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(54) Title: USE OF TLR3 AGONISTS FOR THE TREATMENT OF NEURODEGENERATIVE DISORDERS

(57) Abstract: The present invention relates to a method for the treatment and/or prophylaxis of a degenerative inflammatory process in a tissue of a subject, said method comprising increasing the activity of Toll-like receptor 3 (TLR3) in cells of said tissue. In a preferred embodiment, the TLR3 agonist is stathmin or stathmin-like proteins such as SCGIO, SCLIP or RB3.

USE OF TLR3 AGONISTS FOR THE TREATMENT OF NEURODEGENERATIVE DISORDERS

FIELD OF THE INVENTION

The invention relates to methods for the treatment and/or prophylaxis of degenerative inflammatory processes in a subject and to pharmaceutical preparations for use therein. The invention in a preferred aspect relates to methods for treating

5   degenerative inflammatory disorders such as neurodegenerative disorders and stimulating neuroprotective processes. The invention further relates to the use of TLR3-agonists for the preparation of a medicament for the prevention and/or treatment of an acute or chronic degenerative inflammatory process in a subject, particularly in brain or joint tissue of said subject. In a particularly preferred aspect,

10   the invention relates to the use of stathmins for that purpose.

BACKGROUND OF THE INVENTION

Human neurodegenerative disorders such as stroke, Alzheimer's disease and Parkinson's Disease pose an ever increasing threat to an aging population. Effective

15   methods of treatment are lacking as is a clear understanding of the molecular mechanisms involved. Drugs currently used to treat neurological disease and injuries provide at the most a temporary relief of symptoms but do not stop or slow the underlying neurodegenerative process. Drugs that are believed to ameliorate the disease by intervening in the molecular cascades that lead to accelerated cell death or

20   apoptosis are still under investigation.

Neurodegenerative central nervous system (CNS) disorders are associated with exaggerated local inflammatory responses that ultimately result in tissue damage. It is known that Toll-like receptors or TLR play key roles in the control of innate immune responses to pathogens in many vertebrates. TLR are transmembrane

25   proteins with extracellular leucine-rich repeat domains, and cytoplasmic signalling domains that are similar to the cytoplasmic domain of the interleukin-1 receptor. In the human genome ten TLR family members have been identified to date. TLR1 is expressed in lymphoid organs in monocytes, leukocytes, T cells, B cells and NK cells. TLR2, TLR4 and TLR5 are widely expressed in myelomonocytic elements. Toll-like

30   receptor 3 (TLR3) has a more restricted pattern of expression. A recent study in mice

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indicates that among the different TLR family members TLR3 is strikingly
predominant in CNS.

TLR have been shown to recognise a wide variety of invariant pathogen-
associated molecular patterns (PAMP). They mediate the production of pro-
inflammatory mediators, a generally suitable response associated with the first line of
defence against pathogens. Accordingly, TLR have drawn significant attention as
potential targets to inhibit diseases associated with exaggerated inflammation, as
targets for adjuvants to stimulate immune responses upon vaccination to prevent
infectious diseases, or to promote anti-tumour immunity.

Recent data show that in particular TLR3-mediated responses can be distinct
from responses mediated by other TLR family members due to a different TLR3-
specific intracellular signalling pathway. In response to most PAMP TLR signalling
typically activates the NF-κB-mediated pathway and leads to production of products
including TNF-α, IL-1β, CXCL8 (IL-8), IL-6 and nitric oxide. Accordingly, the TLR-
mediated response is generally seen as a typically antimicrobial host defence response
designed to start pro-inflammatory innate immune responses and –eventually-
antimicrobial adaptive immune responses.

With reference to TLR3 activation, it has been reported that TLR3 signalling
in human mast cells not only fails to trigger TNF-α, IL-1β or IL-5 but instead, inhibits
degranulation of these cells as well as their attachment to extracellular matrix
components like fibronectin and vitronectin.

In the case of TLR3 and TLR4 ligation, production of type I interferons is also
stimulated, which is generally perceived as an antiviral response since it promotes
expression of major histocompatibility complex molecules and cross-presentation of
cellular antigens to MHC class I-restricted cytotoxic T cells. Such a typically antiviral
response appears to be mediated more efficiently by TLR3 as compared to TLR4. This
particular property of the TLR3-mediated response appears to make sense in view of
the fact that the only currently known agonist for TLR3 is double-stranded RNA, in
many cases a structural intermediate of viral replication. It should be pointed out,
however, that double-stranded RNA emerging during viral replication is generally
intracellular and will only be available as an extracellular ligand upon lysis of the
infected cell before viral replication is complete. As this will only be the case as an
exception, current knowledge fails to fully provide a rationale for activation of

extracellular, surface-exposed TLR3 as abundantly found on some cell types including fibroblasts and astrocytes.

Despite these data on the role of TLR in health and disease, it has hitherto remained unknown how interfering in or controlling the activity of any TLR, either by activation or by inactivation of these receptors, could play a role in the treatment of neurodegenerative disorders.

It is an aim of the present invention to provide methods for the treatment of degenerative inflammatory disorders in general and neurodegenerative disorders in particular. It is a further aim to control local inflammatory reactions via the identification of mechanisms and targets involved in these disorders and by subsequent therapeutic intervention. It is yet a further aim of the present invention to provide therapeutic compositions for the use in such methods.

SUMMARY OF THE INVENTION

The present inventors have now surprisingly found that activation of TLR3 on human astrocytes and fibroblasts results in a repair response which consists of enhanced production of a variety of anti-inflammatory, anti-fibrotic, pro-angiogenic, chemotactic and neuroprotective mediators that -together- support regenerative responses.

In a first aspect, the present invention relates to a method for the treatment and/or prophylaxis of a degenerative inflammatory process in a tissue of a subject, said method comprising increasing the activity of TLR3 in cells of said tissue.

