody produced from the HG-HMGB1 hybridoma.

The present invention further relates to a monoclonal antibody, for use as a diagnostic agent.

The principal diagnostic agents are X-ray-contrast preparations, radioactive isotopes, and dyes. A diagnostic agent can be used for making images of many different body parts.

The monoclonal antibody of the present invention can be used in immunoassays for detection and/or quantification of HMGB1 since the monoclonal antibody has the advantage of recognizing an epitope that is identical in mouse, human and other mammals, for example in a sample suspected of containing HMGB1 protein, either unmodified or post-translationally modified. Such immunoassay can be used in clinical diagnosis of pathologies associated with trauma, tissue damage, acute and chronic inflammation, autoimmune diseases including lupus and in the

screening of blood products.

The present invention still further relates to a monoclonal antibody, for use as a therapeutic agent.

Targeted therapies are the focus of much research in medicine. Biological therapies that target pathology-associated antigens give hope for improvement of survival in many types of illnesses. Tumor specific antigens have been for example widely studied in the hope for cancer treatment.

The mAb according to the present invention is useful in the treatment of pathologies including but not limited to hepatitis, trauma, infection, arthritis, ischemia, organ transpantation, in which the inhibition of the chemoattractant activities of HMGB1 is implicated.

A further aspect is a pharmaceutical composition comprising the monoclonal antibody of the present invention as an active ingredient and a pharmaceutically bioactive excipient.

The pharmaceutical composition of the present invention has the advantages of being specific for the inhibition of the chemoattractant activities of HMGB1.

EXAMPLES

Example 1

Preparation of monoclonal antibody: Immunization of mouse and hybridoma generation

The mouse monoclonal IgG1 DPH1.1 Ab specific for mouse HMGB1 was generated by injecting C57BL/6 6-week-old female mice at two-week intervals with three subcutaneous doses (0.2 mg/mouse) of the 17-mer peptide P1 (KGKPDAAKKGVVKAEKS) (SEQ ID NO.1) derived from HMGB1.

Hybridomas according to the present invention (HG-HMGB1) were generated from splenocytes and from a hypoxanthine-aminopterin-thymine (HAT) sensitive myeloma P3/NSI/1-AG4-1 cells by standard techniques and tested by ELISA against the immunogen.

Example 2

Preparation of monoclonal antibody: Culture of the hybridoma

Hybridoma colonies are grown in RPMI 1640 medium additioned with 15%FCS and HAT for 10 days, and manually selected after microscopic analysis.

HMGB1 positivity of the colonies was monitored by ELISA analysis of the colony supernatant.

Positive colonies were transferred to larger wells and subjected to 2 rounds of limiting dilution.

Stable and immortalized DPH1.1 hybridoma clones were obtained.

The DPH1.1 clone was grown for 3 months in a disposable bioreactor for efficient protein expression, using standard techniques, supernatant was collected.

Purification of the supernatant to obtain the monoclonal antibody was performed on a PROT A chromatographic column.

The monoclonal antibody anti HMGB1 was therefore obtained with a percentage of purity of 95%. The monoclonal antibody anti HMGB1 was further purified to 100% purity with further chromatographic purification passages.

Example 3

Anti-HMGB1 Ab (DPH1.1 mAb) characterization and specificity testing by immunofluorescence

Specificity of DPH1.1 mAb was monitored by both Western blot analysis (Figure 1) and by immunofluorescence (Figure 2).

Anti-HMGB-1 Ab (DPH1.1 Ab) characterization and specificity testing by Western blot

Western blots of the recombinant BoxA domain of HMGB1 (negative control) incubated with either the anti-HMGB-1 mAb DPH1.1 or a control anti-BoxA mAb control were performed.

Briefly, 500 ng or 100 ng of recombinant HMGB1 and the (negative control) recombinant BoxA domain of HMGB1 (HMGBiotech, Milan, Italy) were separated by gel electrophoresis and transferred onto membranes as described (Scaffidi et al., 2002).

Results

As can be seen in Figure 1, the anti-HMGB1 mAb DPH1.1 according to the present invention specifically binds the recombinant HMGB1, but not the BoxA domain.

