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(54) **Title**: PEPTIDES DERIVED FROM CARDIAL TROPONIN I AND THEIR USE AS PROGNOSTIC AND DIAGNOSTIC MARKERS FOR HEART FAILURE

(57) **Abstract**: The present invention relates to peptides derived from cardiac troponin I (cTnl) which are the antigenic determinant(s) or pathogenic epitope(s) of cardiac troponin I (cTnl) and antibodies and antibody fragments specific for these peptides. The present invention furthermore relates to the use of these peptides, antibodies and antibody fragments for the prognosis and/or diagnosis of heart insufficiency, in particular of an inflammation of the myocardium and/or fibrosis. The present invention further relates to methods for the prognosis and/or diagnosis of heart insufficiency, in particular of an inflammation of the myocardium and/or fibrosis, utilizing the peptides.

Peptides derived from cardial troponin I and their use as prognostic and diagnostic markers for heart failure

The present invention relates to peptides derived from cardiac troponin I (cTnI) which are the antigenic determinant(s) or pathogenic epitope(s) of cardiac troponin I (cTnI) and antibodies and antibody fragments specific for these peptides. The present invention furthermore relates to the use of these peptides, antibodies and antibody fragments for the prognosis and/or diagnosis of heart insufficiency, in particular of an inflammation of the myocardium and/or fibrosis. The present invention further relates to methods for the prognosis and/or diagnosis of heart insufficiency, in particular of an inflammation of the myocardium and/or fibrosis, utilizing the peptides.

BACKGROUND OF THE INVENTION

Heart failure has become an increasingly prevalent disorder with considerable morbidity and mortality. While many causal mechanisms such as inherited cardiomyopathies, ischemic cardiomyopathy or muscular overload are easily identified in clinical practice, the molecular mechanisms that determine the progression of heart failure or ventricular remodeling are largely unknown. There is compelling evidence that inflammatory mechanisms may contribute to progressive heart failure. Thus, myocardial infiltration of lymphocytes and mononuclear cells, increased expression of pro-inflammatory chemokines and cytokines and circulating autoantibodies are frequently observed in myocarditis and DCM (dilated cardiomyopathy). The antibodies identified in DCM patients are directed against various myocardial constituents (1, 2).

Cardiac troponins in blood are the preferred biomarkers of myocardial injury. The fact that they are strictly intracellular proteins which are not found in the circulation of healthy individuals provides a high level of clinical sensitivity and specificity even when cardiac lesions are small. Thus, any significantly detectable troponin in circulation is considered a sign of acute myocardial cell damage (3, 4).

Nishimura et al. reported that PD-1 receptor deficient mice developed autoantibodies against cardiac troponin I (cTnI) and –as a consequence- severe dilated cardiomyopathy (5). They further found that administration of monoclonal anti-cTnI antibodies induced myocardial dysfunction highly likely by facilitating Ca^{2+} influx into cardiomyocytes (6). Others have shown, that autoantibodies to cTnI are present in patients with acute coronary syndrome and that these antibodies may interfere with diagnostic assays leading to unpredictable results (7). These findings indicate that induction of an autoimmune response to cTnI is not a rare event in patients. Recently, we showed that an autoimmune response to murine cardiac troponin I (mcTnI) induces severe inflammation in the myocardium followed by fibrosis and heart failure with increased mortality in mice (8). Furthermore, we demonstrated that mice immunized with mcTnI prior to LAD ligation showed greater infarct size, more fibrosis, higher inflammation scores and reduced fractional shortening (8).

Thus, the present invention aims to provide means and methods for the prognosis and diagnosis of heart insufficiency, including inflammation, fibrosis and heart failure, in particular autoimmune myocarditis.

It is a further objective of the present invention to provide means and methods for risk stratification of patients with an inflammation of the myocardium and/or fibrosis, preferably with autoimmune myocarditis. These methods and means enable to identify patients at risk for myocardial damage induced by autoimmune inflammation.

SUMMARY OF THE INVENTION

According to the present invention this object is solved by providing peptides derived from cardiac troponin I (cTnI) which are antigenic determinants or pathogenic epitopes of cardiac troponin I (cTnI).

The peptide of the present invention comprises an amino acid sequence of SEQ ID NO. 9 or SEQ ID NO. 11 or variants thereof,
wherein variants are selected from
(a) N-terminally and/or C-terminally truncated or elongated peptides comprising at least 10 contiguous amino acids of SEQ ID NO. 9 or SEQ ID NO. 11;

(b) amino acid substitution or deletion variants, preferably at one amino acid position;

(c) variants comprising modified amino acid(s), unnatural amino acid(s) or peptidomimetic(s);

(d) peptides with at least 90% sequence identity.

According to the present invention this object is furthermore solved by providing an antibody or antibody fragment specific for a peptide of the present invention.

According to the present invention this object is furthermore solved by providing an antibody or antibody fragment specific for cardial troponin I (cTnI), which does not bind to a region or an epitope of cTnI which comprises or corresponds to a peptide of the present invention.

According to the present invention this object is furthermore solved by providing the peptide, the antibody or antibody fragment according to the present invention for use in medicine.

According to the present invention this object is furthermore in particular solved by providing the peptide, the antibody or antibody fragment according to the present invention for the prognosis and/or diagnosis of heart insufficiency, in particular of an inflammation of the myocardium and/or fibrosis.

According to the present invention this object is furthermore solved by a method for the prognosis and/or diagnosis of heart insufficiency, in particular of an inflammation of the myocardium and/or fibrosis.

The method of the present invention comprises

a) providing a patient sample, preferably a bodily fluid or tissue,

b) providing at least one peptide according to the present invention,

c) determining whether the patient has an immune reaction against said peptide(s), wherein said immune reaction is determined by determining the presence or absence of antibodies against said peptide(s) or determining the presence or absence of T cell response in said patient sample,

d) optional, determining the presence or absence of further markers in said patient sample.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

Before the present invention is described in more detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. For the purpose of the present invention, all references cited herein are incorporated by reference in their entireties.

Peptides as antigenic determinants/pathogenic epitopes of cardiac troponin I (cTnI)

As outlined above, the present invention provides peptides derived from cardiac troponin I (cTnI) which are antigenic determinants or pathogenic epitopes of cardiac troponin I (cTnI).

An "antigenic determinant" or "pathogenic epitope" of cardiac troponin I (cTnI) refers to a amino acid sequence or sequence motif of the protein sequence of cTnI that

(i) is responsible for the initiation, maintenance and progress of an inflammatory process in the myocardium

(ii) is responsible for the initiation, maintenance and progress of fibrosis in the myocardium

(iii) induces alteration of heart function and heart failure

(iv) causes an immune response, in particular antibodies directed against itself as well as the whole protein cTnI

(for details see below, in particular Example 1).

Preferably, the peptide is derived from murine cTnI, human cTnI, rat cTnI or other cTnI homologues, more preferably from murine cTnI.

Murine cTnI, human cTnI, rat cTnI or other cTnI homologues as well as their amino acid sequences and the nucleotide sequences of the genes encoding them are known in the art.

For an amino acid sequence of murine cTnI see Genbank Accession No. NP_033432 (SEQ ID NO. 28); for an amino acid sequence of human cTnI see Genbank Accession No. CAA62301

(SEQ ID NO. 29); for an amino acid sequence of rat cTnI see Genbank Accession No. CAA41402 (SEQ ID NO. 30).

A preferred amino acid sequence of murine cTnI is [SEQ ID NO. 28]

```
LOCUS       NP_033432               211 aa
DEFINITION  troponin I, cardiac [Mus musculus].
ACCESSION   NP_033432 XP_980780
VERSION     NP_033432.1  GI:6678393

        1 madessdaag epqpapapvr rrssanyray atephakkks kisasrklql ktlmlqiakq
       61 emereaeerr gekgrvlrtr cqpleldglg feelqdlcrq lharvdkvde erydveakvt
      121 kniteiadlt qkiydlrgkf krptlrrvri sadammqall gtrakesldl rahlkqvkke
      181 diekenrevg dwrknidals gmegrkkkfe g
```

A preferred amino acid sequence of human cTnI is [SEQ ID NO. 29]

```
LOCUS       CAA62301                210 aa
DEFINITION  cardiac troponin I [Homo sapiens].
ACCESSION   CAA62301
VERSION     CAA62301.1  GI:1524066

        1 madgssdaar eprpapapir rrssnyraya tephakkksk isasrklqlk tlllqiakqe
       61 lereaeerrg ekgralstrc qplelaglgf aelqdlcrql harvdkvdee rydieakvtk
      121 niteiadltq kifdlrgkfk rptlrrvris adammqallg arakesldlr ahlkqvkked
      181 tekenrevgd wrknidalsg megrkkkfes
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A preferred amino acid sequence of rat cTnI is [SEQ ID NO. 30]

```
LOCUS       CAA41402                211 aa
DEFINITION  cardiac troponin I [Rattus norvegicus].
ACCESSION   CAA41402
VERSION     CAA41402.1  GI:56023

        1 madessdaag epqpapapvr rrssanyray atephakkks kisasrklql ktlmlqiakq
       61 emereaeerr gekgrvlstr cqplvldglg feelqdlcrq lharvdkvde erydveakvt
      121 kniteiadlt qkiydlrgkf krptlrrvri sadammqall gtrakesldl rahlkqvkke
      181 diekenrevg dwrknidals gmegrkkkfe g
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Preferably, a peptide derived from cardiac troponin I (cTnI) comprises an amino acid sequence of SEQ ID NO. 9 or SEQ ID NO. 11 or variants thereof.

SEQ ID NO. 9 refers to amino acid residues 105 to 122 of murine cTnI

VDKVDEERYDVEAKVTKN

SEQ ID NO. 11 refers to amino acid residues 131 to 148 of murine cTnI

QKIYDLRGKFKRPTLRRV.

More preferably, the peptide has the amino acid sequence of SEQ ID NO. 9 or consists of it.

Preferably, *variants* of the peptide are selected from

(a) N-terminally and/or C-terminally truncated or elongated peptides comprising at least 7 contiguous amino acids of SEQ ID NO. 9 or SEQ ID NO. 11;
preferably N-terminally and/or C-terminally truncated or elongated peptides comprising at least 10 contiguous amino acids of SEQ ID NO. 9 or SEQ ID NO. 11
(the N-terminally and/or C-terminally truncated or elongated peptides can comprise any number of amino acid residues from SEQ ID NO. 9 or 11 in the range from 7 to 18 amino acids, such as 7, 8, 9, 10, ..., 17, 18 amino acids);

(b) amino acid substitution or deletion variants, preferably at one amino acid position;

(c) variants comprising modified amino acid(s), unnatural amino acid(s) or peptidomimetic(s);

(d) peptides with at least 90% sequence identity to SEQ ID NO. 9 or SEQ ID NO. 11.

A peptide sequence is considered a variant of the peptides of the present invention when it

(i) is responsible for the initiation, maintenance and progress of an inflammatory process in the myocardium
(ii) is responsible for the initiation, maintenance and progress of fibrosis in the myocardium
(iii) induces alteration of heart function and heart failure
(iv) causes an immune response, in particular antibodies directed against itself as well as the whole protein cTnI,

in particular when it

(i) causes inflammation and/or fibrosis to the heart of mice immunized with the peptide sequence tested (as preferably measured on heart sections as described in Example 1, see also Figure 4);

or

(ii) causes an immune response, in particular antibodies directed against itself as well as the whole protein cTnI (preferably measured as antibody titer measured in mice immunized with the peptide sequence tested, as described in Example 1, see also Figure 5).

A peptide sequence is considered a variant of the peptides of the present invention when it shows at least 80%, preferably 90% activity of the peptide with SEQ ID NO. 9, wherein "activity" refers to (i) induction of inflammation and/or fibrosis to the heart of mice immunized with the respective peptide tested; and/or (ii) induction of an immune response of mice immunized with the respective peptide (antibody titer against cTnI and the peptide tested).

Amino acid substitution variants comprise conservative or non conservative replacement by other amino acids. Conservative amino acid substitutions typically relate to substitutions among amino acids of the same class. These classes include, for example,

- amino acids having uncharged polar side chains, such as asparagine, glutamine, serine, threonine and tyrosine;
- amino acids having basic side chains, such as lysine, arginine, and histidine ;
- amino acids having acidic side chains, such as aspartic acid and glutamic acid; and
- amino acids having nonpolar side chains, such as glycine, alanine, valine, leucine, isoleucine, proline,phenylalanine, methionine, tryptophan, and cysteine.

Preferably, the variants have an amino acid substitution or deletion (only) at one amino acid position.

An example of an amino acid substitution variant comprises or has amino acid residues 104-121 of human cTnI: VDKVDEERYDIEAKVTKN [SEQ ID NO. 17].

Preferably, the variants do not comprise or have the following sequences:

- the residues 73-90 of slow skeletal troponin I (see also Figure 6)
  VEVVDEERYDIEAKCLHN [SEQ ID NO. 18],
- the residues 99-116 of slow skeletal troponin I (see also Figure 6)
  LKVLDLRGKFKRPPLRRV [SEQ ID NO. 19], and
- the residues 98-115 of fast skeletal troponin I (see also Figure 6)
  QKLFDLRGKFKRPPLRRV [SEQ ID NO. 20].

Examples of variants that are N-terminally and/or C-terminally elongated peptides comprise or have amino acid residues of both peptides of SEQ ID NO. 9 and SEQ ID NO. 11 or at least 7 (preferably 10) contiguous amino acids of both SEQ ID NO. 9 and SEQ ID NO. 11,

such as a peptide that comprises or has amino acid residues of murine cTnI from the start of peptide 9/SEQ ID NO. 9 to the end of peptide 11/SEQ ID NO. 11 (i.e. amino acid residues 105 to 148 of murine cTnI):

VDKVDEERYDVEAKVTKNITEIADLTQKIYDLRGKFKRPTLRRV [SEQ ID NO. 21]

In a further embodiment, *variants* of the peptide are selected from

(a) N-terminally and/or C-terminally truncated or elongated peptides of SEQ ID NO. 21; preferably N-terminally and/or C-terminally truncated or elongated peptides comprising at least 10, preferably 12 contiguous amino acids of SEQ ID NO. 21;

(the N-terminally and/or C-terminally truncated or elongated peptides can comprise any number of amino acid residues of SEQ ID NO. 21 in the range from 10 to 44 amino acids, such as 10, 11, 12 ..., 43, 44 amino acids);

(b) amino acid substitution or deletion variants, preferably at one amino acid position (as described above);

(c) variants comprising modified amino acid(s), unnatural amino acid(s) or peptidomimetic(s) (as described above);

(d) peptides with at least 90% sequence identity to SEQ ID NO 21.

Preferably, the peptide of the present invention comprises 10 contiguous amino acids of SEQ ID NO. 9 or SEQ ID NO. 11, preferably selected from

DEERYDVEAK (SEQ ID NO. 24);
EERYDVEAKV (SEQ ID NO. 25); or
ERYDVEAKVT (SEQ ID NO. 26).

Preferably, the peptides according to the present invention have a length of at least 7 amino acids, preferably have a length from about 10 to about 50 amino acids, wherein at least 7, preferably at least 10 amino acids are contiguous amino acid residues of SEQ ID NO. 9 or SEQ ID NO. 11 (or wherein at least 10, preferably 12 amino acids are contiguous amino acid residues of SEQ ID NO. 21).

In one embodiment, the peptides consist of any length in the range from 7 to about 100 amino acids, preferably from 7 to about 50 amino acids, such as 7, 8, 9, 10, ..., 44, 45, 46, 47, 48, 49, 50 amino acids. It should be noted that "..." denotes every integer in the respective range.

Preferably, the peptides according to the present invention do not comprise the full-length protein cardiac troponin I (cTnI), such as murine cTnI (SEQ ID NO. 28), human cTnI (SEQ ID NO. 29), rat cTnI (SEQ ID NO. 30) or other cTnI homologues.

In a preferred embodiment, the peptides according to the present invention comprise *N-terminal and/or C-terminal modifications*.

Preferred modifications are
- hydrophobic modification,
such as

acylation (e.g. with carboxylic acids, fatty acids, C8 to C22 fatty acids, amino acids with lipophilic side chains);

addition of hydrophobic moieties (e.g. with cholesterol, derivatives of cholesterol, phospholipids, glycolipids, glycerol esters, steroids, ceramids, isoprene derivatives, adamantane, farnesol, aliphatic groups, polyaromatic compounds);

- modification with a moiety that protects from degradation (e.g. amide, D-amino acid, modified amino acid, cyclic amino acid; natural and synthetic polymer, such as PEG, glycane);
- modification with a moiety that boosts the immune response (e.g. bovine serum albumin, keyhole limpet hemocyanin).

Acylation is preferably selected from acylation with carboxylic acids, fatty acids, amino acids with lipophilic side chains. Preferred fatty acids are saturated or unsaturated fatty acids, branched or unbranched fatty acids, preferably with 8 to 22 carbon atoms (C8 to C22). The hydrophobic modification by acylation can be selected from acylation with myristoyl (C14), palmitoyl (C16) or stearoyl (C18).