In addition, the present inventors have now surprisingly found that stathmin and stathmin-like proteins can act as activator or agonist for TLR3 or can activate TLR3-mediated signalling. The inventors discovered this activity in astrocytes, a major cell type in the mammalian CNS. The stathmin-activated TLR3-mediated response of astrocytes includes production of a range of neuroprotective, anti-inflammatory, angiogenic and chemotactic mediators that -together- support regenerative responses rather than polarised pro-inflammatory host-defence responses. A very similar response was found for synoviocytes (joint fibroblasts) in response to TLR3 activation since also this cell type responds by enhanced production of a wide range of anti-inflammatory mediators and molecules that promote tissue-repair. Controlled TLR3 activation by stathmin or stathmin-like proteins on

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fibroblast-like and other cells, notably including CNS astrocytes and synoviocytes, may therefore have broad therapeutic potential in degenerative and other disorders. This is relevant to therapeutic intervention in a potentially wide range of human disorders. In particular such disorders that benefit form activation of TLR3 on human
5   astrocytes and fibroblasts, and in macrophage-like or dendritic cells are envisioned in prophylactic and therapeutic aspects of the present invention.

The present invention therefore provides the use of a TLR-3 agonist in the manufacture of a medicament for treating and/or preventing a degenerative inflammatory condition in a mammalian subject, wherein said antagonist is a
10   stathmin or a stathmin-like protein.

The invention also provides a TLR-3 agonist for use in the treatment and/or prophylaxis of a degenerative inflammatory condition in a mammalian subject.

In a preferred embodiment of a method of the present invention the method comprises the step of administering to the subject in need of the treatment or
15   prophylaxis:

- a therapeutically effective amount of at least one TLR3-agonist;

- a therapeutically effective amount of at least one compound that promotes *in vivo* expression of a TLR3-agonist;

- a therapeutically effective amount of at least one compound that promotes
20   TLR3 expression, and/or

- a therapeutically effective amount of at least one compound that promotes TLR3-mediated signalling.

In another preferred embodiment of a method of the present invention the tissue is nervous tissue, preferably brain tissue and/or synovial tissue.
25   In yet another preferred embodiment of a method of the present invention the cells are glial cells, preferably astrocytes, and/or synovial cells, preferably synoviocytes.

In still another preferred embodiment of a method of the present invention the degenerative inflammatory process is associated with a chronic or acute
30   neurodegenerative disorder. The neurodegenerative disorder is preferably selected from the group consisting of stroke, hypovolemic shock, traumatic shock, reperfusion injury, multiple sclerosis, AIDS-associated dementia, neuron toxicity, Alzheimer's disease (AD), head trauma, acute spiral cord injury, Huntington's disease (HD),

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Parkinson's Disease (PD) and related synucleinopathies, dystonia, Tourette

Syndrome, Frontotemporal Dementias (FTDs) and related tauopathies, prion

disorders such as Fatal Familial Insomnia (FFI) and other disorders related to

Creutzfeldt-Jakob disease, amyotrophic lateral sclerosis (ALS), trinucleotide repeat

5   diseases, Motor Neurone disease, progressive supranuclear palsy, REM Sleep

Behavior Disorder (RBD) and cancer. Particularly preferred neurogenerative

disorders that may be treated or prevented by the method of the invention include

stroke, multiple sclerosis, AD, PD and dementias.

Preferably the treatment in a method of the invention is accompanied by

10   stimulation of a neuroprotective response in cells of said nervous tissue and/or

wherein said treatment is accompanied by stimulation of a repair functions in cells of

said synovial tissue.

The TLR3-agonist that may be used in methods of the present invention is

preferably selected from the group consisting of:

15   - stathmin, TLR3-activating derivatives thereof and precursors thereof;

            - stathmin-like proteins, TLR3-activating derivatives thereof and precursors

              thereof, and

            - proteinaceous compounds comprising a polypeptide chain (preferably in alpha

              helix form) having an amino acid sequence similarity of at least 60% with an

20   amino acid sequence selected from the group consisting of the amino acid

              sequences of stathmin (SEQ ID NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ

              ID NO: 3) and RB3 (SEQ ID NO: 4).

In general, the amino acid sequence similarity relates to the similarity of the

polypeptide to the complete reference sequences. In a preferred embodiment, however,

25   the amino acid sequence similarity relates to the stathmin-like alpha helix domain

corresponding to residues 44 to 138 of SEQ ID NO: 1.

The stathmin-like protein used in aspects of the present invention is preferably

selected from SCG10, SCLIP and RB3; human stathmin is most preferred, and

combinations of stathmins and stathmin-like proteins are also envisaged.

30   A method of the present invention may further comprise the use of the TLR3-

agonist in combination with compounds that promote stabilisation of the stathmin-

like alpha helix domain corresponding to residues 44 to 138 of SEQ ID NO: 1.

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It should be noted that the method of the invention is not necessarily medical, but may also comprise non-medical embodiments. For instance, the method may be for preventing or retarding the onset of a neurodegenerative disorder in a subject, in the absence of any disorder, disease, or injury.

5   In another aspect, the present invention relates to a pharmaceutical composition for the treatment and/or prophylaxis of a degenerative inflammatory process in a tissue of a subject, wherein said composition comprises a therapeutically effective amount of at least one compound capable of increasing the activity of TLR3 in cells of said tissue; and b) a pharmaceutically acceptable carrier.

10   The pharmaceutically active compound in a composition of the invention may for instance be:

        - a TLR3-agonist;

        - a compound that promotes *in vivo* expression of a TLR3-agonist;

        - a compound that promotes TLR3 expression, and/or

15   - a compound that promotes TLR3-mediated signalling.