Immunofluorescence

DPH1.1 mAb, anti-BoxA mAb (HMGBiotech, Milan, Italy) and goat anti-mouse IgG1 Ab (BD PharMingen) were applied at 1 µg/ml dilution. Immunofluorescence was performed as described (Scaffidi et al., 2002) on mouse embryonic fibroblasts (MEFs) derived from either wild type mice or *Hmgb1*$^{-/-}$ mice. DPH1.1 mAb and AlexaFluor 633-labelled goat anti-mouse IgG1 Ab (BD PharMingen) were applied at a 50 µg/ml dilution.

Results

As can be seen in Figure 2: only mouse embryonic fibroblasts derived from *Hmgb1-/-* mice were stained with the anti-HMGB1 monoclonal antibody DPH1.1.

Example 4

Transmigration assay

The *in vitro* activity of DPH1.1 mAb was monitored in trans-well migration assays as described (Palumbo et al., 2009).

Briefly, recombinant HMGB1 was added to the lower chamber at the concentration of 30 ng/ml. Increasing concentrations of DPH1.1 mAb (or of an irrelevant isotype-matched mAb) were added to fifty thousand 3T3 cells. Cell migration was assessed by a modified Boyden chamber assay.

Boyden chambers were incubated at 37°C in 5% CO_2 for 3 hrs. Cells remaining on the upper section of the filters were removed mechanically. Cells that migrated to the lower section of the filters were fixed with ethanol, stained with Giemsa (Sigma-Aldrich), and counted in 10 random fields/filter. Each assay was performed in duplicate and repeated at least three times, independently.

Results:

As can be seen in Figure 3, increasing concentrations of DPH1.1 mAb unexpectedly inhibit cell migration. These results show the surprising effect of the DPH1.1 mAb, which blocks HMGB1 elicited cell migration.

Example 5

In vivo experiments: inflammatory cell recruitment

We had previously shown that inflammatory cells are recruited into the liver by the release of HMGB1 protein (Sitia et al, 1997).

Recruitment of inflammatory cells can be amplified by removal of Kupffer cells (KCs) from the liver, which can be obtained by treatment of the mice with clodronate (Clo-L).

In order to amplify the effect of HMGB1, we removed Kupffer cells (KCs) from HBV transgenic mice, and we injected them with CD8 T cells from mice immunized against HBV (this causes acute hepatitis in the mice). In these mice, we found far larger numbers intrahepatic PMNs than in mice not treated with clodronate (Figure 4). We also found many more hepatocytes expressing cytoplasmic HMGB1 juxtaposed to or surrounded by polymorphonucleate cells (PMNs) in comparison to mice that were not treated with clodronate (Figure 5A abd Figure 5B).

Results

Removal of KCs promoted accumulation of cytoplasmic HMGB1 in hepatocytes and intrahepatic PMN infiltration over what normally seen in mice where KCs were not removed.

Effect of DPH1.1 administration

We tested the effect of administration of the DPH1.1 mAb.

The livers of mice treated with DPH1.1 mAb contained many more hepatocytes expressing cytoplasmic HMGB1 (not shown), higher sALT values (Figure 6A), but reduced numbers infiltrating PMNs (Figure 6B).

Results

These results show that HMGB1 release from hepatocytes contributes to recruit PMNs into the liver, and that neutralization of HMGB1 with DPH1.1 reduces the recruitment of PMNs and hepatitis.

The DPH1.1 mAb according to the present invention blocks cell migration towards HMGB1 *in vivo*, as well as *in vitro*.

Methods:

Mice. HBV replication-competent transgenic mouse (lineage 1.3.32) have

been previously described (Guidotti et al., 1995). Lineage 1.3.32 (inbred C57BL/6, H-2b) was crossed with B10.D2 mice (H-2d) to produce H-2bxd F1 hybrids prior to injection of H-2d-restricted hepatitis B surface antigen (HBsAg)-specific CD8 T cell lines. C57BL/6 and B6.PL-Thy1a/CyJ (Thy-1.1) mice were purchased from The Scripps Research Institute breeding colony or from Charles River Laboratories (Calco, Italy). Bone marrow chimeric phosphoglycerate kinase (PGK)-GFP mice replicating HBV were created by transplanting BM cells derived from PGK-GFP (H-2bxd F1 hybrids, a kind gift of Michele De Palma, San Raffaele Scientific Institute, Milan, Italy) into irradiated 1.3.32 HBV mice. Thy-1.1 mice were crossed once with B10.D2 mice prior to immunization with plasmid DNA- and vaccinia virus-encoding HBsAg as previously described (Iannacone et al., 2005). In all experiments mice were matched for age (8 weeks), sex (males) and, in case of lineage 1.3.32, for serum hepatitis B e antigen (HBeAg) levels before experimental manipulation. All animals were housed in pathogen-free rooms under strict barrier conditions. These studies were approved by the Animal Review Board of the San Raffaele Scientific Institute.

Injection of HBV-specific CD8 T cell lines. HBV-specific CD8 T cell lines were derived from spleen cells of immunized nontransgenic Thy-1.1 x B10.D2 male mice as described (Iannacone et al., 2005). After 3 weeks of in vitro stimulation, the cells were tested for antigen specificity by flow cytometry. CD8+ cells that were over 95% specific for the immunodominant peptide epitope Env 28-39 of HBsAg (Ando et al., 1994) were injected intravenously at different doses (0.5×10^7 cells/mouse, 10^7 cells/mouse or 5×10^7 cells/mouse) into 1.3.32 mice. One, 2, 3, or 5 days later mice were killed and their livers were perfused and harvested for histological and flow cytometry analyses, or they were snap-frozen in liquid nitrogen and stored at -80°C for subsequent molecular analyses.

Depletion of KCs. KC depletion was achieved by intravenous injection of 200 µl of clodronate-containing liposomes (Clo-L, a gift of Roche Diagnostics GmbH, Mannheim, Germany) 3 days before CD8 T cell transfer.

From the above description and the above-noted examples, the advantage attained by the product described and obtained according to the present invention are apparent.

REFERENCES

Ando K, Guidotti LG, Cerny A, Ishikawa T, Chisari FV (1994), CTL access to tissue antigen is restricted in vivo. J Immunol. 1994 Jul 15;153(2):482-8.

Bianchi ME (2009) HMGB1 loves company. J Leukoc Biol 86: 573-6

Bianchi ME and Manfredi AA. (2009) Immunology. Dangers in and out. Science 323:
1683-4

Ferrari S, Finelli P, Rocchi M, Bianchi ME (July 1996). "The active gene that encodes human high mobility group 1 protein (HMG1) contains introns and maps to chromosome 13". Genomics 35 (2): 367–71.

Guidotti, L.G., B. Matzke, H. Schaller, and F.V. Chisari. 1995. High-level hepatitis B
virus replication in transgenic mice. J Virol 69:6158-6169

Iannacone, M., G. Sitia, M. Isogawa, P. Marchese, M.G. Castro, P.R. Lowenstein, F.V. Chisari, Z.M. Ruggeri, and L.G. Guidotti. 2005. Platelets mediate cytotoxic T lymphocyteinduced liver damage. Nature medicine 11:1167-1169

Palumbo, R., F. De Marchis, T. Pusterla, A. Conti, M. Alessio, and M.E. Bianchi. 2009. “Src family kinases are necessary for cell migration induced by extracellular HMGB1.” J Leukoc Biol 86:617-623.

Scaffidi, P., T. Misteli, and M.E. Bianchi. 2002. “Release of chromatin protein HMGB1 by necrotic cells triggers inflammation.” Nature 418:191-195.

Sitia G, Iannacone M, Müller S, Bianchi ME and Guidotti LG (2007) Treatment with HMGB1 inhibitors diminishes CTL-induced liver disease in HBV transgenic mice. J Leukoc Biol 81: 100-7

Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ (July 1999). "HMG-1 as a late mediator of endotoxin lethality in mice". Science 285 (5425): 248–51.

Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, Lu B, Chavan S, Rosas-Ballina M, Al-Abed Y, Akira S, Bierhaus A, Erlandsson-Harris H, Andersson U, Tracey KJ (June 2010). "A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release". Proc. Natl. Acad. Sci. U.S.A. 107 (26): 11942–7.

Yang H, Tracey KJ (2010). "Targeting HMGB1 in inflammation". Biochim. Biophys. Acta 1799 (1-2): 149–56.