The addition of hydrophobic moieties is preferably selected from addition of cholesterol, derivatives of cholesterol, phospholipids, glycolipids, glycerol esters, steroids, ceramids, isoprene derivatives, adamantane, farnesol, aliphatic groups, polyaromatic compounds. The attachment of the hydrophobic moieties is preferably by covalent binding, which can be achieved via carbamate, amide, ether, disulfide or any other linkage that is within the skill of the person skilled in the art.

The acylation or addition of hydrophobic moieties is preferably N-terminal, wherein "N-terminal" refers to the hydrophobic modification at the N-terminus, i.e. the respective first amino acid residue, but comprises also the hydrophobic modification in close proximity to the N-terminus. Thus, the hydrophobic modification can furthermore be obtained by an attachment of a hydrophobic moiety at a site close to the N-terminus of the peptide of the invention.

Furthermore preferred is a modification with a moiety that protects from degradation, such as *in vivo* degradation.

Such a modification is preferably C-terminal, wherein "C-terminal" refers to the modification at the C-terminus, i.e. the respective last amino acid residue, but comprises also the modification in close proximity to the C-terminus, such as the last but one amino acid residue, the last but two amino acid residue or more amino acid residues (e.g. introduction of one D-amino acid that protects the carrier from enzymatic degradation e.g. by the action of carboxypeptidases).

The skilled artisan will be able to select the respective suitable moiety(s) depending on the respective application.

Preferred moieties that protect from degradation are selected from amides, D-amino acids, modified amino acids, cyclic amino acids; natural and synthetic polymers, such as PEG, glycane.

Preferred moieties that boost the immune response are selected from bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). The skilled artisan will be able to select the respective suitable moiety(s) depending on the respective application.

In a preferred embodiment, the peptides according to the present invention comprise
- *label(s)*, such as fluorescent dye(s), radioisotope(s) and contrast agent(s),
and/or
- *drug(s)*.

The label(s) are preferably suitable for an *in vivo* medical use in diagnostics and prognostics, as known to the skilled artisan.
Preferred radioisotopes are ^{131}I, ^{125}I, ^{99m}Tc, ^{18}F, ^{68}Ga, ^{111}In, ^{90}Y, ^{177}Lu.

Preferred fluorescent dyes are Alexa dyes, derivatives of rhodamine and fluorescein, Cy-dyes, green fluorescent protein (GFP) and its derivatives (EGFP, CFP, ECFP, YFP, EYFP, etc), zoanFP or the red fluorescent protein drFP583 and their derivates.

Preferred contrast agents are Gadolinium (Gd) complexes, supramagnetic iron (Fe) complexes and particles, compounds containing atoms of high atomic number, i.e. iodine for computer tomography (CT), microbubbles and carriers such as liposomes that contain these contrast agents.

The peptides of this invention can be prepared by a variety of procedures readily known to those skilled in the art, in general by synthetic chemical procedures and/or genetic engineering procedures.

Synthetic chemical procedures include more particularly the solid phase sequential and block synthesis (Erickson and Merrifield, 1976). The solid phase sequential procedure can be performed using established automated methods such as by use of an automated peptide synthesizer. The peptides of the present invention may also be obtained by coupling (poly)peptide fragments that are selectively protected, this coupling being effected e.g. in a solution. The peptides can further be produced by genetic engineering techniques as known to the skilled artisan, utilizing eukaryotic and/or prokaryotic expression systems.

Antibodies and antibody fragments
As outlined above, the present invention provides antibodies and antibody fragments.

An antibody or antibody fragment of the present invention is specific for a peptide of the present invention as defined herein, i.e. it specifically binds thereto.

An "antibody" refers to monoclonal antibodies, polyclonal antibodies, recombinant antibodies, genetically engineered antibodies, such as humanized antibodies, chimeric antibodies etc.

An "antibody fragment" refers to a fragment of antibody that specifically binds to the respective epitope/antigen and comprises Fab, single chain Fv, genetically engineered antibody fragments (such as diabodies), etc.

A further antibody or antibody fragment of the present invention is specific for cardial troponin I (cTnI) and does not bind to a region or an epitope of cTnI which comprises or corresponds to a peptide of the present invention as defined herein.

The antibodies and/or antibody fragments of this invention can be prepared by a variety of procedures known to those skilled in the art, preferably by genetic engineering procedures, but also synthetic chemical procedures.

Use of the peptides and antibodies as prognostic and/or diagnostic marker

As outlined above, the present invention provides the peptide(s), antibody(ies) and antibody fragment(s) of the present invention for use in medicine.

The present invention provides the first medical use of the peptide(s), antibody(ies) and antibody fragment(s) of the present invention.

The present invention also provides second medical use(s) of the peptide(s), antibody(ies) and antibody fragment(s) of the present invention.

As outlined above, the present invention provides the peptide(s), antibody(ies) and antibody fragment(s) of the present invention for the prognosis and/or diagnosis of heart insufficiency.

"Heart insufficiency" or "heart failure" refers to a condition that can result from any structural or functional cardiac disorder that impairs the ability of the heart to fill with or pump a sufficient amount of blood throughout the body. Causes and contributing factors to congestive heart failure include the following (with specific reference to left (L) or right (R) sides): genetic family history of CHF, ischemic heart disease/myocardial infarction (coronary artery

disease), thyrotoxicosis (hyperthyroidism), hypothyroidism, anemia, arrhythmia, hypertension, infection, cardiac fibrosis, coarctation of the aorta (L), aortic stenosis/regurgitation (L), mitral regurgitation (L), pulmonary stenosis/pulmonary hypertension/cor pulmonale/pulmonary embolism (R), mitral valve disease (L), cardiomyopathy, including noncompaction cardiomyopathy (L&R) and inflammation.

The peptide(s), antibody(ies) and antibody fragment(s) of the present invention are in particular suitable for the prognosis and/or diagnosis of an inflammation of the myocardium and/or fibrosis.

Preferably, the peptide, the antibody or the antibody fragment of the present invention is a suitable marker for the prognosis and/or diagnosis of heart insufficiency, in particular of an inflammation of the myocardium and/or fibrosis,.

In a preferred embodiment, the peptide, the antibody or the antibody fragment of the present invention is used for determining a patient group, in particular a patient risk group, with an inflammation of the myocardium and/or fibrosis.

In a preferred embodiment the inflammation of the myocardium and/or fibrosis is autoimmune myocarditis.

In a preferred embodiment, the peptide, the antibody or the antibody fragment of the present invention is used for risk stratification in acute cardiac disorders of patients.

"Risk assessment" or "risk stratification" of subjects with heart failure according to the present invention refers to the evaluation of factors, such as biomarkers, in order to predict the risk of future events or even death and in order to decide about the type, manner, dosis, regimen of therapy and treatment for the individual subject.

The present invention demonstrates the use of the peptides of the invention (preferably peptide 9 and 11 sequences; SEQ ID NO. 9 and 11) instead of the whole troponin molecule in testing patient samples (preferably sera) to identify patients at risk for myocardial damage induced by autoimmune inflammation. Thus, the use of the peptides of the invention in tests is more specific for identifying the risk of inflammation than using the whole troponin protein

(TnI) since the inventors demonstrated that immunization with the other peptide sequences of troponin I (namely SEQ ID NOs. 1-8, 10, 12-16) did not induce an inflammation or fibrosis in the myocardium. (In contrast: the use of whole troponin protein will identify all antibodies directed against different sequences of troponin, including those that might have no pathogenic effect. For further details see below.)

Troponin autoantibodies are present in patients and can produce false negative results by blocking the binding of troponin antibodies used in analytical assays to the target protein. The presence of autoantibodies against cTnI in patients with acute coronary syndrome points to an early induction of an autoimmune response to cTnI in these patients. In addition to the analytical interference in the assays, troponin I autoantibodies may have clinical consequences. Troponin autoantibodies induce dilation and cardiac dysfunction by the chronic stimulation of calcium influx in cardiomyocytes. Provoking an autoimmune response to cardiac troponin I induces severe inflammation in the myocardium followed by fibrosis and heart failure with increased mortality in mice. These results suggest that some of the patients who develop an autoimmune response to released cTnI may have a higher risk of heart failure due to inflammation in the myocardium. These autoimmune responses may also explain the discrepancy observed in some patients with involvement of one or two coronary arteries while the entire heart is diffusely hypokinetic. Furthermore, the role of autoantibodies in heart failure has been supported by many clinical studies demonstrating that removing immunoglobulins by immunoadsorption can improve the ejection fraction and reduce the morbidity in patients with dilated cardiomyopathy.

The present invention identifies one major and one minor epitope of troponin I (peptide 9 and 11, respectively) that are responsible for disease induction. Using these newly identified epitope sequences of troponin I instead of the whole troponin molecule; a more specific screening test of patients with high risk for progressive autoimmune inflammatory heart failure is possible.

Thus, the above uses preferably comprises

i. determining if a patient has an immune reaction against peptide(s) of the present invention, wherein said immune reaction is determined by
    - o determining the presence or absence of antibodies against said peptide(s)

    or
    - o determining the presence or absence of T cell response

    in a patient sample or the patient,

ii. determining the presence or absence of
    - o auto-antibodies against a peptide according to the present invention

    and/or
    - o auto-antibodies against cardial troponin I (cTnI)

    in a patient sample or the patient,

iii. determining cell proliferation and/or production of cytokine(s) and/or chemokine(s) and/or changes in expression of chemokine/cytokine receptors by different cells after stimulation with the peptide(s).

Preferably, cell proliferation and/or production of cytokine(s) and/or chemokine(s) and/or changes in expression of chemokine/cytokine receptors by different cells is determined in cells of the immune system,
such as (all subsets of) T cells, (all subsets of) B cells, monocytes/macrophages, endothelial cells, (all subsets of) dendritric cells, NK cells, mast cells, thrombocytes and (all subsets of) stem cells.

Preferably, the production of different cytokine(s), such as Interferon-alpha, Interferon-beta, Interferon-gamma, TNF-α, IL-1, IL-2, IL-6, IL-5, IL-4, IL-10, IL-13, IL-17, and others
and/or chemokine(s), such as RANTES, MCP-1, MIP-1α, MIP-1β, MIP-2, TCA-3, eotaxin, is determined.
Preferably, the change in expression of chemokine receptors, such as CCR1, CCR2, CCR5 is determined.

Methods and technologies to carry out the above are known in the art and comprise *in vivo* and *in vitro* methods, for example, MRI, ELISA assays, scintigraphy, Western blots, PET, CT, immune adsorption.

Prognostic and/or diagnostic methods for heart insufficiency
As outlined above, the present invention provides a method for the prognosis and/or diagnosis of heart insufficiency or heart failure.

The method of the present invention is in particular suitable for the prognosis and/or diagnosis of an inflammation of the myocardium and/or fibrosis, more particularly of autoimmune myocarditis.

The method of the present invention comprises the following steps:

a) providing a patient sample,

b) providing at least one peptide according to the present invention,

c) determining whether the patient has an immune reaction against said peptide(s), wherein said immune reaction is determined by determining the presence or absence of antibodies against said peptide(s) or determining the presence or absence of T cell response in said patient sample,

d) optional, determining the presence or absence of further markers in said patient sample.

In a preferred embodiment, the method of the present invention comprises
determining cell proliferation and/or production of cytokine(s) and/or chemokine(s) and/or changes in expression of chemokine/cytokine receptors (such as upregulation) by different cells after stimulation with the peptide(s).

Preferably, cell proliferation and/or production of cytokine(s) and/or chemokine(s) and/or changes in expression of chemokine/cytokine receptors by different cells is determined in cells of the immune system,
such as (all subsets of) T cells, (all subsets of) B cells, monocytes/macrophages, endothelial cells, (all subsets of) dendritric cells, NK cells, mast cells, thrombocytes and (all subsets of) stem cells.
Preferably, the production of different cytokine(s), such as Interferon-alpha, Interferon-beta, Interferon-gamma, TNF-α, IL-1, IL-2, IL-6, IL-5, IL-4, IL-10, IL-13, IL-17, and others
and/or chemokine(s), such as RANTES, MCP-1, MIP-1α, MIP-1β, MIP-2, TCA-3, eotaxin is determined.
Preferably, the change in expression of chemokine receptors, such as CCR1, CCR2, CCR5 is determined.

The patient sample is preferably a bodily fluid or tissue, more preferably whole blood, serum, a biopsy (preferably of the heart).

In step c), preferably, the presence or absence of auto-antibodies against cardial troponin I (cTnI) and/or of auto-antibodies against a peptide according to the present invention is determined.

Methods and techniques for determining whether a patient has an immune reaction against peptide(s) (as in step b)) are known in the art,
such as
- ELISA,
- proliferation assay,
- assays measuring cytokine production and/or chemokine production,
- FACS,
- RNA protection assay,
- immune staining,
- immune adsorption.

Preferably, the method comprises the use of an antibody or antibody fragment according to the present invention.

Preferably, the method according to the present invention is for determining a patient group, in particular a patient risk group, with an inflammation of the myocardium and/or fibrosis, preferably with autoimmune myocarditis.

Preferably, the method according to the present invention is for risk stratification in acute cardiac disorders of patients.

Further markers for heart failure or heart insufficiency (as determined in step d)) are known in the art, such as brain natiuretic peptide (BNP), NT-pro-BNP, ANP, troponin I, troponin T, hsCRP, heart-type fatty acid binding protein (H-FABP), myosin light chain-1(MLC-1),creatinine, soluble CD40 ligand (sCD40L), PAPP-A, MPO, VEGF or PlGF.

Determining the antigenic determinants of cardiac troponin I

- Identity of peptides

To identify the antigenic sequences of murine cardiac troponin I triggering inflammation and fibrosis, sixteen overlapping 16-18mer peptides covering the entire amino acid sequence of murine troponin I (211 residues) were synthesized [SEQ ID NOs. 1-16], followed by HPLC-purification, see Table 1 and Figure 3.

Table 1

Peptide	Amino acid sequence	Incidence	Severity	SEQ ID NO.
1	MADESSDAAGEPQPAPAP	0 (0/5)	0	1
2	PAPAPVRRRSSANYRAYA	0 (0/5)	0	2
3	YRAYATEPHAKKKSKISA	0 (0/5)	0	3
4	SKISASRKLQLKTLMLQI	0 (0/5)	0	4
5	LMLQIAKQEMEREAEERR	0 (0/5)	0	5
6	AEERRGEKGRVLRTRCQP	0 (0/5)	0	6
7	TRCQPLELDGLGFEELQD	0 (0/5)	0	7
8	EELQDLCRQLHARVDKVD	0 (0/5)	0	8
9	VDKVDEERYDVEAKVTKN	80% (4/5)	1,4 (3-1-0-2-1)	9
10	KVTKNITEIADLTQKIYD	0 (0/5)	0	10
11	QKIYDLRGKFKRPTLRRV	20% (4/5)	0,2 (1-0-0-0-0)	11
12	TLRRVRISADAMMQALLG	0 (0/5)	0	12
13	QALLGTRAKESLDLRAHL	0 (0/5)	0	13
14	LRAHLKQVKKEDIEKENR	0 (0/5)	0	14
15	EKENREVGDWRKNIDALS	0 (0/5)	0	15
16	IDALSGMEGRKKKFEG	0 (0/5)	0	16
17	Positive control (murine TnI)	80% (4/5)	1,6 (3-2-0-1-2)	28
18	Negative control (control buffer)	0 (0/4)	0	

- Immunization and humoral immune response

The mice developed significant autoantibody titers against all injected peptides by day 28. However, total IgG autoantibody titers were significantly higher in mice immunized with the peptides 7, 8, 10 and 14 [SEQ ID NOs. 7, 8, 10 and 14] (data not shown).

- Immunization with the residues 105-122 (peptide 9) and 131-148 (peptide 11) of murine cTnI induces myocardial inflammation

Four of five mice immunized with the residues 105-122 of mcTnI (peptide 9: VDKVDEERYDVEAKVTKN; SEQ ID NO. 9) showed inflammation with a histoscore of ≥

1 (Figures 3 and 4a-d). One out of five mice immunized with the residues 131-148 of mcTnI (peptide 11: QKIYDLRGKFKRPTLRRV; SEQ ID NO. 11) showed signs of inflammation (histoscore=1) (Figures 3 and 4e). No inflammation was observed in the myocardium of mice immunized with the other synthesized mcTnI residues or control buffer and adjuvant alone (Figures 3 and 4f).

- Immunization with the residues 105-122 (peptide 9) of murine cTnI causes myocardial fibrosis

Comparable to the observed inflammation four out of five mice immunized with the residues 105-122 of mcTnI (peptide 9; SEQ ID NO. 9) showed significant deposition of collagen in the myocardium with a fibrosis score of ≥ 1 (Figures 4g-i) whereas no fibrosis was detected in the mice immunized with the other peptides or control buffer and adjuvant alone. Myocardial fibrosis was indicated by bright blue staining for collagen deposition and was associated with myocardial inflammation.