        The active compound in a preferred embodiment has the capacity of stimulating a neuroprotective response in cells of nervous tissue and/or of stimulating repair functions in cells of synovial tissue.

        Highly preferred active compounds are TLR3-agonists, preferably one or more

20   selected from the group consisting of:

        - stathmin, TLR3-activating derivatives thereof and precursors thereof,

        - stathmin-like proteins, TLR3-activating derivatives thereof and precursors
          thereof, and

        - proteinaceous compounds comprising a polypeptide chain having an amino

25   acid sequence similarity of at least 60% with an amino acid sequence selected
          from the group consisting of the amino acid sequences of stathmin (SEQ ID
          NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3 (SEQ ID
          NO: 4).

        Again, in a preferred embodiment, the amino acid sequence similarity relates

30   to the stathmin-like alpha helix domain corresponding to residues 44 to 138 of SEQ
        ID NO: 1.

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A composition of the invention may further comprise compounds that promote stabilisation of the stathmin-like alpha helix domain corresponding to residues 44 to 138 of SEQ ID NO: 1.

The pharmaceutical composition is meant specifically for use in methods of the
5   invention as described above and for treatment or prevention of the disorders referred to above. In addition, the compound may be used in combination with other drugs, such as drugs that cause inflammation, in order to ameliorate the inflammatory effect of such drugs.

In still a further aspects, the present invention relates to the first and second
10 medical indication of a TLR3-agonist selected from the group consisting of:
        - stathmin, TLR3-activating derivatives thereof and precursors thereof,
        - stathmin-like proteins, TLR3-activating derivatives thereof and precursors
          thereof, and
        - proteinaceous compounds comprising a polypeptide chain having an amino
15   acid sequence similarity of at least 60% with an amino acid sequence selected
          from the group consisting of the amino acid sequences of stathmin (SEQ ID
          NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3 (SEQ ID
          NO: 4).

Such first and second medical indications in particular relate, respectively, to
20 the compounds for use as a medicament and to the use of the compounds for the preparation of a medicament for the treatment and/or prophylaxis of a degenerative inflammatory process in a tissue of a subject.

It is an aspect of the present invention that the composition is for preventing or retarding the onset of degenerative inflammatory disorders and neurodegenerative
25 disorders as described above in a subject, in the absence of any disorder, disease, or injury.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates preferential expression of TLR4, and to a lesser extent of
30 TLR1-3 on cultured but otherwise untreated human astrocytes from six different donors, and strong and preferential induction of TLR3 by a variety of cytokines. The data illustrate particularly strong induction of TLR3 by pro-inflammatory cytokines but not by anti-inflammatory cytokines. Panel A shows that in cultured but otherwise

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untreated astrocytes from six different control donors TLR4 is generally dominantly expressed, while TLR1-3 are expressed at lower levels. TLR5-10 were undetectable by RT-PCR in all cultures. Panel B shows that TLR3 is preferentially induced in human astrocytes by pro-inflammatory but not anti-inflammatory cytokines. Panel B depicts

5   mRNA expression levels after 48 h relative to untreated control astrocyte cultures for a single representative culture. Apart from individual cytokines, also a mixture of IL-1ß, TNF-α and IFN-γ (MIX) was tested.

Figure 2 illustrates the preferential induction of TLR3 in cultured adult human astrocytes from six different donors under the influence of the TLR3 agonist

10   poly I:C or the TLR4 agonist LPS. TLR3 is preferentially induced in human astrocyte cultures by TLR3 or TLR4 activation (panel A). Stimulation with poly I:C (a TLR3 agonist) or LPS (a TLR4 agonist) was performed for 24 h. The data in panel A represent individual data for 6 astrocyte cultures derived from different donors, depicting mRNA expressing levels relative to untreated control astrocyte cultures

15   from the same donor. By activating TLR3 or TLR4, also some induction is observed of TLR2, but this is an indirect effects as it is blocked by the protein synthesis-blocking agent cycloheximide, whereas TLR3 is not blocked by such a treatment (Panel B; showing data for a single representative donor).

Figure 3 illustrates that in cultured adult human astrocytes, TLR3 as well as

20   TLR4 can be found exclusively on the cell surface. TLR3 (stained by green fluoresence) is exclusively found on the cell surface of cultured GFAP-positive adult human astrocytes (panel A). TLR3 expression (stained by brown deposits) in human brain during the neuroinflammatory process of multiple sclerosis (panels B and C). Note that TLR3 staining manifests itself in two distinct ways. Astrocytes are

25   exclusively stained in a way consistent with surface expression as found also in cell culture (panel B; open arrow), while additional vesicular staining is associated with TLR3 expressed by microglia (panel C; closed arrow), similar to the exclusively vesicular expression of TLR3 in cultured human microglia.

Figure 4 illustrates the effect of mediators, secreted by astrocytes in response

30   to TLR3 activation, on growth of astrocytes (A) or endothelial cells (B). While astrocytes growth (gliosis) is attenuated by TLR3- but not TLR4-triggered mediators, endothelial cell growth is stimulated by TLR3- but not TLR4-triggered mediators. Poly I:C-conditioned medium inhibits astrocyte proliferation and promotes endothelial

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cell growth. Astrocytes (panel A) or endothelial cells (panel B) were cultured for 3
days in poly I:C- or LPS-conditioned astrocyte medium that had been harvested at
different time points. Proliferation was evaluated by WST-1 staining of viable cells at
the end of the culture period. Bars represent the pooled data ± standard deviations

5   from six (panel A) or two (panel B) independent experiments in which each condition
was assayed in quadruplicate. Significance marked ** indicates p<0.003 by analysis of
variance (ANOVA).