CLAIMS

1. A hybridoma which is deposited under deposit Accession No. PD 10002, with International Deposit Authority Interlab Cell Line Collection, Genoa Italy.
2. A murine anti-HMGB1 monoclonal antibody produced by the hybridoma according to claim 1, or fragments and derivatives thereof.
3. The murine anti-HMGB1 monoclonal antibody according to claim 2, wherein the variable region of the heavy chain has nucleotide sequence which corresponds to SEQ ID NO. 2 and an amino acid sequence which corresponds to SEQ ID NO.4, or fragments and derivatives thereof.
4. The murine anti-HMGB1 monoclonal antibody according to claim 2, wherein the variable domain of the light chain has nucleotide sequence which corresponds to SEQ ID NO. 3 and an amino acid sequence which corresponds to SEQ ID NO.5, or fragments and derivatives thereof.
5. The murine anti-HMGB1 monoclonal antibody according to anyone of claims 2 to 4, for use as a diagnostic agent.
6. The murine anti-HMGB1 monoclonal antibody according to anyone of claims 2 to 4 for use as a therapeutic agent.
7. A pharmaceutical composition comprising the monoclonal antibody according to anyone of claims 2 to 4 as an active ingredient and a pharmaceutically bioactive excipient.

Figure 1

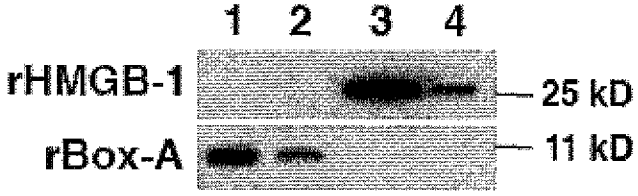


Figure 2

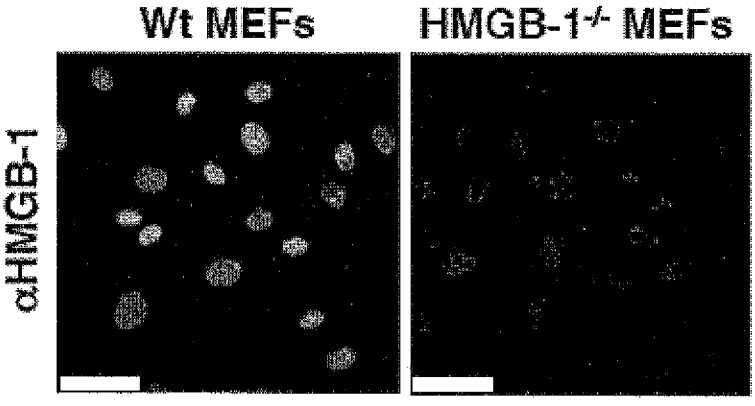


Figure 3

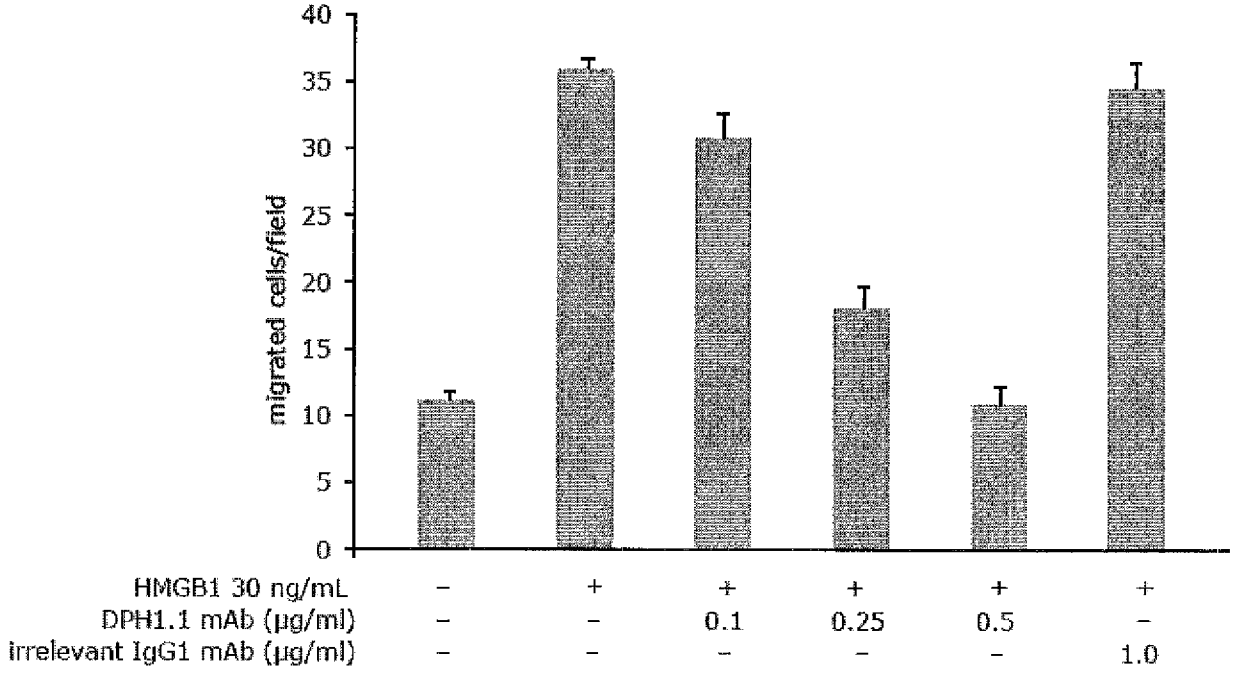

migrated cells/field
40
35
30
25
20
15
10
5
0
HMGB1 30 ng/mL – + + + + +
DPH1.1 mAb (µg/ml) – – 0.1 0.25 0.5 –
irrelevant IgG1 mAb (µg/ml) – – – – – 1.0