- Immunization with the residues 105-122 (peptide 9) of murine cTnI induces expression of chemokines and chemokine receptors in the myocardium

In order to investigate the expression of different chemokines and chemokine receptors the inventors measured the mRNA-expression levels of the chemokines Ltn, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3, eotaxin and of the chemokine receptors CCR1, CCR2, CCR1b, CCR3, CCR4 and CCR5 in the myocardium of the immunized mice. The inventors were able to detect mRNA levels for RANTES, MIP-1β, MIP-1α, MIP-2, MCP-1, TCA-3, eotaxin only in mice immunized with the residues 105-122 of mcTnI (peptide 9; SEQ ID NO. 9) but not in mice immunized with the other peptides or control buffer and adjuvant alone (Figure 5A). In addition, the inventors found that the mRNAs for the chemokine receptors CCR1, CCR2 and CCR5 were solely expressed in the myocardium of mice immunized with the residues 105-122 of mcTnI (peptide 9, SEQ ID NO. 9) (Figure 5B).

- Immunization with the residues 105-122 (peptide 9) of murine cTnI induces production of antibodies reacting with the complete protein mcTnI

First the inventors tested whether immunization with the complete protein mcTnI also induces also antibodies to the residues 105-122 of mcTnI (peptide 9, SEQ ID NO. 9). On day 28, mice immunized with mcTnI developed not only antibody against the whole mcTnI protein but also against the residues 105-122 of mcTnI (peptide 9 SEQ ID NO. 9) (Figure 5C). Conversely,

immunization with the residues 105-122 of mcTnI (peptide 9 SEQ ID NO. 9) induced production of antibodies reacting with the complete protein mcTnI (Figure 5D).

- hcTnI and other proteins with similar amino acid sequences as the antigenic murine amino acid sequences 105-122 and 131-148

When comparing the antigenic residues 105-122 and 131-148 of murine cTnI to corresponding amino acid sequences of hcTnI or other proteins with similar amino acid sequences, we found that the residues 104-121 and residues 130-147 of human cTnI (hcTnI) were different in only 1 amino acid to the major (residues 105-122) and minor epitope (residues 131-148) of mcTnI, respectively (Figure 6). The next protein with amino acid sequences similar to both mcTnI epitopes (105-122 and 131-148) was skeletal troponin I (residues 73-90 and 99-116 in slow skeletal troponin and residues 98-115 in fast skeletal troponin). Residues 73-90 of slow skeletal troponin were already different in 6 amino acids to the main epitope of mcTnI (105-122; peptide 9 SEQ ID NO. 9) (Figure 6). Residues 98-115 of fast skeletal troponin I were different in 3 amino acids and residues 99-116 of slow skeletal troponin I were different in 4 amino acids to the minor epitope of mcTnI (131-148; peptide 11).

- Residues 104-121 of human cTnI but not residues 73-90, 98-115 or 99-116 of skeletal troponin I induce myocardial inflammation and fibrosis

In order to study whether hcTnI or skeletal troponin I, different peptides with the most similarity in amino acid sequence to both pathogenic epitopes of mcTnI (105-122 and 131-148), have a pathogenic effect on the myocardium, mice were immunized with either hcTnI (104-121, SEQ ID NO. 17), or slow skeletal troponin I (residues 73-90 or 99-116, SEQ ID ON. 18 or SEQ ID NO. 19), or fast skeletal troponin I (residues 98-115, SEQ ID NO. 20). Residues 105-122 (peptide 9, SEQ ID NO. 9) of mcTnI were used as a positive control (Figures 7A-G). All mice immunized with residues 105-122 of mcTnI (SEQ ID NO. 9) developed mild to severe inflammation and fibrosis (Figures 7A-E). Four of eight mice immunized with the residues 104-121 of hcTnI (SEQ ID NO. 17) showed mild inflammation and fibrosis (Figures 7A and 7F-G), some located perivascularly (Figure 7G). In contrast, none of the mice immunized with skeletal troponin I residues 73-90 (SEQ ID NO. 18) (Figure 7A), residues 98-115 (SEQ ID NO. 20), or residues 99-116 (SEQ ID NO. 19) (data not shown) showed signs of inflammation.

Here, the inventors showed for the first time that transfer of troponin I-specific T-cells led to severe inflammation and fibrosis in healthy recipient WT mice resulting in enlarged hearts, increased end-systolic and end-diastolic diameters, reduced fractional shortening, inflammation, fibrosis and heart failure. Furthermore, the inventors identified two amino acid sequences of murine troponin I that led to heart failure by inducing inflammation and fibrosis. Hereby, the residues 105-122 (peptide 9, SEQ ID NO. 9) of mcTnI were the strongest inductor of inflammation and fibrosis in the myocardium which is accompanied by increased expression of inflammatory chemokines RANTES, IP-10, MCP-1, MIP-1α, MIP-1β, MIP-2, TCA-3 and eotaxin, and of the chemokine receptors CCR1, CCR2, CCR5. McTnI residues 131-148 (peptide 11, SEQ ID NO. 11) were a minor epitope inducing milder inflammation. While the corresponding human cTnI residues 104-121 which were different in one amino acid from the murine cTnI residues 105-122 also induced milder inflammation in mice, none of the mice immunized with skeletal troponin I showed significant signs of inflammation. Furthermore, the inventors demonstrated that mice immunized with mcTnI also developed antibodies directed against the residues 105-122 of mcTnI (peptide 9, SEQ ID NO. 9). Conversely, immunization with the residues 105-122 of mcTnI (peptide 9, SEQ ID NO. 9) induced production of antibodies reacting with the complete protein mcTnI. Thus, the residues 105-122 of mcTnI (SEQ ID NO. 9) must be immunogenic in the complex structure of the whole mcTnI protein as well. This epitope becomes more interesting, since it forms a parallel helix (residues 90-135 of TnI) with the helix of TnT (residues 226-271) and it has been suggested that this coiled coil between TnI and TnT has important physiological roles that are characteristic of troponin (12).

It has been reported that autoantibodies against cTnI induced heart failure by chronic stimulation of Ca^{2+}-influx in cardiomyocytes (5, 6). Recently, the inventors reported that a humoral and cellular autoimmune response against mcTnI induced severe inflammation and fibrosis in the myocardium of mice with persistent, prominent inflammation and fibrosis over 270 days and reduced long-term survival (8). The inventors also demonstrated that mice pre-immunized with TnI prior to LAD ligation showed greater infarct size, more fibrosis, higher inflammation scores and reduced fractional shortening than mice without pre-immunization (8). These results indicate that an autoimmune response against troponin I aggravates the outcome of acute cardiac damage and may have a significant influence on post-infarct remodeling. Now the inventors demonstrate that the troponin induced heart failure is T-cell dependent. Furthermore, CD4+ T-cells are thereby necessary for successful transfer of

disease. As opposed to CD8+ T-cells which seem not to play a significant role. In addition, the inventors found significantly elevated levels of mc-TnI specific TNF-α, IL-1, IL-4, IL13, IL-17 production. This cytokine profile suggests that mcTnI induced myocarditis in A/J mice exhibits a Th2 (IL-4, IL13) and Th17 (IL-17) like phenotype. In the past, the inventors demonstrated that EAM in A/J mice induced by cardiac myosin has a Th2 phenotype (13). Others described an important role for Th1 subsets in EAM, so that the relative contributions of the CD4+ Th1 and Th2 subsets are still unclear (13-15). Recently, Rangachari et al. (16) demonstrated a significant role for another subset of CD4+ T-cells characterized by IL-17 production in EAM Balb/c mice induced by cardiac myosin peptide. It appears that the strain of mice used in the experiments is a crucial factor in deciding which subsets of CD4+ cells are involved in disease induction and progression.

Proinflammatory cytokines are important in the development of autoimmune myocarditis. The inventors previously showed that administration of either IL-1 or TNF-α promoted virus- and myosin-induced myocarditis in genetically resistant B10.A mice (17). Recently, the inventors demonstrated that the presence of myocarditis is associated with increased levels of TNF-α from cardiac myosin-stimulated splenocytes in culture (18). Furthermore, when A/J mice were infected with CB3 and treated with an IL-1 receptor antagonist, myocardial injury was diminished (19). Thus, IL-1 and TNF-α are clearly critical in the pathogenesis of autoimmune myocarditis.

The inventors have identified *two pathogenic epitopes of the troponin I molecule responsible for the induction of inflammation, fibrosis and heart failure*. All of the peptide sequences used to immunize mice led to an increase in total IgG autoantibody titers. Even though mice immunized with peptide sequences 7, 8 ,10 and 14 (SEQ ID NOs. 7, 8, 10, 14) showed higher total IgG antibody titers compared to the other peptides used in the experiments only mice immunized with peptide 9 and 11 (SEQ ID NO. 9 and 11) showed inflammation and fibrosis in the myocardium. The inventors conclude that in their model the initiation of the inflammatory process in the myocardium, followed by fibrosis and alteration of heart function is primarily T-cell dependent. This is supported by i) their findings that disease can be transferred to healthy WT mice with isolated T-cells alone; ii) the findings by Smith et al. (20), where Smith et al. demonstrated that myosin induced myocarditis is a T-cell-mediated disease; iii) the fact that Okazaki, et al. (6) and Nishimura, et al. (5) described no inflammation in the myocardium in mice treated with antibodies against troponin. These

authors demonstrated that antibodies to cTnI (humoral immune response only) induced heart dysfunction due to chronic stimulation of Ca2+ influx in cardiomyocytes by these antibodies. The inventors also recognize the possibility that once the inflammatory process is induced, antibodies may have additional effects in the broadening this ongoing inflammatory process that is followed by fibrosis and alteration of heart function.

When mice were immunized with the whole troponin protein (mcTnI) the inventors expect to induce an immune response (cellular and humoral) directed against different peptide sequences (epitopes) of this protein. By testing the sera of mice immunized with the whole troponin protein for presence of antibodies directed against the pathogenic peptide 9 (SEQ ID NO. 9) the inventors demonstrated that an immune response against peptide 9 can be induced not only by immunization with the synthesized peptide 9 alone but also when the whole troponin protein is used for immunization. This demonstrates that the peptide 9 sequences are immunodominant in the whole troponin protein. On the contrary, when mice were immunized with peptide 9 alone the inventors would expect initially an immune response directed only against the amino acid sequences of peptide 9. These antibodies are able to bind to the whole protein TnI as well. This major finding suggests that these amino acid sequences can be bound by antibodies even in the complex structure of the whole protein. These results document that the peptide 9 sequence is not cryptic (i.e. buried in the core of the whole troponin protein complex). That was confirmed with the actual 3-D structure of troponin I (data not shown). Furthermore, mice immunized with peptide 9 show at day 21 only antibodies binding itself (peptide 9) and the whole troponin protein TnI but do not have antibodies binding the other peptides used in our experiments.

The utility of the present peptide study indicated the use of peptide 9 (+11) sequences (SEQ ID NO. 9 and 11) instead of the whole troponin molecule in testing patient sera to identify patients at risk for myocardial damage induced by autoimmune inflammation. Thus the use of the peptide 9 (+11) sequences in tests would be more specific for identifying the risk of inflammation than using the whole troponin protein (TnI) since the inventors demonstrated that immunization with the other peptide sequences of troponin I did not induce an inflammation or fibrosis in the myocardium. The use of whole troponin protein will identify all antibodies directed against different sequences of troponin including those that might have no pathogenic effect.

Chemokines, such as RANTES, MCP-1, MIP1-α and their major receptors, CCR2 and CCR5, play important roles in the pathogenesis of many inflammatory diseases (21-23). MCP-1 mRNA expression has been shown in endomyocardial biopsy specimens from patients with dilated cardiomyopathy and suggested a significant role of this chemokine in the regulation of inflammatory cell infiltration into the myocardium (23). Recently, the inventors described an important role for MCP-1 and MIP1-α and their major receptors CCR2 and CCR5, in the initiation of autoimmune myocarditis (22). In the present experiments, only mice immunized with residues 105-122 of mcTnI (peptide 9) showed increased expression of these chemokines, which correlated with increased myocardial inflammation.

Eriksson et al. have shown that *troponin autoantibodies* are present in patients and can produce false negative results by blocking the binding of troponin antibodies used in analytical assays to the target protein (7). The report of the presence of autoantibodies against cTnI in patients with acute coronary syndrome (7) points to an early induction of an autoimmune response to cTnI in these patients. In addition to the analytical interference in the assays, troponin I autoantibodies may have clinical consequences. Okazaki et al. showed that troponin autoantibodies induced dilation and cardiac dysfunction by the chronic stimulation of calcium influx in cardiomyocytes (6). Recently, the inventors showed that provoking an autoimmune response to cardiac troponin I induces severe inflammation in the myocardium followed by fibrosis and heart failure with increased mortality in mice (8). These results suggest that some of the patients who develop an autoimmune response to released cTnI may have a higher risk of heart failure due to inflammation in the myocardium. These autoimmune responses may also explain the discrepancy observed in some patients with involvement of one or two coronary arteries while the entire heart is diffusely hypokinetic. Furthermore, the role of autoantibodies in heart failure has been supported by many clinical studies demonstrating that removing immunoglobulins by immunoadsorption can improve the ejection fraction and reduce the morbidity in patients with dilated cardiomyopathy (24). Now the inventors identified one major and one minor epitope of troponin I (peptide 9 and 11, respectively) that seem to be responsible for disease induction. Using these newly identified epitope sequences of troponin I instead of the whole troponin molecule; a more specific screening test of patients with high risk for progressive autoimmune inflammatory heart failure is possible.

Furthermore, the inventors demonstrated the important role of troponin-specific T-cells in inducing the autoimmune inflammation. These findings aid in developing new approaches to the early treatment of heart failure in some patients and in initiating further (clinical) studies to investigate the role of troponin I release, induction of an autoimmune response to released troponin I during acute cardiac damage, its role on post-infarct remodeling and heart failure.

Auto-antibodies against cardiac troponin I

The inventors furthermore showed that the absence of auto-antibodies against cardiac troponin I predicts improvement of left ventricular function after acute myocardial infarction. For further details, see Example 2.

Aims: Application of antibodies against cardiac troponin I (cTnI-Ab) can induce dilation and dysfunction of the heart in mice. Recently, the inventors demonstrated that immunization with cTnI induces inflammation and fibrosis in myocardium of mice. Others have shown that autoantibodies to cTnI are present in patients with acute coronary syndrome. But little is known about the clinical relevance of detected cTnI-Ab.

Methods and results: First, anti-cTnI and anti-cTnT antibody titers were measured in sera from 272 patients with dilated- (DCM) and 185 with ischemic- (ICM) cardiomyopathy. Secondly, 108 patients with acute myocardial infarction (AMI) were included for a follow-up study. Heart characteristics were determined by magnetic resonance imaging 4 days and 6-9 months after AMI. Altogether, in 7,0% of patients with DCM and in 9,2% with ICM an anti-cTnI IgG antibody titer ≥1:160 was measured. In contrast, only in 1,7% of patients with DCM and in 0,5% with ICM an anti-cTnT IgG antibody titer ≥1:160 was detected. Ten out of 108 patients included in the follow-up study were tested positive for cTnI-Ab with IgG Ab titers ≥1:160. TnI-Ab negative patients showed a significant increase in LVEF and stroke volume 6-9 months after AMI. In contrast, there was no significant increase in LVEF and stroke volume in TnI-Ab positive patients.

Conclusion: The inventors demonstrate for the first time that the prevalence of cTnI-Abs in patients with AMI has an impact on the improvement of the LVEF over a study period of 6-9 months.

These results, which are described in detail in Example 2, show findings concerning auto-antibodies against the whole cTnI protein. However, in combination with the findings of the inventors about peptide 9 and peptide 11 (being the antigenic determinants/epitopes and thus responsible for the effects of the whole protein) as disclosed herein and found in an animal model and described in detail in Example 1, it can be concluded that what is shown here and in Example 2 will also be induced by these peptides. Thus, the absence or presence of auto-antibodies against peptide 9 and 11 and variants thereof has an influence on the disease progression and can be used e.g. for risk stratification of the respective patient.

The following examples and drawings illustrate the present invention without, however, limiting the same thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. *Transfer of T-cells into WT mice.*

Mice were immunized with mcTnI or adjuvant alone. On day 21 T-cells were isolated from splenocytes and re-stimulated in vitro in the presence of dendritic cells and monocytes with 10µg/ml of mcTnI (mcTnI/mcTnI or no/mcTnI) for 48h or were not re-stimulated with mcTnI (mcTnI/no or CFA/no). Then 10^6-10^7 T-cells per mouse were transferred to WT mice irradiated with 600 rad. Effects on production of mcTnI specific autoantibodies (total IgG **(A)**, mean ± SEM) and cytokines **((B)**, mean ± SEM), on inflammation and fibrosis **(C)**, on left ventricular end-diastolic **((D)**, mean ± SEM) and end-systolic diameter **((E)**, mean ± SEM) on fractional shortening **((F)**, mean ± SEM), and on expression of cytokines and cytokine receptors in the myocardium **(G)**. Antibody endpoint titers for each individual mouse were calculated as the greatest positive dilution of antibody above normal mouse serum levels for day 21. *: $p<0,05$ (Kruskal-Wallis test and *U* test).

(H): Schematic illustration of the experimental design.

Figure 2:

(A) WT recipient mice irradiated with 600 rad were injected i.p. with either 10^6-10^7, 10^5-10^6 or 10^4-10^5 T-cells.

(B) WT recipient mice irradiated with 600 rad were injected i.p. with either 10^6-10^7 CD90+ T-cells, 10^6-10^7 CD4+ or with 10^6-10^7 CD8+ T-cells.

(Kruskal-Wallis test and *U* test).