       Figure 5 illustrates the survival-promoting effect on neurons in organotypic
human brain slice cultures of mediators, secreted by astrocytes in response to TLR3

10   activation but not TLR4 activation. Poly I:C-conditioned but not LPS-conditioned
astrocyte medium promotes survival of neurons in organotypic human brain slice
cultures. Organotypic human cortical brain slice cultures were kept for 1 week in poly
I:C- or LPS-conditioned astrocyte medium that had been harvested after 48 h. In
panels A and B colour images of calcein/AM-stained brain slices are shown,

15   illustrating markedly reduced numbers of dead cells (marked by red nuclei) in slices
kept in poly I:C-conditioned astrocyte medium (panel B) as compared to control
medium (panel A). In panel C percentages are given of either live or dead neurons
that are the mean ± standard deviation of four individual slices examined for each
condition using live/dead staining with calcein/AM. Significance marked * indicates

20   p< 0.03 by analysis of variance (Kruskal-Wallis test).

       Note that both poly I:C-conditioned astrocyte medium and poly I:C alone
significantly promotes survival of neurons relative to other culture conditions,
suggesting that also when part of a culture brain slice, astrocytes activated by poly
I:C can produce neuroprotective mediators.

25   Figure 6 illustrates the results of a screening of cDNA bank-derived gene
products on their ability to induce CXCL8 secretion in astrocytes, a sensitive albeit
not specific marker for TLR3 engagement. This screening led to identification of a
stathmin-derived sequence as one of two major CXCL8 inducing products encoded by
this cDNA bank. The other product has no relevance to TLR3-mediated signalling by

30   astrocytes. Screening of a cDNA bank derived from human brain tumours on products
that trigger CXCL8 secretion by human astrocytes in culture. cDNAs were made
using mRNA templates from a mixture of human brain tumour cell lines and these
were individually cloned into *E. coli* using a construct that promotes expression and

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secretion of the product in mammalian cells. In a first step (panel A) 768 different pools of 50 to 100 individual plasmids containing the different cDNAs were transfected into COS-7 cells. Culture supernatants from those transfected cells were collected after 48 h and fed to human astrocytes in culture. After another 48 h,

5   CXCL8 release by stimulated astrocytes was assessed by enzyme-linked immuno sorbent assays. In panel A, the results are shown for the first screening of 768 pools of cDNA, illustrating that one pool (marked by an arrow) induced markedly higher than average CXCL8 release by astrocytes. Subsequent testing of all individual cDNAs contained in that pool (panel B) reveals two cDNA candidate agonists. Candidate 1

10   was found to be irrelevant to the current invention while candidate 2 (clone F5) contains the sequence corresponding to residues 44-149 of human stathmin (SEQ ID NO.1) .

Figure 7 illustrates the ability of stathmin to induce secretion of CXCL8 (interleukin-8; referred to as IL-8) in human astrocytes or IL-6 in murine astrocytes,

15   sensitive albeit not specific markers for TLR3 engagement, but the failure of stathmin to do so in astrocytes isolated from TLR3-deficient mice, confirming the notion that the astrocyte response to stathmin is TLR3-mediated. Also shown is the failure of stathmin to activate either TLR2 or TLR4, ruling out any confounding effects by contaminant endotoxins in the stathmin preparation. Activation of astrocytes by

20   recombinant human stathmin is TLR3-dependent.

In Panel A, the averaged response of 4 individual human adult astrocyte cultures is shown to increasing doses of recombinant stathmin; release levels of CXCL8 in response to poly I:C in these experiments is around 200 ng/mL. Possibly due to the fact that only part of the stathmin molecules in solution adopt the full

25   alpha helical conformation required for TLR3 binding, the astrocyte response to stathmin is still not maximal at 100 μg/mL. In panel B the effect of three different small interfering RNAs (siRNA) specific for human TLR3 are shown, revealing that the stathmin-induced CXCL8 response in human astrocytes is strongly reduced or even fully ablated by these siRNAs. In panels C and D, it is shown that at levels of at

30   least 50 μg/mL the preparation of recombinant stathmin used does not contain any endotoxin contaminants that trigger responses of HEK293 transfectant cells either via TLR2 (panel C) or via TLR4 (panel D). In Panel E, it is shown that for astrocytes

of TLR3-deficient mice, both the stathmin- and the poly I:C-induced response is ablated, while the response to LPS (TLR4 agonist) is still intact.

Figure 8 summarises the currently documented expression of TLR3 in different human tissues and cell types under normal (non-diseased) conditions.

Figure 9 lists all gene products represented on a macroarray used in the example study whose expression levels were found to be reproducibly increased at least two-fold following stimulation of astrocytes with either the TLR3 agonist poly I:C, the TLR4 agonist LPS or the mixture of both. Numerical values represent the level of mRNA expression in the stimulated astrocyte population relative to their expression in unstimulated astrocytes from the same donor, cultured for the same time under the same culture conditions but without the specific TLR agonist. The numerical values represent the maximum stimulation found after analysing levels of expression after 2, 6, 24 and 48 h. Ratios are given of the gene expression signals found in agonist-treated astrocytes cultures relative to untreated control cultures after a 48-h culture period. Data represent average ratios ± standard deviation for 4 cultures derived from different donors. Only data are given for those gene products that show at least 2-fold induction (in bold) under any of the three experimental conditions. ND: not reliably detectable; average gene expression signal below 10% threshold relative to housekeeping gene expression signals. Note that about half of the mediators induced by poly I:C in astrocytes are also induced by poly I:C in human synoviocytes (see Figure 10). Figure 10 lists all gene products induced by poly I:C in cultured human synoviocytes

Figure 11 shows the nucleotide sequence of the cDNA clone (clone F5) found in the screening experiments described below in the experimental section and its corresponding protein sequence encoding a protein capable of activating TLR3-mediated signalling. The protein sequence encoded by F5 (excluding the plasmid sequence) is identical to residues 44 (D, aspartic acid) to the C-terminal residue 149 (D, aspartic acid) of human stathmin (SEQ ID NO.1), which stretch fully comprises the conserved stathmin-like alpha helical domain that is located between residues 44 and 138.