Figure 4

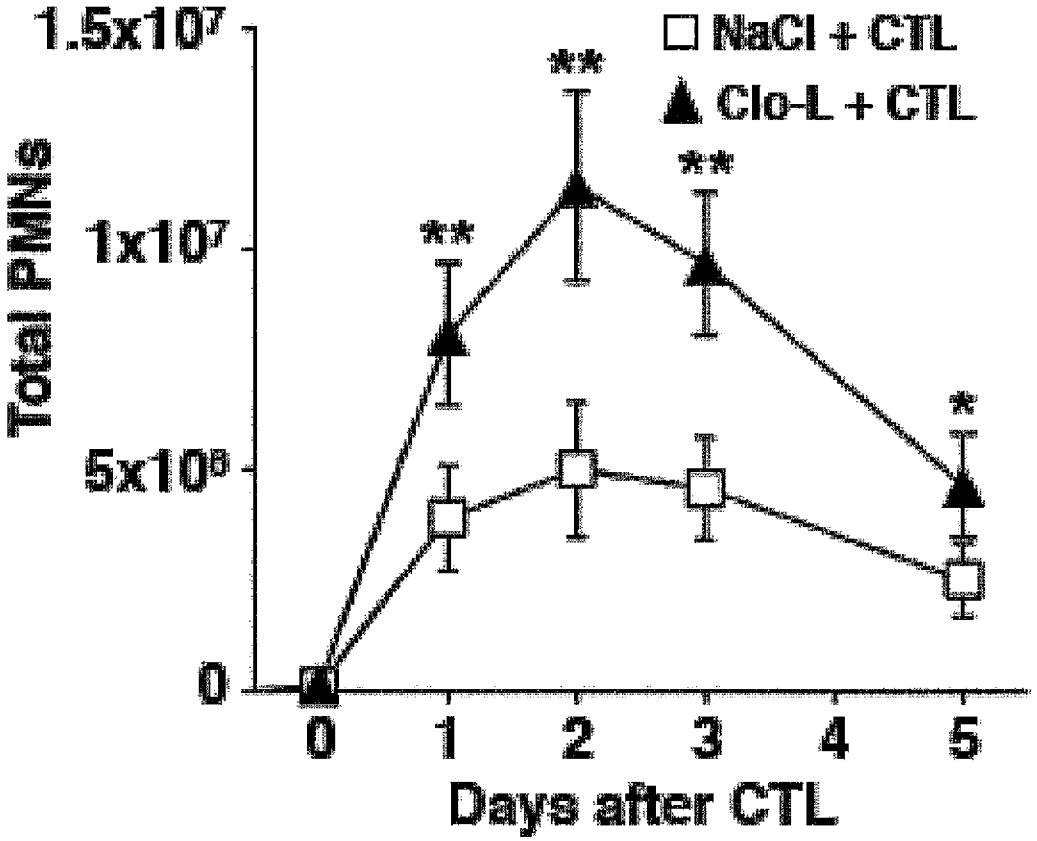

1.5x10⁷
1x10⁷
5x10⁶
0
Total PMNs
NaCl + CTL
Clo-L + CTL
**
**
**
*
0
1
2
3
4
5
Days after CTL