Figure 3:

Sixteen overlapping 16-18 mer peptides covering the entire amino acid sequence of murine troponin I (211 residues) were synthesized followed by HPLC-purification (SEQ ID NO. 1-16).

Figure 4: *Histological examination of the hearts.*

Histological examination of the hearts on day 28 (stain with haematoxylin and eosin **(a-f)** or Masson's trichrome **(g-i)**). Representative heart section of the four mice immunized with the peptide 9 with the inflammation scores of 3 **(a)**, 2 **(b-c)**, and 1 **(d)**. The heart section of the one mouse immunized with peptide 11 and a histoscore of 1 **(e)**. Representative heart section of mice without significant inflammation **(f)**. Representative heart sections of mice immunized with the peptide 9 and fibrosis scores of 1 **(g)**, 3 **(h)**, and 2 **(i)**. Fibrosis was indicated by bright blue staining for collagen deposition. (**(a and b)**: 5x magnification; **(c-i)**: 20x magnification).

Figure 5: *RNA protection assay and antibody titers.*

mRNA expression levels of some tested chemokines **(A)** and mRNA expression levels of the chemokine receptors CCR1-5 **(B)** in the myocardium of mice immunized with the different peptides and mcTnI as a positive control on day 28. Antibody titers directed against the whole mcTnI protein and against the peptide 9 were measured in mice immunized with either mcTnI (**(C)**, 10,000±500 and 1,110±285) or peptide 9 (**(D)**, 4,320±1,098 and 4,676±1,143). Antibody endpoint titers for each individual mouse were calculated as the greatest positive dilution of antibody above normal mouse serum levels for day 21.

Figure 6:

Corresponding proteins with similar sequences to the residues 105-122 and 131-148 of mcTnI.

Figure 7:

(A) Groups of mice were immunized with either mcTnI (residues 105-122), or hcTnI (residues 104-121), or skeletal troponin (residues 73-90). Effects on severity of inflammation and fibrosis on day 28 (Kruskal-Wallis test and *U* test).

(B-G) Histological examination of the hearts (stain with haematoxylin and eosin). Representative heart section of mice immunized with mcTnI (105-121) (peptide 9) with the inflammation scores of 3,5 **(B and D)** and 2,5 **(C and E)**. Representative heart section of mice immunized with hcTnI (104-121) and inflammation score of 1 **(F)** and with perivascular inflammation **(G)** on day 28. (**B and C**: 10x magnification; **D-G**: 20x magnification).

Figure 8:

(A) Five overlapping 10 mer peptides covering the entire amino acid sequence of peptide 9 (18 residues) [SEQ ID NOs. 22-24, 26, 27] were synthesized followed by HPLC-purification. The purity of the peptides was >90%.

(B-D) Histological examination of the hearts on day 28 (stained with haematoxylin and eosin). Representative heart section of mice immunized with the peptide 9.3 (inflammation scores of 2) **(C)** and with the peptide 9.4 (inflammation score of 1) **(D)**. (20x magnification)

EXAMPLES

Example 1: Identification of Cardiac Troponin I Sequence Motifs Leading to Heart Failure by Inducing Myocardial Inflammation and Fibrosis

Methods

Mice

Female A/J mice (5-6 weeks of age) obtained from Harlan Winkelmann GmbH (33176 Borchen, Germany), female SCID mice (5-6 weeks of age) obtained from Charles River (Sulzfeld, Germany) were maintained in the animal facility at the University of Heidelberg and used in all experiments. The animal work was approved by the Animal Care and Use Committee of the University of Heidelberg.

Preparation of recombinant murine cardiac troponin I

The murine cardiac troponin subunit mcTnI was expressed in *E.coli* and purified as previously described[9]. In addition to purification via ion exchange chromatography, mcTnI was applied to a cardiac troponin C affinity column as second purification step (10). Isolated mcTnI-fractions were dialysed extensively against 1 mM HCl, then lyophilised and stored at – 80 °C.

Cell sorting

CD90+, CD8+ and CD4+ T-cells were enriched to 90% purity from the spleen by magnetically activated cell sorting using anti-CD90, anti-CD8, anti-CD4- conjugated microbeads (Miltenyi-Biotec, Auburn, CA).

Transfer of T-cells

For the transfer experiments four groups of mice treated differently were used. Two groups of mice were first immunized with mcTnI on days 0 and 7. On day 21 purified T-cells from one group of mice were re-stimulated in vitro in the presence of dendritic cells and monocytes with 10µg/ml of mcTnI for 48h whereas T-cells from the second group were not re-stimulated with mcTnI. Additionally, two other groups of mice were immunized first with adjuvant alone on days 0 and 7. On day 21 purified T-cells from one group of mice were re-stimulated in vitro in the presence of dendritic cells and monocytes with 10µg/ml of mcTnI for 48 h whereas T-cells from the second group were not re-stimulated with mcTnI. Then 10^6-10^7 of stimulated T-cells were injected intraperitoneally (i.p.) to WT recipient mice irradiated with 600 rad or to non irradiated SCID mice. In order to survey the effect of the number of T-cells transferred, three additional groups of WT recipient mice irradiated with 600 rad were injected i.p. with either 10^6-10^7, 10^5-10^6 or 10^4-10^5 T-cells. Finally CD4+ and CD8+ subsets were isolated from the spleens of immunized mice and were re-stimulated in vitro in the presence of 10µg/ml of mcTnI for 48 h whereas CD8+ T-cells were re-stimulated in the presence of additional 50 IU/ml IL-2 (R&D Systems, 65205 Wiesbaden-Nordenstadt, Germany).

Determination of autoantibody titers

Antibody titers were essentially determined as described before (11). In brief, to measure serum anti–peptide or troponin I titers, plates were coated either with 100µl/well of each peptide or cardiac troponin I (5µg/ml) in bicarbonate buffer (pH 9.6) and incubated overnight. Anti-mouse secondary antibody diluted to 1:5000 for IgG (Sigma) was used for detection. Serum samples from test mice were diluted to 1:100, 1:500, 1:2500, and 1:12500. Normal mouse serum was used as control. Optical densities were determined at 450nm. Antibody endpoint titers for each individual mouse were calculated as the greatest positive dilution of antibody yielding a positive signal.

Cardiac-troponin I dependent cytokine production by splenocytes

For *in vitro* cytokine production, the splenocytes were cultured at $5x10^6$ per well in RPMI 1640 complete medium in the presence of 10µg/ml of either cTnI or medium alone for 48 h. Supernatant was collected, aliquoted and frozen at -20 °C. Cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, IFN-γ, and TNF-α) were measured by DuoSet ELISA Development Systems (R&D Systems, 65205 Wiesbaden-Nordenstadt, Germany), according to the manufacturer's instructions.

Histopathological evaluation

For the histopathological evaluation of myocardium, mice were sacrificed on day 21 after transfer of T-cells and on day 28 after immunization with peptides respectively. Sections of 5µm thickness were cut at various depths in the myocardial tissue section and stained with haematoxylin and eosin to determine the level of inflammation and with Masson's Trichrome to detect collagen deposition. Evidence of myocarditis and fibrosis was evaluated in a blinded manner by two independent investigators who used light microscopy, according to a scoring system: grade 0, no inflammation; grade 1, cardiac infiltration in up to 5% of the cardiac sections; grade 2, 6-10%; grade 3, 11–30%; grade 4, 31-50%; and grade 5, >50%.

RNA protection assay

The mCR-5 cytokine receptor multi-probe template set (BD Biosciences Pharmingen, Heidelberg, Germany) was used to measure mouse mRNAs encoding CCR1, CCR2, CCR1b, CCR3, CCR4, and CCR5. The mCK-5c multi-probe template set (BD Biosciences Pharmingen, Heidelberg, Germany) was used to measure mouse mRNAs encoding Ltn, RANTES, MIP-1b, MIP-1a, MIP-2, IP-10, MCP-1, TCA-3, and eotaxin. The measurement was done according to the manufacturer's guidelines.

Echocardiography

Transthoracic echocardiography was performed as previously described (8). The investigator who conducted the echocardiography was unaware of the treatment status.

Synthesis of peptides

The following peptides were synthesized and purified by HPLC (Peptide Specialty Laboratories, Heidelberg, Germany):

- sixteen overlapping 16-18mer peptides covering the entire amino acid sequence of mcTnI (211 residues) (see Figure 3) [SEQ ID NOs. 1-16]

MADESSDAAGEPQPAPAP [SEQ ID NO. 1],
PAPAPVRRRSSANYRAYA [SEQ ID NO. 2],
YRAYATEPHAKKKSKISA [SEQ ID NO. 3],
SKISASRKLQLKTLMLQI [SEQ ID NO. 4],
LMLQIAKQEMEREAEERR [SEQ ID NO. 5],
AEERRGEKGRVLRTRCQP [SEQ ID NO. 6],
TRCQPLELDGLGFEELQD [SEQ ID NO. 7],
EELQDLCRQLHARVDKVD [SEQ ID NO. 8],
VDKVDEERYDVEAKVTKN [SEQ ID NO. 9],
KVTKNITEIADLTQKIYD [SEQ ID NO. 10],
QKIYDLRGKFKRPTLRRV [SEQ ID NO. 11],
TLRRVRISADAMMQALLG [SEQ ID NO. 12],
QALLGTRAKESLDLRAHL [SEQ ID NO. 13],
LRAHLKQVKKEDIEKENR [SEQ ID NO. 14],
EKENREVGDWRKNIDALS [SEQ ID NO. 15],
IDALSGMEGRKKKFEG [SEQ ID NO. 16],

- the corresponding human cTnI residues 104-121 (see also Figure 6)

VDKVDEERYDIEAKVTKN [SEQ ID NO. 17],

- the residues 73-90 of slow skeletal troponin I (see also Figure 6)

VEVVDEERYDIEAKCLHN [SEQ ID NO. 18],

- the residues and 99-116 of slow skeletal troponin I (see also Figure 6)

LKVLDLRGKFKRPPLRRV [SEQ ID NO. 19], and

- the residues 98-115 of fast skeletal troponin I (see also Figure 6)

QKLFDLRGKFKRPPLRRV [SEQ ID NO. 20].

The purity of the peptides was >90%.

Antigen preparation and administration

Each mouse was injected subcutaneously with an emulsion of 120µg of one of the peptides, or troponin I as positive control, or control buffer and adjuvant as negative control. The peptide/protein was supplemented with CFA containing 5 mg/ml of *Mycobacterium tuberculosis H37Ra* (Sigma, St. Louis, MO, USA) on days 0, 7 and 14. The mice were

sacrificed on day 28 for histopathological evaluation, RNA protection assay analysis, and measurement of cytokines and antibody titers.

Statistical analysis

Results are expressed as mean ± SEM. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney *U* test to explore the significance between the treatment groups. P values of <0.05 were considered significant. The statistical software SPSS (ver 15.0) was used for all calculations.

Results

Transfer of T-cells

Mice receiving T-cells from mcTnI immunized mice that have been re-stimulated *in vitro* with mcTnI showed high mcTnI-specific total IgG antibody titers (Figure 1A), severe inflammation (histoscore: 1.8±0.4) and fibrosis (histoscore: 1.9±0.4) in myocardium (Figure 1C), and increased mcTnI-induced production of the cytokines IL-1β , IL-4, IL-13, IL-17, and TNF-α (Figure 1B) compared to mice receiving T-cells from mcTnI immunized mice that have not been re-stimulated *in vitro* with mcTnI or receiving T-cells from mice given adjuvant alone irrespective of their in vitro re-stimulation. There was no significant difference in the production of the other cytokines (IL-2, IL-6, and IFN-γ). Transfer of T-cells from mcTnI immunized mice that have been re-stimulated *in vitro* with mcTnI led to enlarged hearts with significantly increased left ventricular end-diastolic (3.6±0.2mm) (Figure 1D) and end-systolic diameter (2.3±0.2mm) (Figure 1E). The fractional shortening was significantly reduced (35.6±3.1%) (Figure 1F). In contrast, mice receiving T-cells from mice given adjuvant alone irrespective their *in vitro* re-stimulation did not show any signs of inflammation, fibrosis, or alteration of heart function. One of six mice receiving T-cells from mcTnI immunized mice without *in vitro* re-stimulation showed slight inflammation and fibrosis but no significant alteration of heart function.

To examine the expression of different chemokines and chemokine receptors, the inventors measured the mRNA expression levels of the chemokines Ltn, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3 and eotaxin as well as the levels of the chemokine receptors CCR1, CCR2, CCR1b, CCR3, CCR4 and CCR5 in the myocardium of the recipient mice. The inventors were able to detect mRNA levels for RANTES, MIP-1β, MIP-1α, MIP-2, MCP-1, TCA-3 and eotaxin only in mice receiving mcTnI-specific T-cells unlike the

otherwise treated mice (Figure 1G). In addition, the inventors found that the mRNAs for CCR1, CCR2 and CCR5 were expressed only in the myocardium of mice receiving mcTnI-specific T-cells but not in the other groups of mice (Figure 1G).

Further on, in order to study the effect of the number of T-cells transferred, three additional groups of WT recipient mice irradiated with 600 rad were injected i.p. with either 10^6-10^7, 10^5-10^6 or 10^4-10^5 T-cells. When the inventors reduced the number of T-cells transferred the severity of disease decreased. Transfer of 10^4-10^5 or lower number of T-cells disclosed no significant inflammation in the recipient mice (Figure 2A). On the other hand, transfer of 10^6-10^7 T-cells in non-irradiated SCID mice induced in 10 out of 11 recipients an inflammation in the myocardium. Overall, the histoscores in SCID mice were lower compared to irradiated WT recipient mice (histoscore: 0.8±0.1, data not shown).

In order to study the effect of T-cell subsets, CD4+ and CD8+ T-cells were isolated from the spleens of immunized mice and were re-stimulated *in vitro* with mcTnI or in the case of CD8+ T-cells were re-stimulated with mcTnI and supplemental IL-2. Transfer of whole T-cells (CD90+; histoscore: 2.1±0.5) and transfer of CD4+ subset of T-cells (histoscore: 2.4±0.4) induced moderate to severe disease, whereas transfer of CD8+ subset of T-cells alone did not induce any signs of inflammation in the recipient mice (Figure 2B).

3-D structure of troponin I

The peptide 9 sequences (SEQ ID NO. 9) was localized in an actual 3-D structure of troponin I in association to troponin T (with kind permission of 'Nature Publishing Group', data not shown) (12).

Example 2: Absence of Auto-Antibodies against Cardiac Troponin I Predicts Improvement of Left Ventricular Function after Acute Myocardial Infarction

Aims: Application of antibodies against cardiac troponin I (cTnI-Ab) can induce dilation and dysfunction of the heart in mice. Recently, the inventors demonstrated that immunization with cTnI induces inflammation and fibrosis in myocardium of mice. Others have shown that autoantibodies to cTnI are present in patients with acute coronary syndrome. But little is known about the clinical relevance of detected cTnI-Ab.

Methods and results: First, anti-cTnI and anti-cTnT antibody titers were measured in sera from 272 patients with dilated- (DCM) and 185 with ischemic- (ICM) cardiomyopathy. Secondly, 108 patients with acute myocardial infarction (AMI) were included for a follow-up study. Heart characteristics were determined by magnetic resonance imaging 4 days and 6-9 months after AMI. Altogether, in 7,0% of patients with DCM and in 9,2% with ICM an anti-cTnI IgG antibody titer ≥1:160 was measured. In contrast, only in 1,7% of patients with DCM and in 0,5% with ICM an anti-cTnT IgG antibody titer ≥1:160 was detected. Ten out of 108 patients included in the follow-up study were tested positive for cTnI-Ab with IgG Ab titers ≥1:160. TnI-Ab negative patients showed a significant increase in LVEF and stroke volume 6-9 months after AMI. In contrast, there was no significant increase in LVEF and stroke volume in TnI-Ab positive patients.

Conclusion: The inventors demonstrate for the first time that the prevalence of cTnI-Abs in patients with AMI has an impact on the improvement of the LVEF over a study period of 6-9 months.

Methods

Study population

Patients with cardiomyopathies

To get a general impression of antibodies against troponin I and T in patients with cardiomyopathies, the inventors performed a retrospective analysis measuring antibody-titers in sera from a total of 457 patients, among which 272 had a dilated cardiomyopathy, while 185 had an ischemic cardiomyopathy (ICM). Inclusion criteria for ICM-patients were previously documented MI and coronary artery disease with a LVEF less than 40 %. For DCM-patients inclusion criteria were exclusion of coronary artery disease and an LVEF under 40 %.[2] Blood was collected in our cardiac catheter laboratory. All patients gave informed consent.

Patients with acute myocardial infarction and follow-up

Another 108 patients with acute myocardial infarction were separately considered in our observational follow-up study. Their inclusion criteria were as follows: all patients presented with acute myocardial infarction to the chest pain unit of the University Hospital of Heidelberg, Germany. Patients were included from October 2005 until August 2006. Through

a 12-lead ECG and measuring cardiac troponin T (cTnT) in serum, they were either identified as ST-elevation myocardial infarction (STEMI) or as non-ST-elevation myocardial infarction (NSTEMI). All patients underwent percutaneus transluminal coronary angiography and were treated according to the ACC/AHA guidelines. Heart characteristics were determined by MRI on day 2-4 after MI. After 6-9 months MRI was repeated. Furthermore, laboratory parameters were evaluated. Patients with autoimmune diseases, active infections, cancer or heart failure of other known origins than MI were excluded. All patients received stable oral medication according to the ACC/AHA guidelines from 2004 (25) (acetylsalicylic acid, clopidogrel, renin-angiotensin-aldosterone-system-blocker, beta blocker, statin). The local ethic commissions approved the study protocol and written informed consent was obtained from all patients.