Figure 12 provides the amino acid sequences of all four currently known human members of the family of stathmin-like proteins.

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Figure 13 highlights the sequence homologies within the conserved stathmin-like alpha helical domains of the four human stathmin-like family members stathmin, SCG10, SCLIP and RB3. Sequence similarities among the four family members of human stathmin-like proteins. Note that most of the sequence differences reside in

5   the N-terminal domain that controls intracellular trafficking and subcellular localisation. The highly conserved stathmin-like alpha helix domain corresponding to residues 44 to 138 of stathmin is boxed. The percentage amino acid identity of this helical part of the sequence of stathmin with the other family members is between 73 and 82.

10   Figure 14 shows the IL-6 release by astrocytes isolated from either wild-type or TLR3-deficient mice as described in Example 2. In astrocytes from wild-type mice, stathmin, poly I:C or LPS all lead to marked IL-6 release. In astrocytes from TLR3-deficient animals, the response to stathmin as well as poly I:C is fully abrogated, while the response to LPS remains intact.

15   Figure 15 shows the gene response profiles of stathmin- or poly I:C-stimulated human astrocytes as described in Example 3. Gene profiling was performed using a cDNA array containing 268 probes for cytokines, chemokines and growth factors. In panel A, the gene profile is compared between a stathmin-treated astrocyte culture and an untreated control culture. In panel B the response in a poly I:C-treated culture

20   is shown. In both cases, a comprehensive stimulatory effect is obvious on a variety of genes. When the stathmin-induced profile is compared to the poly I:C-induced profile (panel C), all hybridization signals aggregate along the diagonal implying that the expression profiles in either situation are essentially the same. The Pearson correlation coefficient (R) between either data set is > 0.95; the corresponding p value

25   < 10^{-35}.

Figure 16 shows the TLR3 agonist activity of stathmin depends on its alpha helix as described in Example 4. In Fig. 16A, CD spectra are given for stathmin preparations pre-treated either with TFA or TFE to produce a different helical content in the two preparations, which were otherwise identical. The TFE-treated

30   preparation contains 37% alpha helix, a 3-fold higher level than the TFA-treated preparation which contains only 12% alpha helix. In Fig. 16B it is shown that the stathmin preparation pre-treated with TFE is also 3-fold more active in triggering TLR3-mediated release of IL-8 in human astrocytes than the TFA-treated

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preparation. As a reference, the CD spectrum is also shown of a stathmin preparation subjected to CD spectroscopy in the presence of 50% TFE. Under such conditions, stathmin adopts an alpha helical conformation over essentially the entire length of the protein chain (helical content 98%).

5   Figure 17 illustrates the response of human peripheral blood mononuclear cells (PBMC), pre-dendritic cells (pre-DC) and mature plasmacytoid dendritic cells (DC) from two healthy donors to various TLR agonists as described in Example 5. CXCL8 accumulation in the culture medium was used as a measure of both TLR3 and TLR4 activation. Final concentrations of the TLR3 agonists poly I:C (PIC) and stathmin

10   (STM) are given in □g/ml, those of the TLR4 agonist LPS are given in ng/ml.

Figure 18 lists the results of accumulation after 48 h of cytokines and chemokines in a culture of stathmin-treated human pre-dendritic cells as compared to untreated control cells described in Example 6.

Figure 19 illustrates CXCL8 induction by stathmin in purified cultures of

15   human microglia as described in Example 7. Microglia were isolated from three different donors (A, B and C) and in one case, from different anatomical areas of the brain and stimulated with different TLR agonists. In all cases, all TLR agonist trigger marked accumulation of CXCL8 as compared to control cultures (C).

20   DETAILED DESCRIPTION OF THE INVENTION
Definitions

"Treatment and/or prophylaxis" as used herein includes retarding and/or reversing the progression of tissue deterioration or destruction, as well as inhibiting or preventing such deterioration or destruction and reversing the degradation process

25   into a regeneration process. In case the deterioration or destruction takes place or is directed at nervous tissue, the treatment and/or prophylaxis may involve neuroprotective processes as described herein. As used herein the term is specifically used in connection with the TLR3-mediated repair response which consists of enhanced production of a variety of anti-inflammatory, anti-fibrotic, pro-angiogenic,

30   chemotactic and neuroprotective mediators that -together- support regenerative responses. The term "regenerative responses" indicates the opposite of tissue degeneration processes, i.e. it indicates the tissue regeneration process, including the

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provision of protection against cell damage. Thus, when the term "TLR3-mediated repair response" is used herein it is meant to indicate all of the above effects.

As used herein the terms "tissue degeneration", "tissue deterioration" or destruction" are used interchangeably and describes any internal or external process

5   which causes damage or injury to a tissue or an impaired function of a body tissue of tissue failure, which may be characterised by impaired cellular activity or death of cells in the tissue and tissue necrosis, and includes in general histological alteration. This degeneration can be caused by medication, by viral infection, by trauma, by anoxia or by starvation or any other process or disorder that results in chronic or

10   acute degeneration of cells in the tissue or in a supporting tissue, such as by the detrimental effect of exaggerated inflammation.