Figure 5A

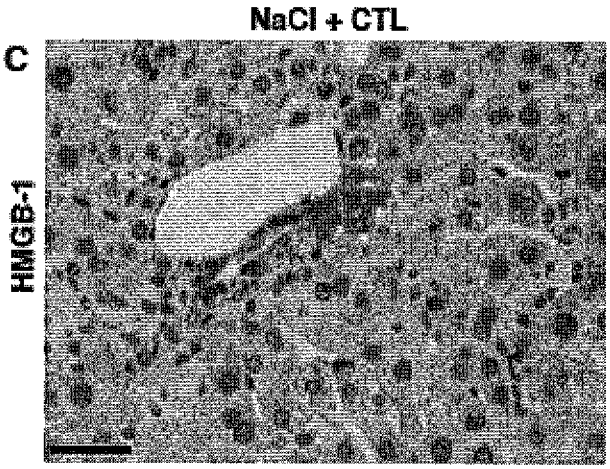


Figure 5B

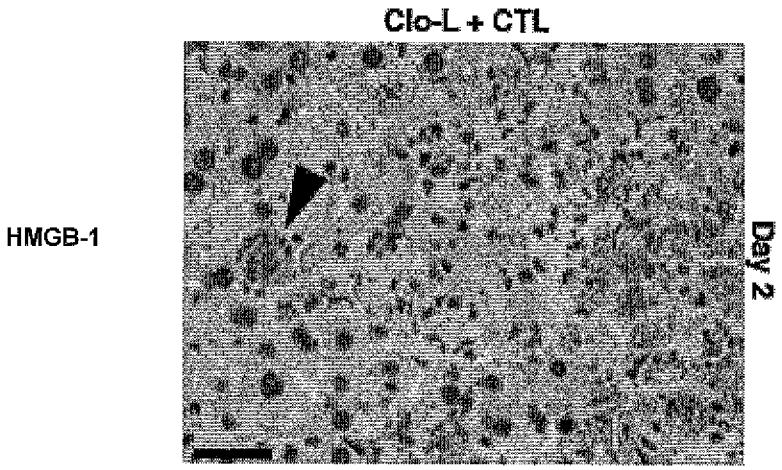


Figure 6A

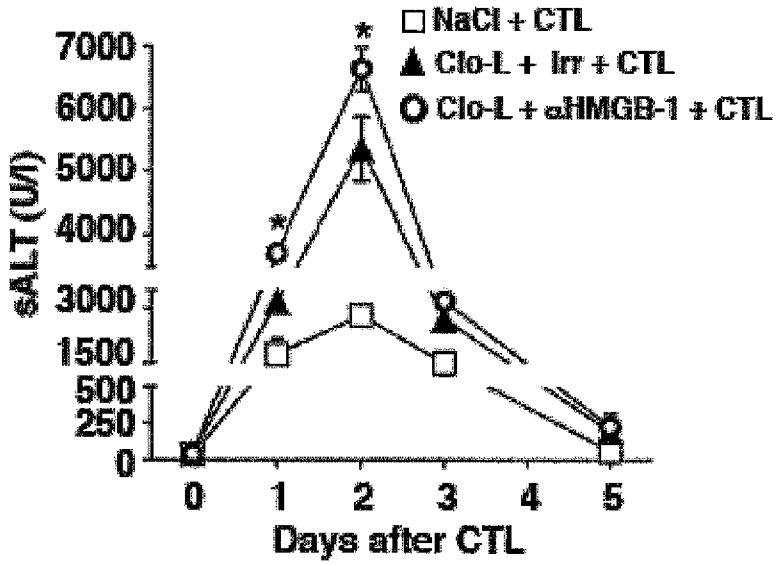
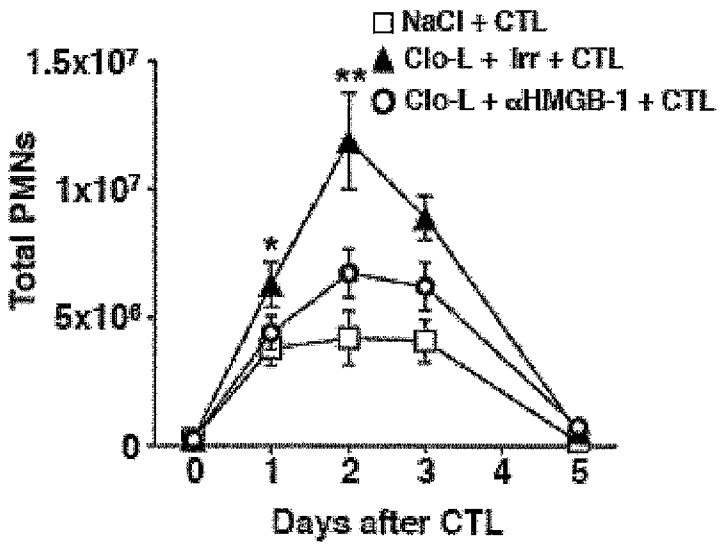


Figure 6B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/055274

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/055274

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/24
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

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 practical, search terms used)
EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/138429 A2 (THE FEINSTEIN INST MEDICAL RES [US]; MESSMER DAVORKA [US]) 28 December 2006 (2006-12-28) the whole document	1-7
A	EP 2 105 447 A1 (SHINO TEST CORP [JP]) 30 September 2009 (2009-09-30) the whole document	1-7

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 September 2011	10/10/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hix, Rebecca

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YAMADA S ET AL: "HIGH MOBILITY GROUP PROTEIN 1 (HMGB1) QUANTIFIED BY ELISA WITH A MONOCLONAL ANTIBODY THAT DOES NOT CROSS-REACT WITH HMGB2", CLINICAL CHEMISTRY, AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY, WASHINGTON, DC, vol. 49, no. 9, 1 September 2003 (2003-09-01), pages 1535-1537, XP001205689, ISSN: 0009-9147, DOI: 10.1373/49.9.1535 the whole document -----	1-7