Healthy subjects and athletes with general trauma to the skeletal muscular system

Sera from ten healthy male athletes (median (25th/75th percentile)) aged 52 (43/57) years, height 177 (170/182) cm, weight 75 (71/78) kg and body mass index 24 (23/25) kg/m^2 who participated in the Badwater ultra-marathon 2004 were analysed and screened for the presence of antibodies against troponin I and T. Runners were initially included for a cardiospecifity study of the 3rd generation cardiac troponin T assay (26). Ethical approval was obtained from the ethics committee, Münster, Germany. Written informed consent from each athlete prior to study participation was obtained. The Badwater marathon is a continuous 216 km (134 miles) race, which takes place in July under extreme heat in Death Valley, California, USA. The start line is at Badwater, Death Valley, which marks the lowest elevation in the Western hemisphere at 282 feet (85,5 m) below sea level. The race finishes at the Mt. Whitney Portals at 8,360 feet (2,533 m).

Each of the ten experienced athletes had previously completed 43,4 marathon and 20,4 ultra-marathon races. No athlete had a history of cardiovascular disease, hypertension or any significant comorbidity. None of the subjects was taking cardiovascular drugs. One athlete suffers from type I diabetes since childhood with continuously low HbA1c values < 6%. Stationary laboratories fully equipped, ensuring proper preanalytical conditions like specimen centrifugation and specimens freezing at –80 C (dry-ice) were installed at Stovepipe Wells (Lab1) and Lonepine (Lab2). Blood was collected at the start (E0), after the first half-marathon distance (E1), after each full marathon distance (E2–E6), and at the end (E7). Blood samples (whole blood, EDTA-blood) were kept on crushed ice in the supporters' car until the

shuttle service took over the specimens transferring them to the stationary laboratories for preanalytical preparation. One EDTA-blood sample was transferred to a laboratory in Las Vegas on a daily basis on crushed ice for blood-count testing. The samples were transferred by WorldCourier® that documented an uninterrupted cold chain to the Laboratory in Heidelberg, Germany. No sample showed any sign of haemolysis.

During the run, concentrations of CK, CK-MBact and CK-MBmass significantly increased, while cTnT-concentration remained below detectable levels in all runners (26).

Enzyme-linked immunoabsorbent assay (ELISA)

Peripheral venous blood samples were collected from all patients. 96-well plates were coated with anti-cTnI or anti-cTnT diluted in coating buffer (0,1M $NaHCO_3$/34mM Na_2CO_3, pH 9,5) and then incubated over night at 4°C. All washing steps were performed with 1xPBS/0.05% Tween 20 three times each. 1% Gelatine (Cold Water Fish, Sigma)/1xPBS was used for blocking. After 2h incubation at 37°C half of the plate was coated with human cTnI or cTnT for another 2h at room temperature (RT) while the other half served as control, thus, only coated with 1xPBS/1%BSA/0.1%Tween 20. The dilution series of the serum samples were as follows: 1:40, 1:80, 1:160, 1:320. For 1h30min the plates were incubated at RT. HRP anti-human IgG or anti-human IgM (diluted 1:7500 with 1xPBS/1%BSA/0.1% Tween 20, 1h incubation at RT) was used as detection-antibody. Blue Star HRP-Substrate from Diarect was applied for 45 min at RT. The reaction was stopped with 0.3M H_2SO_4. Finally, the absorbance was measured at 450 nm. We used a hybrid antibody construct (Fc-fragment=human IgG + Fab-fragment=mouse antihuman cTnI or cTnT; provided by Roche Diagnostics, Mannheim, Germany) as a positive control. Sera from healthy persons, mouse serum and buffer alone served as negative controls. To calculate the cTnI titers the optical densities on both halves of the plate of each sample and dilution was subtracted. Total IgG antibody titers were measured in all patients. Additionally, we measured total IgM antibody titers in the follow-up study patients. Total antibody endpoint titers for each sample were calculated as the highest positive dilution of antibody.

Cardiac magnetic resonance imaging (MRI)

MRI examinations were performed with a 1,5 Tesla whole-body MRI system (Philips Medical Systems, 1,5 Tesla). Functional and morphological cardiac assessment was adequately executed in all 108 MI-patients. Duration of MRI scan was approximately 30 to 60 minutes.

They were examined for several parameters such as the left ventricular ejection fraction (LVEF), the end-systolic volume (ESV), the end-diastolic volume (EDV) and the stroke volume (SV). Infarct size was calculated as previously described (27). All data were provided by independent examiners, on hospital admission and 6-9 months after MI.

Statistical Analysis

For the DCM/ICM study population, the McNemar test was used to evaluate the relationship between autoantibodies against cTnI and cTnT, separately for each group.

As for the patients undergoing MRI investigations, they were compared with respect to their initial demographic data including cardiovascular risk factors and oral medication. The two-sided Mann-Whitney U test was adopted to compare the continuous parameters, while the two-sided Chi-square test was applied for the categorical data. The main criterion for the MRI examination was the LVEF. For this parameter a confirmatory comparison controlling the α-error was carried out to compare the difference of the MRI data on day 3-4 and 6-9 months later between the titer positive and negative group. All other MRI data were exploratory analyzed and the resulting p-values cannot be interpreted in a confirmatory sense. To compare the difference in the MRI parameters, an univariate analysis of covariance was applied considering the value on admission as covariate in order to account for the slight imbalance in the distribution of the parameters between the groups in the baseline MRI. Due to the small sample size of the titer positive group, we dispensed with a multivariable analysis of covariance including further parameter. A p-value < 0.05 was considered significant though only the analysis of the LVEF can be interpreted in a confirmatory sense. All calculations were carried out with SPSS, version 15.0. Analysis was done blinded.

Results

cTnI- and cTnT- autoantibodies in DCM and ICM patients

Out of 457 patients, 27,9% DCM-patients (n = 272) and 28% ICM-patients (n = 185) showed a positive total IgG antibody titer against cTnI $\geq$ 1:40. A total IgG titer $\geq$ 1:80 was present in 14,3% of the DCM-patients and in 17% of the ICM-patients and a titer $\geq$ 1:160 was present in 7,0% of the DCM-patients and in 9,2% of the ICM-patients. In contrast, only 5,2% of DCM-patients and 2% of ICM-patients had cTnT total IgG antibodies with a titer $\geq$ 1:40, 1,8% of DCM-patients and 1% of ICM-patients had a titer $\geq$ 1:80, and 1,7% of DCM-patients and 0,5% of ICM-patients had a titer $\geq$ 1:160 (Table 2). Further subdivision according to gender presented the following pattern: Overall, 28,5% of the anti-cTnI antibody titer $\geq$ 1:40 were

male, 26,3% were female (no significance (NS)). The age distribution revealed a positive titer ≥ 1:40 in 35,1% of the patients over 65 years and 35,4% of those younger than 65 (NS, data not shown).

Table 2: *cTnI- and cTnT-autoantibodies in DCM and ICM patients*

	cTnI-Ab ≥	cTnT-Ab ≥		cTnI-Ab ≥	cTnT-Ab ≥		cTnI-Ab ≥	cTnT-Ab ≥	
DCM n=27	27,9	5,2	<0,0	14,3	1,8	<0,0		1,7	<0,0
ICM n=18	27,6	1,6	<0,0	17,3	0,5	<0,0	9,2%	0,5	<0,0

Baseline characteristics of patients with acute myocardial infarction and follow-up

All 108 MI-patients (mean age 60,1 ± 11,3 years, 82% males) were classified into 2 groups regarding their antibody titers: 98 patients (mean age 59,9 ± 11,6 years, 82% males) were found negative for cTnI antibodies, while in 10 patients (mean age 62,9 ± 8,0 years, 80% males) total IgG antibody titers ≥ 1:160 were detected. Their heart rates (mean HR 72,1 ± 13,2/min) were considered as well as their blood pressures at hospital presentation (mean RR 131,2/80,1 ± 19,0/14,2 mmHg). The infarct size measured by MRI was 17,3 g ± 15,3 in average. Cardiac Troponin T and NTproBNP levels 72-96 hours after the acute event were comparable for both groups (1,7 ± 1,4 ng/ml vs. 1,5 ± 0,8 ng/ml; p=0,96 and 1076,2 ± 989,0 vs. 2515,6 ± 4515,3). There were no significant differences in baseline characteristics between patients with positive and negative cTnI titers (Table 3). Two patients were not treated with clopidogrel due to severe bleeding complications.

Table 3: *Baseline data of patients, risk factors and medication at discharge of both cTnI-Ab negative and positive patients*

	All Patients (n = 108)		Patients TnI-Ab neg (n = 98)		Patients TnT-Ab pos (n = 10)		p
	Mean	SD	Mean	SD	Mean	SD	
Data of patients							
Age [years]	60.1	11.3	59.9	11.6	62.9	8.0	0.50
Male [%]	82 (88)		82 (80)		80 (8)		0.86
STEMI [%]	60 (65)		61 (60)		50 (5)		0.30
Heart rate [min^{-1}]	72.1	13.2	72.5	13.4	68.2	10.1	0.38
cTnT* [µg/l]	1.7	1.4	1.7	1.4	1.5	0.8	0.96
NTproBNP*[ng/l]	1196.2	1590.5	1076.2	989.0	2515.6	4515.3	0.61
Infarct size [g]	17.3	15.3	17.4	15.8	16.6	8.4	0.60
Cardiovascular risk factors							
BMI	27.1	3.6	27.0	3.7	27.6	2.6	0.46
RR syst [mmHg]	131.2	19.0	131.5	19.1	128.6	18.8	0.41
RR diast [mmHg]	80.1	14.2	80.1	14.5	79.2	11.4	0.71
Total cholesterol [mg/dl]	190.2	39.3	190.2	39.9	190.4	41.3	0.98
LDL [mg/dl]	116.6	34.0	116.5	33.4	117.5	40.5	0.89
HbA1c [%]	5.9	0.9	5.9	0.9	6.0	0.9	0.54
Nicotine [%]	56 (60)		56 (55)		50 (5)		0.56
Family history [%]	44 (48)		43 (42)		60 (6)		0.09
Medication at discharge							
Diuretics[%]	26 (28)		28 (27)		10 (1)		0.01
ASS [%]	100 (108)		100 (98)		100 (10)		0.89
Clopidogrel [%]	97 (105)		99 (97)		80 (8)		0.20
Beta-Blocker [%]	98 (106)		98 (96)		100 (10)		0.78
ACEI/AT1-bl. [%]	97 (105)		99 (97)		80 (8)		0.20
Statin [%]	100 (108)		100 (98)		100 (10)		0.89

SD: standard deviation;
ASA: Acetylsalicylic acid; ACEI/AT1-bl.: ACE-inhibitor/AT1-blocker
*: 72-96h post-MI
( ): absolute counts

Presence of cardiac troponin antibodies and LVEF

For assessment of development of cardiac function, each patient underwent examination through MRI on day 4 after admission and 6-9 months thereafter. Blood samples from all patients were analyzed on both time-points. Patients that were cTnI-Ab-positive (titer ≥ 1:160) at the time of acute MI remained positive, 3 of them showed even higher optical densities. Only one patient had total IgM antibodies against cTnI with a titer ≥ 1:160 and one patient had total IgG antibodies against cTnT with a titer ≥ 1:160.

Patients with a negative total IgG antibody titer against cTnI revealed an increase in LVEF from 54,6 ± 9,5 % to 59,7 ± 9,0 % (Table 4) in contrast to the group of patients with total IgG

antibody titers ≥ 1:160 against cTnI with no comparable improvement in LVEF (from 57,4 ± 10,9 % to 56,0 ± 11,6 %, p = 0,031) (Table 4).

Table 4: *MRI-data on admission (3-4 days post-MI) and on follow-up (6-9 months post-MI)*

	MRI data on day 3-4		MRI data 6-9 months after MI	
	Mean	**SD**	**Mean**	**SD**
All Patients (n = 108)				
LVEF [%]	54.9	9,6	59.4	9.2
EDV [ml]	173.4	39.9	176.8	42.6
ESV [ml]	79.7	28.4	75.0	31.2
SV [ml]	93.6	20.0	101.9	22.4
Patients TnI.Ab neg. (n = 98)				
LVEF [%]	54.6	9.6	59.7	9.2
EDV [ml]	173.7	39.2	177.3	43.4
ESV [ml]	80.2	28.2	74.8	31.3
SV [ml]	93.5	20.1	102.5	22.8
Patients TnI.Ab pos. (n = 10)				
LVEF [%]	57.4	10.9	56.0	11.6
EDV [ml]	170.5	46.8	172.2	34.2
ESV [ml]	75.3	31.9	76.5	31.8
SV [ml]	95.1	20.3	95.7	17.4

SD: standard deviation;
MRI: magnetic resonance imaging; MI: myocardial infarction

Measurement of antibody titers in healthy subjects and athletes with general trauma to the skeletal muscular system

In none of the ten athletes running the ultra marathon antibodies against troponin I or T with a titer ≥ 1:160 were detectable. One runner developed exercise-induced rhabdomyolysis with spontaneous recovery with a peak CK of 27,951 U/L.

Discussion

After a myocardial infarction cardiac troponins are released into the systemic circulation. As their localization is thought to be strictly intracellular, the immune system of the MI patient is then being exposed to previously unknown antigens, whereupon the immune system is triggered to produce antibodies against cardiac troponins. Earlier studies have indeed described anti-cTnI autoantibodies in patients, while to our knowledge no studies exist investigating the presence of antibodies against cTnT in patients. Furthermore, the clinical

impact of existing anti-troponin antibodies has never been explored so far (28). To answer these questions, the inventors first screened over 400 serum samples collected from DCM and ICM patients for the presence of cTnI- and cTnT- autoantibodies. The results provided evidence for the presence of anti-cTnI as well as for anti-cTnT antibodies, even though of much lesser extent. The characterization of their titer pattern revealed no differences regarding gender or age (cut-off 65 years) of the patients.

Recently, the inventors showed that induction of an immune response to cTnI leads to the development of severe myocardial inflammation in mice, in contrast to cTnT, which induces little or no effect in myocardium (8). Okazaki et al. demonstrated with PD1-deficient mice that autoantibodies against cTnI induce the development of cardiac dysfunction and dilatation by stimulating the Ca^{2+} influx in cardiomyocytes (6). Therefore, the inventors further investigated the clinical relevance of cTnI autoantibodies in humans with ischemic heart disease. The inventors followed up MI-patients regarding the presence of cTnI antibody titers and their heart function on admission as well as 6-9 months later. Out of 108 patients 98 were considered as titer negative (total IgG antibody titer < 1:160) whereas 10 had a titer ≥ 1:160. Both groups showed comparable demographic data and cardiovascular risk factor constellation. Patients with antibodies against troponin I remained positive on the follow-up visit, three of them even showing higher optical densities. In contrast, none of the initially negative patients developed auto-antibodies against troponin during the follow-up-phase. This could be due to the shortness of our observation-time and a general susceptibility towards an autoimmune-reaction. The inventors believe that those patients presenting with a positive titer at the time of the acute infarction had experienced "silent" release of troponins before the actual AMI and thus had developed the antibodies therefore prior to the event. This hypothesis is supported by a report from Zethelius et al, who detected cTnI in men free from coronary heart disease, which was shown to predict death and first CAD event (29). Our data collected from MRI revealed a significant improvement in LVEF through time to the anti-cTnI positive group ($p = 0{,}031$). These antibodies as of a titer ≥ 1:160 seem to have a restraining effect on the recovery of the heart after myocardial injury, as the patients with a lower titer or no titer showed amelioration in heart function after 6-9 months.

One might assume that smaller lesions in skeletal muscle can prompt an immunization or cross-reactivity. We therefore analyzed blood samples from athletes running a 216 km ultra-endurance marathon. These well-trained athletes all had great experience and a history of marathons run before the time-point of the analysis. It is therefore very likely that their

immune system had already been confronted with the release of cardiac proteins (and skeletal troponins) before. However, none of them showed elevated titers for antibodies against cardiac troponin I or T. Also, the documented rhabdomyolysis in one of the runners did not induce cTnI- or cTnT antibodies.

These findings may open new opportunities for therapeutic approaches. Comparable to previous studies on patients with DCM in which cardiac hemodynamics improved after immunoadsorption, the removal of anti-cTnI antibodies in patients with a titer $\geq$ 1:160 may enhance their outcome as well (30, 31). Likewise, the autoantibodies represent possible therapeutic targets to be withdrawn from circulation, with the intention to prevent further inhibiting effects on the repairing process of the myocardium. In contrast, earlier trials applying immunosuppressants such as glucocorticoids and biologicals in patients after MI failed with dissatisfying results (32-34). Despite multiple attempts to characterize the antibodies responsible for the cardiodepressive effect in DCM-patients, no definite actor could be isolated so far (35).