As used herein, the term "neurodegenerative disorder", refers to a disease that result from deterioration of neurons, including chronic and acute neurodegenerative disorders such as stroke, hypovolemic shock, traumatic shock, reperfusion injury,

15   multiple sclerosis, AIDS-associated dementia, neuron toxicity, Alzheimer's disease (AD), head trauma, acute spiral cord injury, Huntington's disease (HD), Parkinson's Disease (PD) and related synucleinopathies, dystonia, Tourette Syndrome, Frontotemporal Dementias (FTDs) and related tauopathies, prion disorders such as Fatal Familial Insomnia (FFI) and other disorders related to Creutzfeldt-Jakob

20   disease, amyotrophic lateral sclerosis (ALS), trinucleotide repeat diseases, Motor Neurone disease, progressive supranuclear palsy, REM Sleep Behavior Disorder (RBD) and cancer. Other neurodegenerative disorders include Alexander disease, Alper's disease, Amyotrophic lateral sclerosis, Ataxia telangiectasia, Batten disease (also known as Spielmeyer-Vogt-Sjogren-Batten disease), Bovine spongiform

25   encephalopathy (BSE), Canavan disease, Cockayne syndrome, Corticobasal degeneration, Huntington disease, Kennedy's disease, Krabbe disease, Lewy body dementia, Machado-Joseph disease (Spinocerebellar ataxia type 3), Multiple sclerosis, Multiple System Atrophy, Pelizaeus-Merzbacher Disease, Pick's disease, Primary lateral sclerosis, Refsum's disease, Sandhoff disease, Schilder's disease,

30   Schizophrenia, Spinocerebellar ataxia (multiple types with varying characteristics), Spinal muscular atrophy, Steele-Richardson-Olszewski disease, and Tabes dorsalis.

A "neuroprotective" effect is defined herein as an inhibition, prevention or generally control of processes that cause damage or injury to cells of the nervous

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tissue, in particular to the neurons. The neuroprotective effect of TLR3 stimulation by stathmin was observed in astrocytes as described in more detail in the Examples below.

The term "nervous tissue" is used herein in it's the art-recognized meaning of a
5   tissue composed of impuls-conducting neurons (ganglion cells) and their supporting glial cells. The supporting glial cells cells of the nervous system include for instance the capsule or satellite cells of peripheral ganglia, ependyma, neuroglia (astrocytes, oligodendroglia and microglia), and Schwann cells.

As used herein, the term "degenerative inflammatory condition" refers to any
10   inflammatory disease with which is associated the degeneration of tissues. Examples are rheumatoid arthritis, osteoarthritis and chronic obstructive pulmonary (lung) disease, including any neurodegenerative disorder associated with inflammation.

Stathmin, originally also named oncoprotein 18 (or Op-18), is a cytosolic phosphoprotein originally characterized as an intracellular relay molecule that
15   integrates various signalling pathways. Combinatorial phosphorylation of four serine residues in the N-terminal region of the molecule is believed to affect at least part of its functions. A major function of stathmin is regulation of microtubule assembly.

As used herein, the term "stathmin-like protein" (*sensu stricto*) refers to the art-recognized term for a particular group of proteins that has amino acid sequence
20   motives in common with stathmin (stathmin-1) and is generally used to address the group of compounds consisting of the stathmin-like proteins SCG10 (Superior Cervical Ganglion 10; stathmin-2), SCLIP (SCG10-like protein; stathmin-3), and RB3 (stathmin-4). The term "stathmin-like protein" is also used herein to refer to a proteinaceous compound comprising a polypeptide chain having an amino acid
25   sequence similarity of at least about 60%, preferably at least 70%, more preferably at least 80%, still more preferably at least 90%, even more preferably at least 95%, and particularly preferably at least 97%, 98% and most preferably at least 99%, with an amino acid sequence selected from the group consisting of the amino acid sequences of stathmin (SEQ ID NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3
30   (SEQ ID NO: 4). Preferably the amino acid sequence similarity relates to a region of at least 30 contiguous amino acids, preferably at least 50, more preferably at least 80 and most preferably at least 90 contiguous amino acids. Most preferably the amino acid sequence similarity relates to the stathmin-like alpha helix domain

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corresponding to residues 44 to 138 of human stathmin (SEQ ID NO: 1), resulting in a
stathmin-like protein wherein the basic structural features consistent with the
stathmin-like alpha helical domain that allows interaction with TLR3 is maintained.

The term "amino acid sequence similarity" as used herein denotes the presence

5   of similarity between two polypeptides or proteins. Polypeptides have "similar"
sequences if the sequence of amino acids in the two sequences is the same when
aligned for maximum correspondence. Sequence comparison between two or more
polypeptides is generally performed by comparing portions of the two sequences over a
comparison window to identify and compare local regions of sequence similarity. The

10   comparison window is generally from about 10 to 100 contiguous amino acids. The
"percentage of sequence similarity" for polypeptides, such as 50, 60, 70, 80, 90, 95, 98,
99 or 100 percent sequence similarity may be determined by comparing two optimally
aligned sequences over a comparison window, wherein the portion of the polypeptide
sequence in the comparison window may include amino acid deletions, modification or

15   addition of single amino acids or groups of amino acids as compared to the reference
sequence (which does not comprise additions or deletions) for optimal alignment of the
two sequences. The percentage is calculated by: (a) determining the number of
positions at which the identical amino acid occurs in both sequences to yield the
number of matched positions; (b) dividing the number of matched positions by the

20   total number of positions in the window of comparison; and (c) multiplying the result
by 100 to yield the percentage of sequence similarity. Optimal alignment of sequences
for comparison may be conducted by computerized implementations of known
algorithms, or by visual inspection. Sequence comparison and multiple sequence
alignment algorithms are readily available on the internet, for instance via the

25   National Center for Biotechnology Information, U.S. National Library of Medicine,
8600 Rockville Pike, Bethesda, MD 20894.