A	WU HUILING ET AL: "HMGB1 contributes to kidney ischemia reperfusion injury.", JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY : JASN NOV 2010 LNKD-PUBMED:20847143, vol. 21, no. 11, November 2010 (2010-11), pages 1878-1890, XP008143389, ISSN: 1533-3450 the whole document -----	1-7
A	LI JUNHUA ET AL: "Neutralization of the extracellular HMGB1 released by ischaemic damaged renal cells protects against renal ischaemia-reperfusion injury.", NEPHROLOGY, DIALYSIS, TRANSPLANTATION : OFFICIAL PUBLICATION OF THE EUROPEAN DIALYSIS AND TRANSPLANT ASSOCIATION - EUROPEAN RENAL ASSOCIATION FEB 2011 LNKD-PUBMED:20679140, vol. 26, no. 2, February 2011 (2011-02), pages 469-478, XP002659978, ISSN: 1460-2385 the whole document -----	1-7
A	WATANABE TAIJI ET AL: "The role of HMGB-1 on the development of necrosis during hepatic ischemia and hepatic ischemia/reperfusion injury in mice.", THE JOURNAL OF SURGICAL RESEARCH MAR 2005 LNKD- PUBMED:15734480, vol. 124, no. 1, March 2005 (2005-03), pages 59-66, XP002659979, ISSN: 0022-4804 the whole document -----	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/055274

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006138429 A2	28-12-2006	EP 1899376 A2	19-03-2008
		EP 2364998 A1	14-09-2011
EP 2105447 A1	30-09-2009	WO 2008075788 A1	26-06-2008
		US 2009297546 A1	03-12-2009