The present follow-up study discloses several limitations. Our study is limited by its relatively small number of anti-cTnI positive patients. The inventors used a cut-off of 1:160 for the anti-cTnI antibody (total IgG) titers and classified the patients accordingly as cTnI-antibody negative or positive. Therefore only 10 persons were found to be anti-cTnI antibody positive. This group of patients showed no improvement of their heart function, while lower titers don't seem to have any significant clinical relevance. Further research is required to clarify the role of the humoral immune system in the post-MI phase, as data on the potential effect of cTnT autoantibodies or IgM subclasses on the myocardial function are lacking. Moreover, studies with larger number of patients with a longer follow-up are needed for more conclusive data towards the patient's prognosis in relation to their antibody titers.

In conclusion, the present study for the first time provides evidence of anti-cTnT antibodies in a small subgroup of patients with dilated and ischemic cardiomyopathy. Furthermore, our findings suggest that autoantibodies against cTnI in patients with myocardial infarction may play a role in the ventricular remodeling process of the myocardium after infarction.

The features disclosed in the foregoing description, in the claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

REFERENCES

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Claims

1. A peptide derived from cardiac troponin I (cTnI) comprising an amino acid sequence of SEQ ID NO. 9 or SEQ ID NO. 11
or variants thereof,
wherein variants are selected from
(a) N-terminally and/or C-terminally truncated or elongated peptides comprising at least 7 contiguous amino acids of SEQ ID NO. 9 or SEQ ID NO. 11;
(b) amino acid substitution or deletion variants, preferably at one amino acid position;
(c) variants comprising modified amino acid(s), unnatural amino acid(s) or peptidomimetic(s);
(d) peptides with at least 90% sequence identity to SEQ ID NO. 9 or SEQ ID NO. 11.

2. The peptide according to claim 1 having the amino acid sequence of SEQ ID NO. 9.

3. The peptide according to claim 1 or 2, wherein the peptide comprises 10 contiguous amino acids of SEQ ID NO. 9 or SEQ ID NO. 11, preferably selected from SEQ ID NOs. 24 to 26,
or wherein the peptide comprises both SEQ ID NO. 9 and SEQ ID NO. 11, such as SEQ ID NO. 21.

4. The peptide according to any of claims 1 to 3, having a length of 7 to 100 amino acids, preferably 10 to 50 amino acids.

5. The peptide according to any of claims 1 to 4, comprising N-terminal and/or C-terminal modifications, preferably
- hydrophobic modification, such as
  acylation (e.g. with carboxylic acids, fatty acids, C8 to C22 fatty acids, amino acids with lipophilic side chains);

addition of hydrophobic moieties (e.g. with cholesterol, derivatives of cholesterol, phospholipids, glycolipids, glycerol esters, steroids, ceramids, isoprene derivatives, adamantane, farnesol, aliphatic groups, polyaromatic compounds);

- modification with a moiety that protects from degradation (e.g. amide, D-amino acid, modified amino acid, cyclic amino acid; natural and synthetic polymer, such as PEG, glycane)
- modification with a moiety that boosts the immune response (e.g. bovine serum albumin, keyhole limpet hemocyanin).

6. The peptide according to any of the foregoing claims, wherein the peptide comprises label(s), such as fluorescent dye(s), radioisotope(s) and contrast agent(s), or drug(s).

7. An antibody or antibody fragment specific for a peptide as defined in any of claims 1 to 6.

8. An antibody or antibody fragment specific for cardial troponin I (cTnI), which does not bind to a region or an epitope of cTnI which comprises or corresponds to a peptide as defined in any of claims 1 to 6.

9. The peptide according to any of claims 1 to 6 or the antibody or antibody fragment according to claim 7 or 8 for use in medicine.

10. The peptide according to any of claims 1 to 6 or the antibody or antibody fragment according to claim 7 or 8 for the prognosis and/or diagnosis of heart insufficiency.

11. The peptide, the antibody or the antibody fragment according to claim 10 as marker for the prognosis and/or diagnosis of heart insufficiency.

12. The peptide, the antibody or the antibody fragment according to claim 10 to 11, wherein the heart insufficiency is an inflammation of the myocardium and/or fibrosis, in particular autoimmune myocarditis.

13. The peptide, the antibody or the antibody fragment according to any of claims 10 to 12 for determining a patient group, in particular a patient risk group, with an inflammation of the myocardium and/or fibrosis.

14. The peptide, the antibody or the antibody fragment according to any of claims 10 to 13 for risk stratification in acute cardiac disorders of patients.

15. A method for the prognosis and/or diagnosis of heart insufficiency comprising

a) providing a patient sample, preferably a bodily fluid or tissue,

b) providing at least one peptide according to any of claims 1 to 6,

c) determining whether the patient has an immune reaction against said peptide(s), wherein said immune reaction is determined by determining the presence or absence of antibodies against said peptide(s) or determining the presence or absence of T cell response in said patient sample,

d) optional, determining the presence or absence of further markers in said patient sample.

16. The method according to claim 15, wherein the heart insufficiency is an inflammation of the myocardium and/or fibrosis, in particular autoimmune myocarditis.

17. The method according to claim 15 or 16, wherein the presence or absence of auto-antibodies against cardial troponin I (cTnI) and/or of auto-antibodies against a peptide according to any of claims 1 to 6 is determined in step c).

18. The method according to any of claims 15 to 17, comprising the use of an antibody or antibody fragment according to claim 7 or 8.

19. The method according to any of claims 15 to 18, further comprising determining cell proliferation and/or production of cytokine(s) and/or chemokine(s) and/or changes in expression of chemokine/cytokine receptors by cells of the immune system after stimulation with the peptide(s).

20. The method according to any of claims 15 to 19 for determining a patient group, in particular a patient risk group, with an inflammation of the myocardium and/or fibrosis.

21. The method according to any of claims 15 to 20 for risk stratification in acute cardiac disorders of patients.

Figure 1 A, B

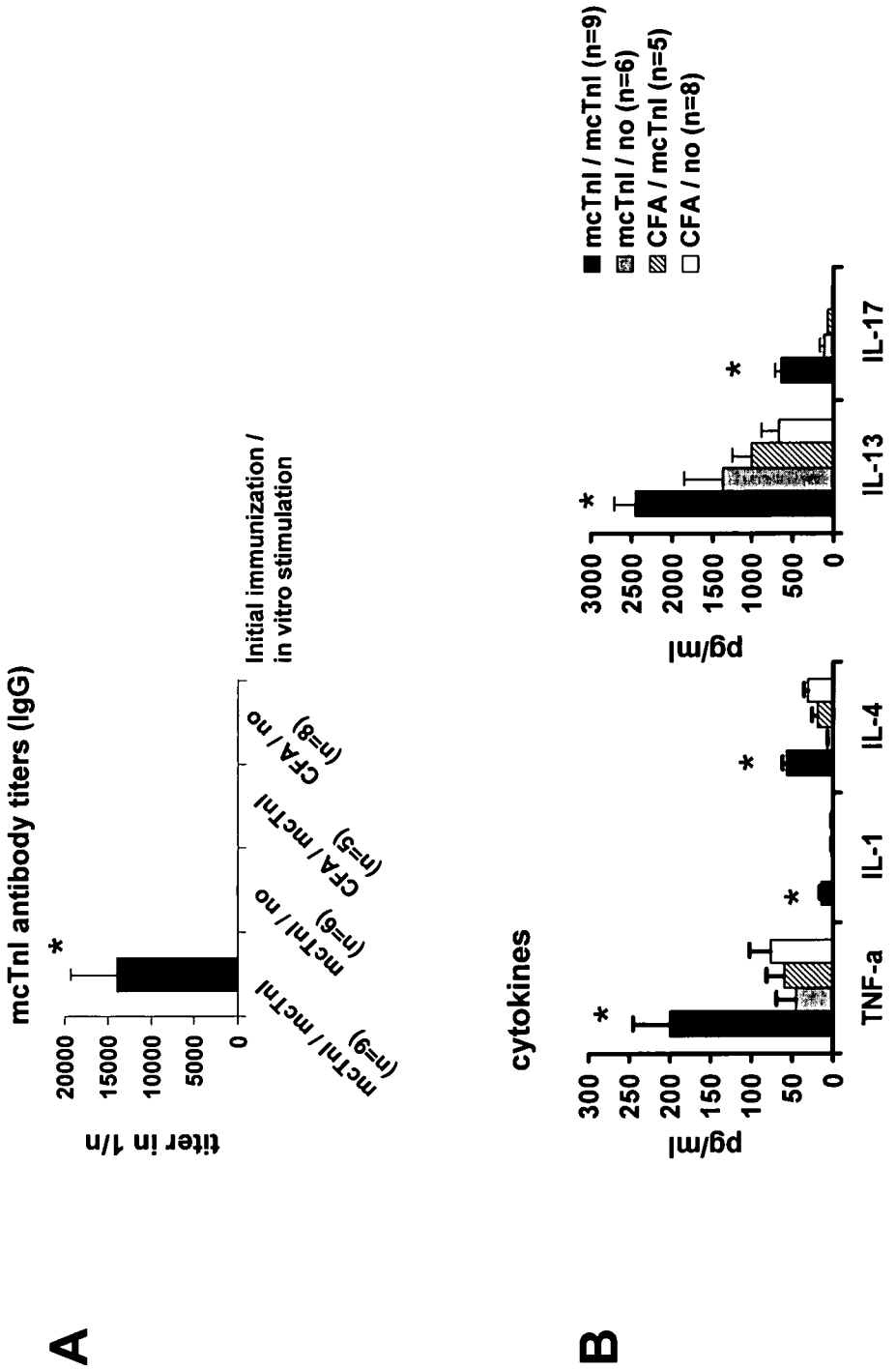

A
mcTnI antibody titers (IgG)
titer in 1/n
20000
15000
10000
5000
0
*
Initial immunization / in vitro stimulation
mcTnI / mcTnI (n=9)
mcTnI / no (n=6)
CFA / mcTnI (n=5)
CFA / no (n=8)
B
cytokines
pg/ml
300
250
200
150
100
50
0
TNF-a
IL-1
IL-4
3000
2500
2000
1500
1000
500
0
IL-13
IL-17
mcTnI / mcTnI (n=9)
mcTnI / no (n=6)
CFA / mcTnI (n=5)
CFA / no (n=8)

Figure 1 C

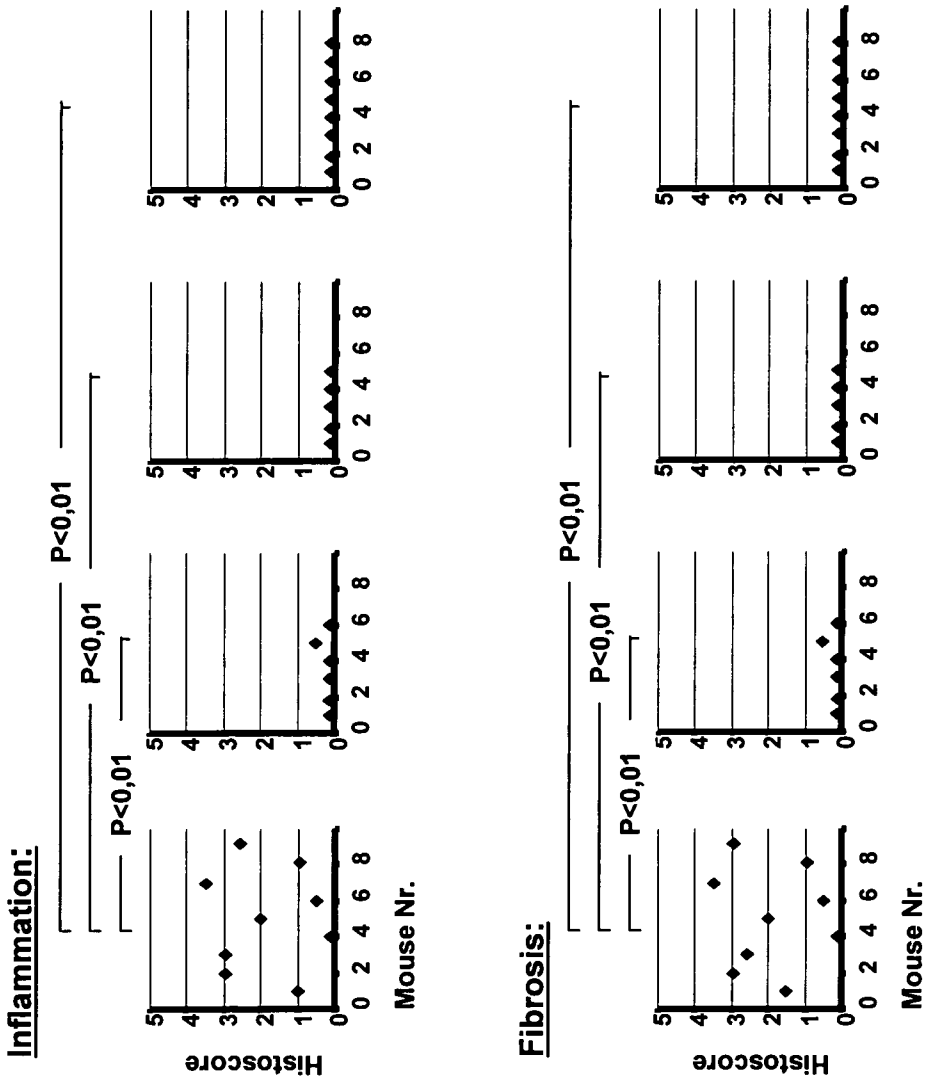
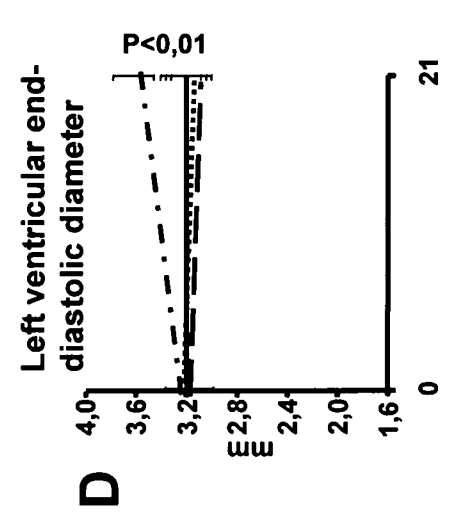
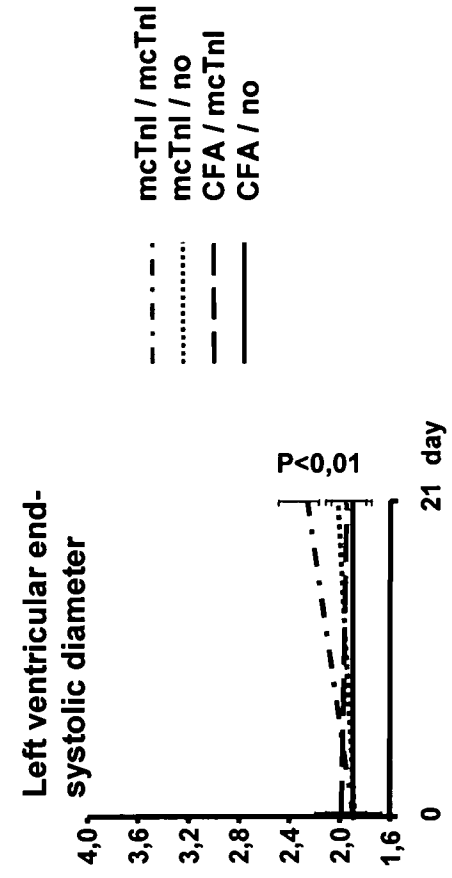
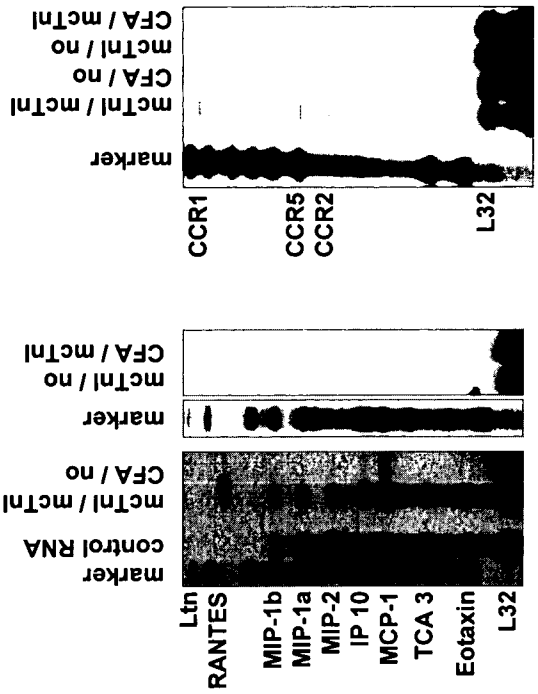

mcTnI / mcTnI
mcTnI / no
CFA / mcTnI
CFA / no
Inflammation:
P<0,01
P<0,01
P<0,01
Histoscore
Mouse Nr.
0 2 4 6 8
0 1 2 3 4 5
Fibrosis:
P<0,01
P<0,01
P<0,01