A "derivative", "analog" or "analogue" is defined herein as a stathmin or
stathmin-like protein as defined herein that is subjected to a (bio)chemical
modification (e.g. organo-chemical or enzymatical). Derivatising may comprise the

30   substitution of certain chemical groups to the protein, e.g. phosphorylation, thereby
retaining the TLR3-agonist character of the compound. Such derivatizations are
known in the art. The derivatives and analogues, by way of substitution of certain
chemical groups, maintain the biological activity of the natural stathmin or stathmin-

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like protein and function in a similar manner in that they increase the activity of Toll-
like receptor 3 (TLR3) (i.e. act as TLR3 agonist), but may provide advantages to the
natural molecule such as longer half-life, resistance to degradation or an increased
activity. Conservative amino acid substitutions may be made, for example within the
5   group of aliphatic non-polar amino acids (Gly, Ala, Pro, Ile, Leu, Val), the group of
polar uncharged amino acids (Cys, Ser, Thr, Met, Asn, Gln), the group of polar
charged amino acids (Asp, Glu, Lys, Arg) or the group of aromatic amino acids (His,
Phe, Tyr, Trp). Stathmin-like proteins of the invention may be chemically modified,
e.g. post-translationally modified. For example, they may be glycosylated or may
10   comprise modified amino acid residues. They may also be modified by the addition of
histidine residues or an epitope tag for example by a repeat of several His residues or
other sequence tags well known to those skilled in the art to assist their purification
or detection. They may be modified by the addition of a signal sequence to promote
insertion into the cell membrane. Such modified proteins also fall within the scope of
15   the term "stathmin-like proteins, or derivatives or precursors thereof". The term
"stathmin-like protein" is preferably to be understood as a protein comprising an α-
helix structure having an amino acid homology of at least 85%, preferably at least
90%, more preferably at least 92 % to that of amino acid residues 44-138 of human
stathmin as displayed in SEQ ID NO.1. Examples of stathmin-like proteins (peptides)
20   are SCG10, SCLIP and RB3, and homology and sequence identity between these
proteins is described in Example 1. In particular derivatives of peptides according to
the present invention include synthetic amino acid analogues (natural or synthetic).
Amino acid analogues are chemical substances having similar molecular structure
and function with normal amino acids. Examples include aliphatic amino acids, single
25   ring aromatic amino acids, polycyclic aromatic amino acids, Single Ring heterocyclic
amino acids, polycyclic heteronuclear amino acids, amino and alkylamino containing
side chains, carboxyl, carboxamide, and carboxyl ester group containing side chains,
guanidine and amidine containing side chains, hydroxy and alkoxy containing side
chains, mercapto and alkylmercapto containing side chains, other heteroatom
30   containing side chains, alpha-imino acids, non-α amino acids (beta-amino acids, etc.),
des-carboxy and des-amino acids. The derivatives include functional equivalents, such
as modified peptides, peptide analogues, or peptidomimetics. Peptidomimetics, in
their broadest scope, are compounds which are in their functional structure more or

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less similar to a peptide, but which may also contain non-peptidic bonds in the backbone, or D-amino acids. In general, peptidomimetics serve as substitutes for native peptides in the interaction with receptors and enzymes (Pharmaceutical Biotechnology, Ed. D. J. A. Crommelin and R. D. Sindelar, Harwood Academic

5   Publishers, 1997, p. 138). Pseudopeptides, a class of peptidomimetics, are compounds containing amide bond isosteres instead of amide bonds (*ibid.*, pp. 137-140). Peptides of the invention also include salts of peptides or functional equivalents, such as pharmaceutically acceptable acid- or base addition salts. They also include multimers of peptides or funtional equivalents. Techniques for selecting appropriate analogues

10   or mimetics of naturally occurring amino acids are well-known to those skilled in the art. For example, the pseudopeptide approach aims at achieving a higher chemical or enzymatic stability of a peptide while retaining its bioactivity. Peptide bonds which undergo rapid degradation *in vivo* are replaced by other functional groups to create bioisosteres or amide bond surrogates. Examples of bioisosteric groups are N-methyl

15   amide, thioester, thioamide, ketomethylene, methyleneamino, retro-inverse amide, methylenethio, and methyleneoxy groups (*ibid.*, pp. 138-139). An approach to improve the selectivity of a peptide is to introduce conformational constraints to the backbone, which decreases the number of potential receptor or enzyme interactions. The constraints are most often achieved by the introduction of carbon-carbon double bonds

20   (olefinic analogues) and ring structures (*ibid.*). A further method to identify analogues which mimic the functional structure of an amino acid such as cysteine (C), methionine (M), or valine (V) is computer-aided modeling. Preferred mimetic structures possess a side chain with similar charge or electronegativity, hydrophobicity and spatial orientation to said amino acids.

25   "Sequence similarity" as used herein refers to the percent similarity between two (amino acid or nucleic acid) sequences. Highly similar sequences may have many identical or homologous positions. An identical position refers to a position wherein both residues are identical, while a homologous position refers to a position wherein an exchange of one amino acid for another amino acid within any one of the following

30   groups has occurred: 1. Small aliphatic, nonpolar or slightly poor residues: alanine, serine, threonine, glycine, proline; 2 Polar, negatively charged residues and their amides: aspartic acid, asparagine, glutamic acid, glutamine; 3. Polar, positively charged residues: histidine, arginine, lysine; 4. Large aliphatic, nonpolar residues:

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methionine, leucine, isoleucine, valine, cysteine; 5. Large aromatic residues: phenylalanine, tyrosine, tryptophan.

A "precursor" is defined herein as a derivative of the active compound with similar, less or no activity, and which can be transformed to the active compound e.g.

5   by the digestive tract or other digestive systems in the body. Such precursors can be obtained by chemical or enzymatic modification of the active molecule.