Figure 1 D - G


D
Left ventricular end-diastolic diameter
P<0,01
mm
4,0
3,6
3,2
2,8
2,4
2,0
1,6
0
21


E
Left ventricular end-systolic diameter
P<0,01
mm
4,0
3,6
3,2
2,8
2,4
2,0
1,6
0
21 day
mcTnI / mcTnI
mcTnI / no
CFA / mcTnI
CFA / no


G
marker
control RNA
mcTnI / mcTnI
CFA / no
marker
mcTnI / no
CFA / mcTnI
Ltn
RANTES
MIP-1b
MIP-1a
MIP-2
IP 10
MCP-1
TCA 3
Eotaxin
L32
CCR1
CCR5
CCR2
L32
marker
mcTnI / mcTnI
CFA / no
mcTnI / no
CFA / mcTnI

F
Fractional shortening
P<0,01
Percent
50
45
40
35
30
25
20
0
21 day

Figure 1 H

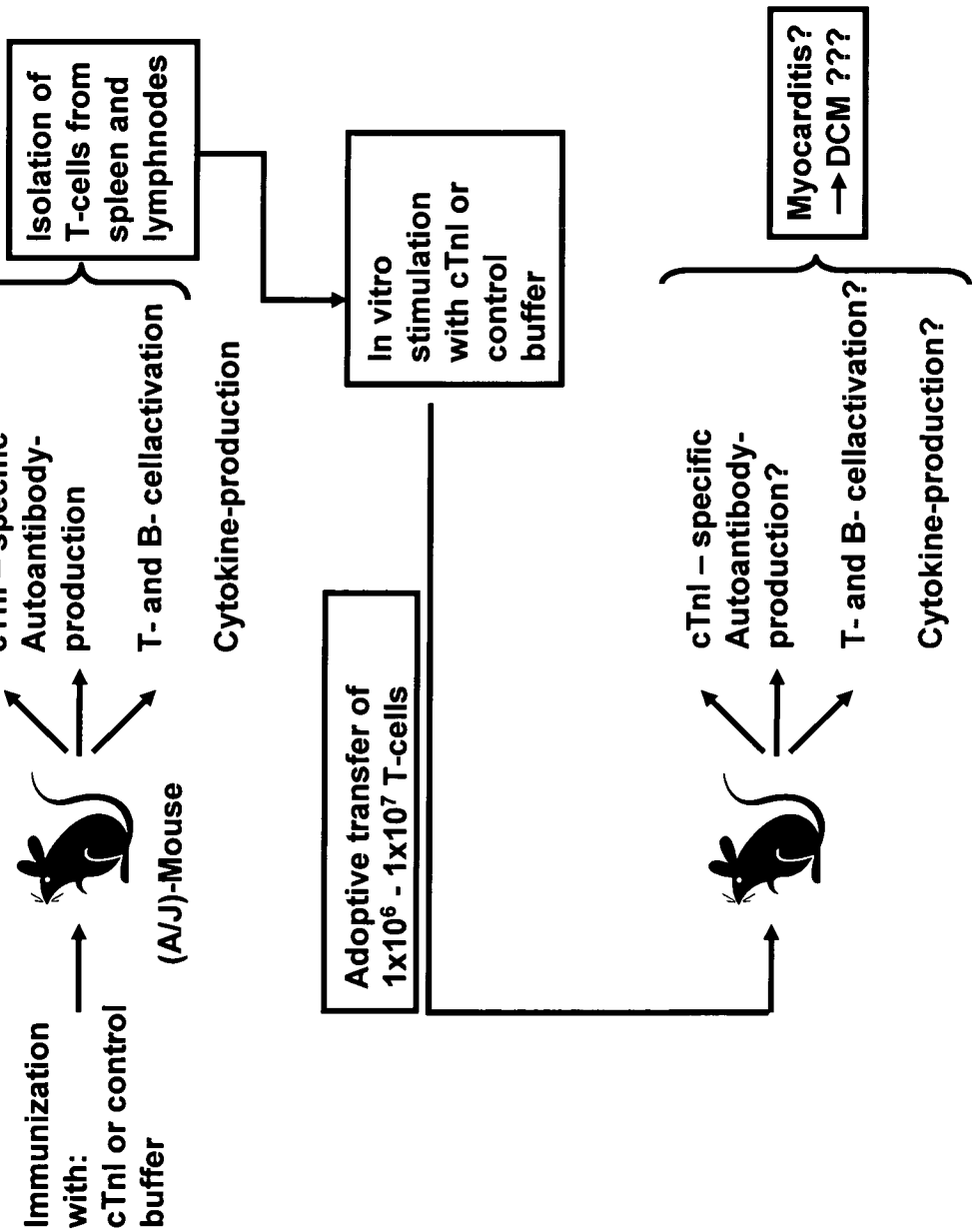

Immunization with: cTnI or control buffer
(A/J)-Mouse
cTnI – specific Autoantibody-production
T- and B- cellactivation
Cytokine-production
Isolation of T-cells from spleen and lymphnodes
In vitro stimulation with cTnI or control buffer
Adoptive transfer of 1x10⁶ - 1x10⁷ T-cells
cTnI – specific Autoantibody-production?
T- and B- cellactivation?
Cytokine-production?
Myocarditis? → DCM ???

Figure 2 A, B

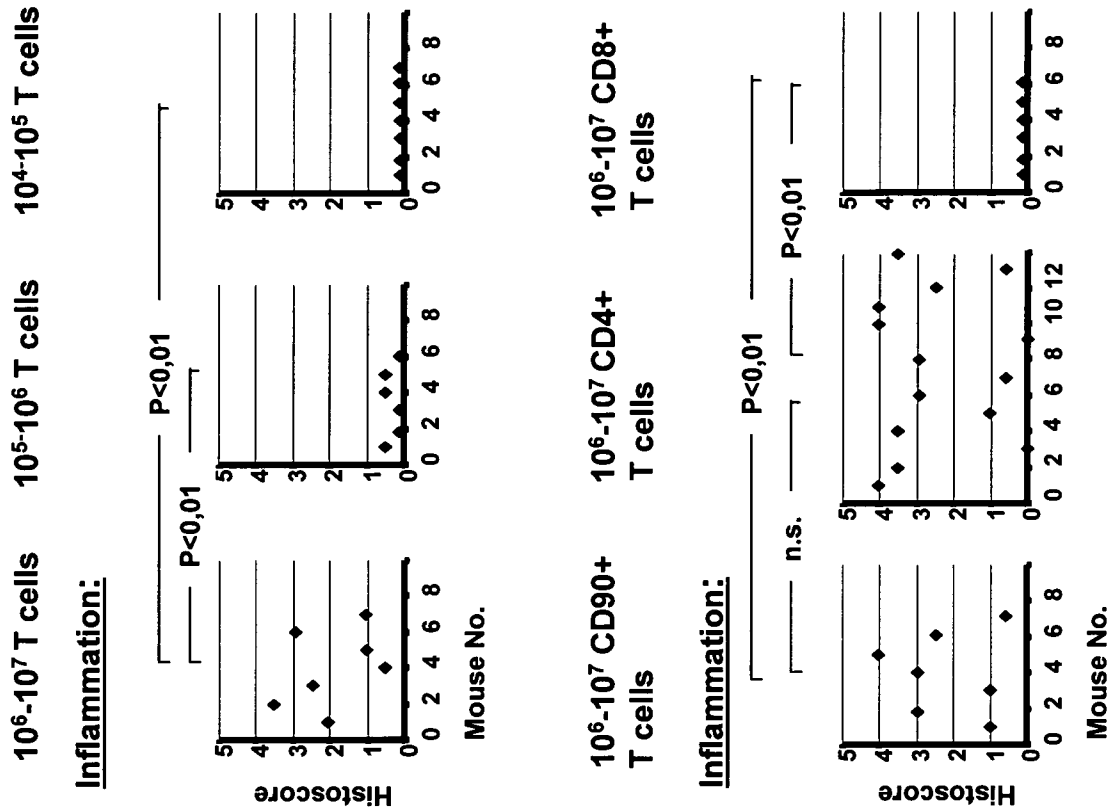

A
10^6-10^7 T cells
10^5-10^6 T cells
10^4-10^5 T cells
Inflammation:
P<0,01
P<0,01
Histoscore
Mouse No.
B
10^6-10^7 CD90+ T cells
10^6-10^7 CD4+ T cells
10^6-10^7 CD8+ T cells
Inflammation:
P<0,01
n.s.
P<0,01
Histoscore
Mouse No.

Figure 3

Peptide:						Incidence:	Severity:	SEQ ID NO.
1	MADES	SDAAG	EPQPA	PAP		0 (0/5)	0	1
2	PAPAP	VRRRS	SANYR	AYA		0 (0/5)	0	2
3	YRAYA	TEPHA	KKKSK	ISA		0 (0/5)	0	3
4	SKISA	SRKLQ	LKTLM	LQI		0 (0/5)	0	4
5	LMLQI	AKQEM	EREAE	ERR		0 (0/5)	0	5
6	AEERR	GEKGR	VLRTR	CQP		0 (0/5)	0	6
7	TRCQP	LELDG	LGFEE	LQD		0 (0/5)	0	7
8	EELQD	LCRQL	HARVD	KVD		0 (0/5)	0	8
9	VDKVD	EERYD	VEAKV	TKN		80% (4/5)	1,4 (3-1-0-2-1)	9
10	KVTKN	ITEIA	DLTQK	IYD		0 (0/5)	0	10
11	QKIYD	LRGKF	KRPTL	RRV		20% (1/5)	0,2 (1-0-0-0-0)	11
12	TLRRV	RISAD	AMMQA	LLG		0 (0/5)	0	12
13	QALLG	TRAKE	SLDLR	AHL		0 (0/5)	0	13
14	LRAHL	KQVKK	EDIEK	ENR		0 (0/5)	0	14
15	EKENR	EVGDW	RKNID	ALS		0 (0/5)	0	15
16	IDALS	GMEGR	KKKFE	G		0 (0/5)	0	16
17	positive control (murine TnI)					80% (4/5)	1,6 (3-2-0-1-2)	28
18	negative control (control buffer)					0 (0/4)	0	

Figure 4

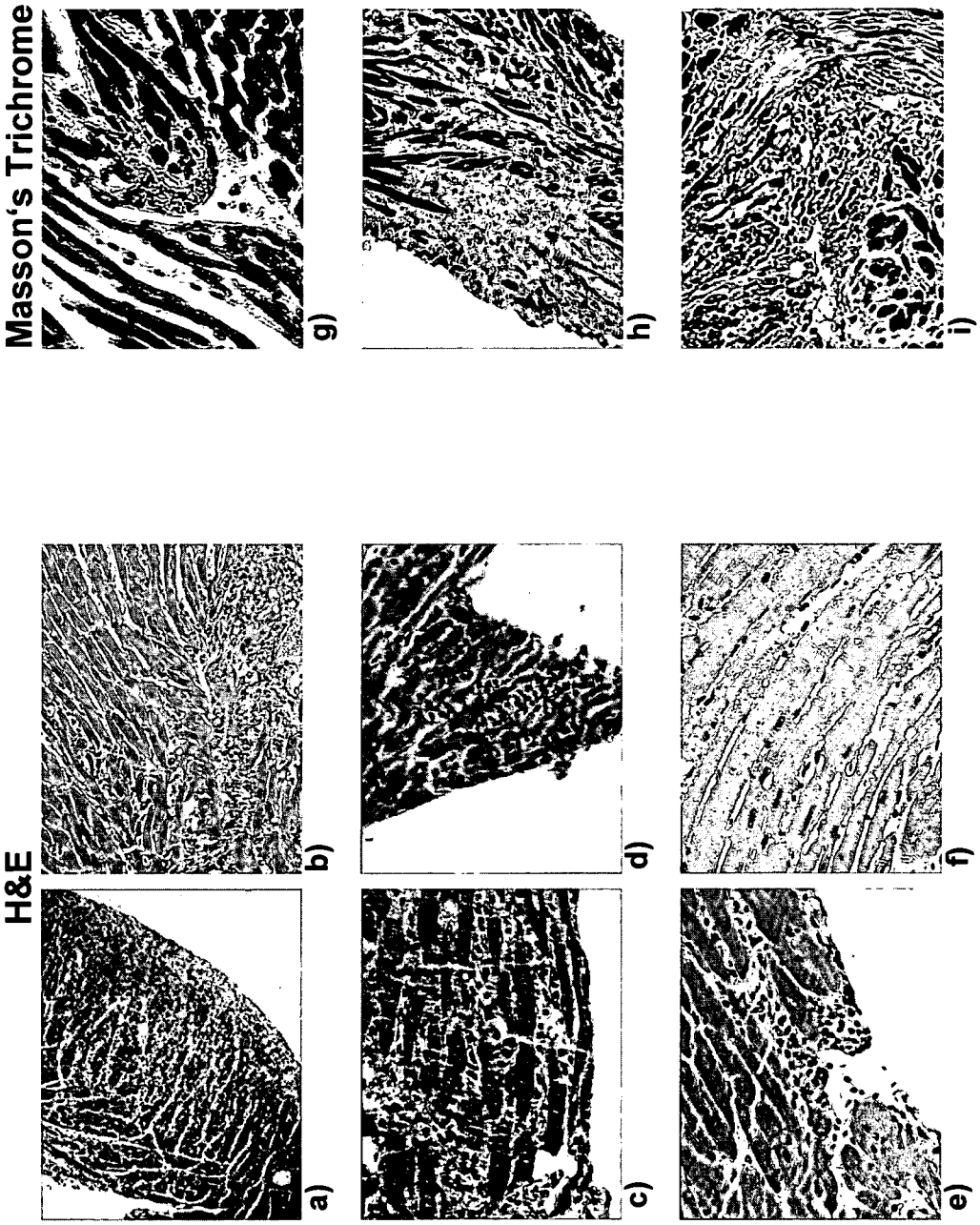


Figure 5

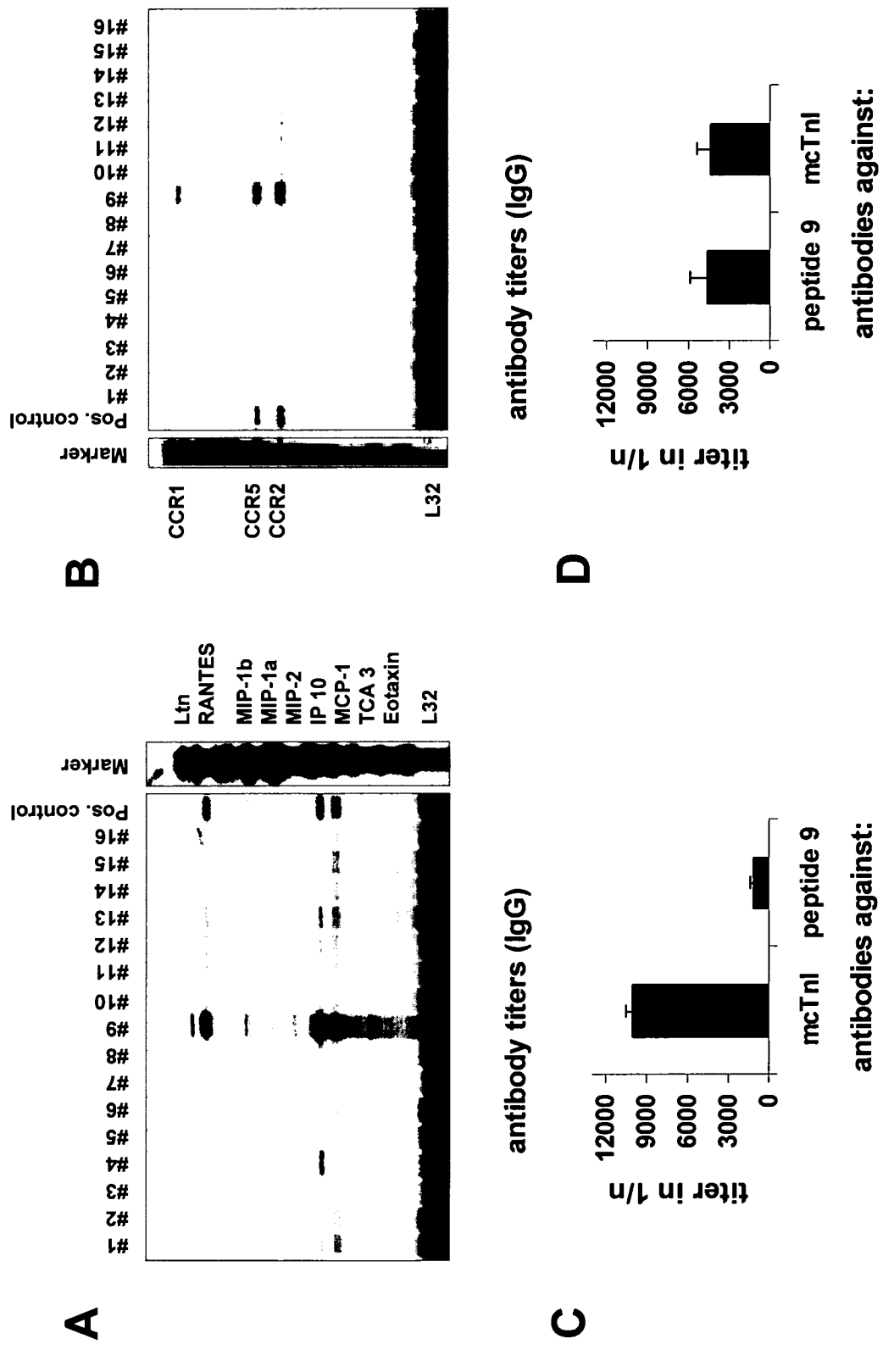

A
Ltn
RANTES
MIP-1b
MIP-1a
MIP-2
IP 10
MCP-1
TCA 3
Eotaxin
L32
Marker
Pos. control
#16
#15
#14
#13
#12
#11
#10
#9
#8
#7
#6
#5
#4
#3
#2
#1
B
CCR1
CCR5
CCR2
L32
Marker
Pos. control
C
antibody titers (IgG)
titer in 1/n
12000
9000
6000
3000
0
mcTnI
peptide 9
antibodies against:
D
antibody titers (IgG)
titer in 1/n
12000
9000
6000
3000
0
peptide 9
mcTnI
antibodies against:

Figure 6

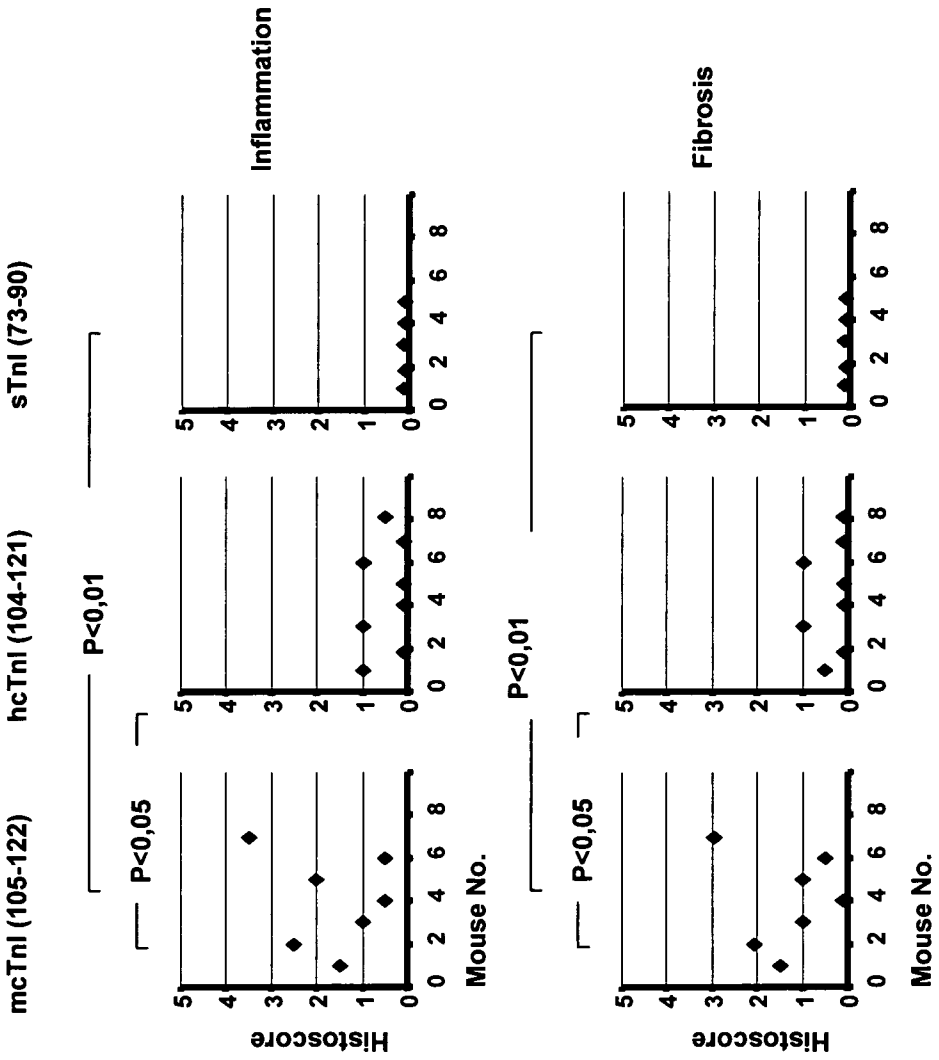
Peptide 9

105 VDKVDEERYDVEAKVTKN 122	**TnI, cardiac [Mouse]**	[SEQ ID NO. 9]
105 VDKVDEERYDVEAKVTKN 122	**TnI, cardiac [Rat]**	
104 VDKVDEERYDIEAKVTKN 121	**TnI, cardiac [Human]**	[SEQ ID NO. 17]
73 VEVVDEERYDIEAKCLHN 90	**TnI, skeletal, slow [Mouse]**	[SEQ ID NO. 18]
73 VEVVDEERYDIEAKCLHN 90	**TnI, skeletal, slow [Rat]**	
73 VEVVDEERYDIEAKCLHN 90	**TnI, skeletal, slow [Human]**	

Peptide 11

131 QKIYDLRGKFKRPTLRRV 148	**TnI, cardiac [Mouse]**	[SEQ ID NO. 11]
131 QKIYDLRGKFKRPTLRRV 148	**TnI, cardiac [Rat]**	
130 QKIFDLRGKFKRPTLRRV 147	**TnI, cardiac [Human]**	
98 QKLFDLRGKFKRPPLRRV 115	**TnI, skeletal, fast [Mouse]**	
98 QKLFDLRGKFKRPPLRRV 115	**TnI, skeletal, fast [Rat]**	
98 QKLFDLRGKFKRPPLRRV 115	**TnI, skeletal, fast [Human]**	[SEQ ID NO. 20]
99 LKVLDLRGKFKRPPLRRV 116	**TnI, skeletal, slow [Mouse]**	
99 LKVLDLRGKFKRPPLRRV 116	**TnI, skeletal, slow [Rat]**	
99 LKVMDLRGKFKRPPLRRV 116	**TnI, skeletal, slow [Human]**	[SEQ ID NO. 19]

Figure 7 A


mcTnI (105-122)
hcTnI (104-121)
sTnI (73-90)
P<0,05
P<0,01
P<0,01
P<0,05
Inflammation
Fibrosis
Histoscore
Mouse No.
0 1 2 3 4 5
0 2 4 6 8

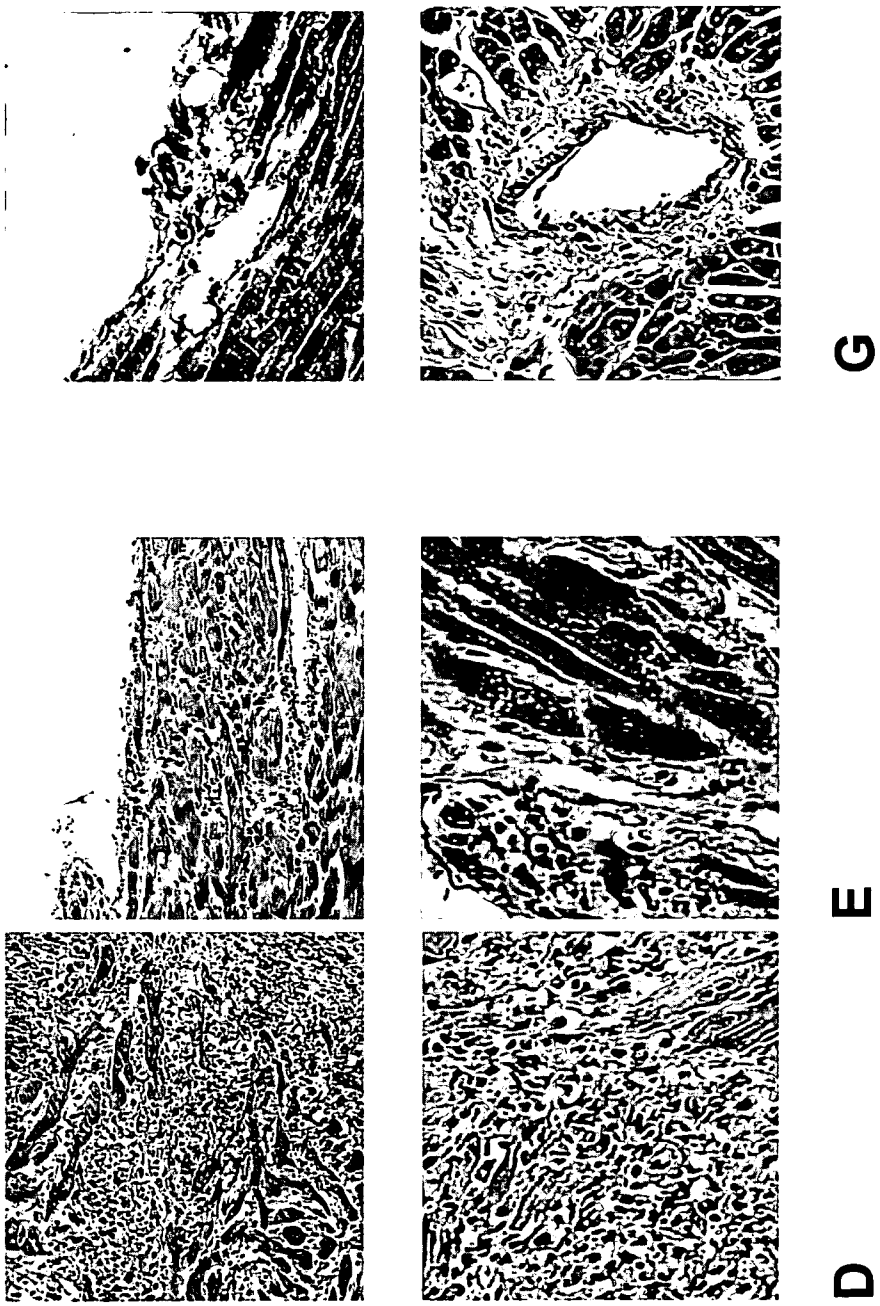


Figure 7 B - G

Figure 8 A

Peptide 9 TnI, cardiac [Mouse]

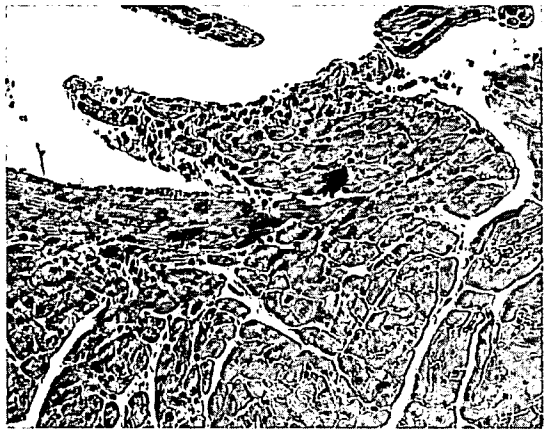
105	**VDKVDEERYDVEAKVTKN**	**122**	[SEQ ID NO. 9]
mcTnI9.1	**VDKVDEERYD**		[SEQ ID NO. 22]
mcTnI9.2	**KVDEERYDVE**		[SEQ ID NO. 23]
mcTnI9.3	**DEERYDVEAK**		[SEQ ID NO. 24]
mcTnI9.4	**ERYDVEAKVT**		[SEQ ID NO. 26]
mcTnI9.5	**YDVEAKVTKN**		[SEQ ID NO. 27]

Figure 8 B - D

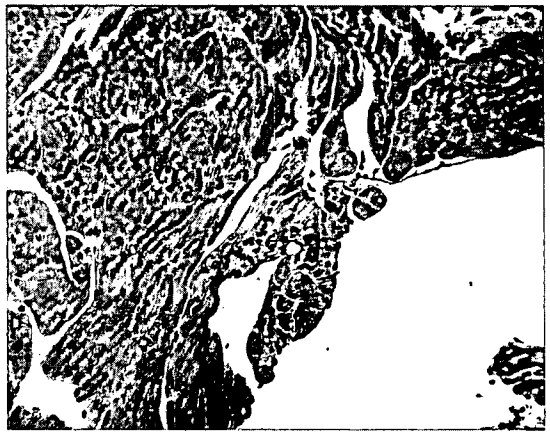
B

Peptide:				Incidence:	Severity:	SEQ ID NO.
mcTnI9.1	VDKVD	EERYD		0 (0/6)	0	22
mcTnI9.2	KVDEE	RYDVE		0 (0/6)	0	23
mcTnI9.3	DEERY	DVEAK		67% (4/6)	0,8 (1,5-1-0-0-1-1)	24
mcTnI9.4	ERYDV	EAKVT		33% (2/6)	0,4 (0-0-1,5-0-0-1)	26
mcTnI9.5	YDVEA	KVTKN		0 (0/6)	0	27

C Peptide mcTnI9.3:



D Peptide mcTnI9.4:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/002237

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/47

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, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GÖSER STEFAN ET AL: "Cardiac troponin I but not cardiac troponin T induces severe autoimmune inflammation in the myocardium." CIRCULATION, vol. 114, no. 16, 17 October 2006 (2006-10-17), pages 1693-1702, XP002528562 ISSN: 1524-4539 cited in the application the whole document	1-3,5-21
X	US 2007/172888 A1 (HALLERMAYER KLAUS [DE] ET AL) 26 July 2007 (2007-07-26) paragraphs [0008] - [0010], [0028], [0102] - [0104]	1-3,5-21

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[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

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"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
25 May 2009	02/07/2009

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	Spindler, Mark-Peter

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	SHMILOVICH ET AL: "Autoantibodies to cardiac troponin I in patients with idiopathic dilated and ischemic cardiomyopathy" INTERNATIONAL JOURNAL OF CARDIOLOGY, vol. 117, no. 2, 24 March 2007 (2007-03-24), pages 198-203, XP022000067 ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL ISSN: 0167-5273 cited in the application the whole document	1-3,5-21
X	FILATOV V L ET AL: "EPITOPE MAPPING OF ANTI-TROPONIN I MONOCLONAL ANTIBODIES" BIOCHEMISTRY AND MOLECULAR BIOLOGY INTERNATIONAL, vol. 45, no. 6, 1 September 1998 (1998-09-01), pages 1179-1187, XP000941112 ACADEMIC PRESS, LONDON, GB ISSN: 1039-9712 page 1181, paragraph 2; figures 1,3; table 1	1-11,14, 15
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	BABUIN LUCIANO ET AL: "Troponin: the biomarker of choice for the detection of cardiac injury." CMAJ : CANADIAN MEDICAL ASSOCIATION JOURNAL = JOURNAL DE L'ASSOCIATION MEDICALE CANADIENNE, vol. 173, no. 10, 8 November 2005 (2005-11-08), pages 1191-1202, XP002528565 ISSN: 1488-2329 the whole document	1-3,5-14
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X	BODOR G S ET AL: "Development of monoclonal antibodies for an assay of cardiac troponin-I and preliminary results in suspected cases of myocardial infarction." CLINICAL CHEMISTRY, vol. 38, no. 11, November 1992 (1992-11), pages 2203-2214, XP002528564 ISSN: 0009-9147 tables 1,5-8	1-3, 5-11,14
X	ABBOTT M B ET AL: "Cardiac troponin I inhibitory peptide: location of interaction sites on troponin C" FEBS LETTERS, vol. 469, no. 2-3, 10 March 2000 (2000-03-10), pages 168-172, XP004261070 ELSEVIER, AMSTERDAM, NL ISSN: 0014-5793 paragraph [02.1]	1,3-9

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AUSONI S ET AL: "Structure and regulation of the mouse cardiac troponin I gene." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 1, 7 January 1994 (1994-01-07), pages 339-346, XP002529073 ISSN: 0021-9258 figure 4	1-3,5-8
P,X	WO 2008/051761 A2 (ABBOTT LAB [US]; MATTINGLY PHILLIP G [US]; ADAMCZYK MACIEJ [US]; BRASH) 2 May 2008 (2008-05-02) paragraphs [0104] - [0107], [0125] - [0129], [0173] - [0175]; figure 16; table 15; sequences 34-37,43-46	1-21
P,X	KAYA ZIYA ET AL: "Identification of cardiac troponin I sequence motifs leading to heart failure by induction of myocardial inflammation and fibrosis." CIRCULATION, vol. 118, no. 20, 11 November 2008 (2008-11-11), pages 2063-2072, XP002528568 ISSN: 1524-4539 the whole document	1-21
P,X	LEUSCHNER FLORIAN ET AL: "Absence of auto-antibodies against cardiac troponin I predicts improvement of left ventricular function after acute myocardial infarction." EUROPEAN HEART JOURNAL, vol. 29, no. 16, August 2008 (2008-08), pages 1949-1955, XP002528567 ISSN: 1522-9645 the whole document	1-3,5-21
A	LI ET AL: "Genetic complexity of autoimmune myocarditis" AUTOIMMUNITY REVIEWS, vol. 7, no. 3, 3 December 2007 (2007-12-03), pages 168-173, XP022413394 ELSEVIER, AMSTERDAM, NL ISSN: 1568-9972 the whole document	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/002237

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US 2007172888 A1	26-07-2007	NONE	
CN 1982337 A	20-06-2007	NONE	
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