"Subject" as used herein includes, but is not limited to, mammals, including, e.g., a human, non-human primate, mouse, pig, cow, goat, cat, rabbit, rat, guinea pig, hamster, horse, monkey, sheep, or other non-human mammal; and non-mammal

10 animals, including, e.g., a non-mammalian vertebrate, such as a bird (e.g., a chicken or duck) or a fish, and an invertebrate. A preferred subject in aspects of the invention is a mammal, most preferably a human.

The terms "pharmaceutical compound", "(pharmaceutically) active compound", "(pharmaceutically) active substance", "therapeutic agent", "pharmaceutical agent",

15 "medicament" and "drug" are used herein interchangeably.

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of the therapeutic agent. The term refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue

20 toxicity. As pharmaceutically acceptable carrier, any solvent, diluent or other liquid vehicle, dispersion or suspension aid, surface active agent, isotonic agent, thickening or emulsifying agent, preservative, encapsulating agent, solid binder or lubricant can be used which is most suited for a particular dosage form and which is compatible with the stathmin or stathmin-like protein. Suitable pharmaceutically acceptable

25 carriers in therapeutic compositions may be liquids such as water, saline, glycerol and ethanol. Liposomes are included within the definition of a pharmaceutically acceptable carrier. Suitable carriers may also be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such

30 alternative carriers are also well known to those of ordinary skill in the art. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough

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discussion of pharmaceutically acceptable excipients is available in Remington's
Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

The term "therapeutically effective amount" as used herein refers to an
amount of a therapeutic agent to treat, ameliorate, or prevent a disease or condition,

5   or to exhibit a detectable therapeutic or prophylactic effect. The precise effective
amount needed for a subject will depend upon the subject's size and health, the nature
and extent of the condition, and the therapeutics or combination of therapeutics
selected for administration. Thus, it is not useful to specify an exact effective amount
in advance. However, the effective amount for a given situation can be determined by

10   routine experimentation.

TLR3 is identified herein as a preferred mediator of tissue repair responses in
mammalian cell types such as for instance astrocytes and synoviocytes. The TLR3-
mediated repair response consists of enhanced production of a variety of anti-
inflammatory, anti-fibrotic, pro-angiogenic, chemotactic and neuroprotective

15   mediators that -together- support regenerative responses rather than polarised pro-
inflammatory host-defence responses.

TLR3 is localised in intracellular vesicles in dendritic cells and CNS microglia,
while it is exclusively found as a surface-exposed membrane protein in fibroblasts and
astrocytes. When intracellular, TLR3 resides in multivesicular bodies associated

20   within the endosomal/lysosomal pathway of endocytosis and phagocytosis. To what
extent such differences in localisation relate to different TLR functions and may be
associated with different functional responses to TLR agonists remains to be
established. One hint that this might be the case has emerged from a recent study
that shows TLR3 signalling to become augmented by an acidic environment, and also

25   requires multimerisation of TLR3 molecules. To certain agonists, therefore, a TLR3-
mediated response may be affected by its presence either in acidic endosomal vesicles
or expression at the cell surface.

TLR3 ectodomain is a horseshoe-like structure assembled from 23 leucine-rich
repeats that is partly glycosylated. Highly conserved TLR3-specific structures exists

30   that are consistent with ectodomain dimer formation. Patches of positively charged
amino acid residues are present on the surface of the TLR3 ectodomains that face
each other in the dimer configuration, and these can be considered suitable contact

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regions for an extended rod-like double-stranded RNA helix lined with negatively charged phosphate groups.

The present inventors have discovered that activation of TLR3 or promotion of the expression of TLR3 or promotion of the TLR3 signalling pathway results in

5   retarding of neurodegenerative processes. In attempting to identify a therapeutically useful agonist for TLR3 it was surprisingly found that stathmin and stathmin-like proteins provide very suitably such agonists.

In testing the efficacy of the agonist the inventors have found that activation of the TLR3-mediated response leads not only to the activation of said repair responses

10   but also to enhanced and selective further expression of TLR3 itself. This renders the TLR3-mediated response self promoting.

Interestingly enough, the use of stathmin as an agonist has further revealed to also enhance and selectively further express stathmin itself, again rendering the stathmin-induced TLR3-mediated response self promoting.

15   The present invention further relates to a method for retarding or inhibiting an acute or chronic degenerative inflammatory process and/or for stimulating a neuroprotective process in a mammal in need thereof, preferably a human, comprising administering to said mammal a therapeutically effective amount of a stathmin or stathmin-like protein.

20   In the Table below, the qualitative effects of stathmin on two different human cell types that express TLR3 either on the cell surface (astrocytes) or intracellularly (dendritic cells) are summarized

Cell type	stathmin-induced mediators	biological relevance
Astrocytes	neurotrophin, BDNF, GGF1 and 2, PTN, TGF-β2, GDF1, EDF1	neuroprotection and cellular growth
	CNTF, LIF	remyelination
	TSG-6, IL-9, IL10, IL-11, IL-5	anti-inflammatory
	VEGF, VEGF-C, CXCL8 CCL2, CCL3, CXCL9	angiogenesis and cell migration
Dendritic cells	IL-10, IL-12p40 subunit	anti-inflammatory
	CXCL-8	angiogenesis and

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CCL2   cell migration

As disclosed herein, TLR3-activating substances, or other substances that

5   specifically promote expression of stathmin and/or stathmin-like proteins and/or

TLR3 and/or that promote the functional interaction between stathmin or stathmin-

like proteins and TLR3 are effective against autoimmunity, transplant or graft

rejection, inflammation, allergic responses, other autoimmune, immune-mediated or

trauma related tissue degeneration, tissue diseases and exaggerated inflammation.

10   Thus, the present invention discloses methods of use for permitting or promoting

wound healing; controlling neurodegenerative disorders, including damage or injury

to neurons.

That controlled TLR3 activation by stathmin or stathmin-like proteins on

fibroblast-like cells and other cells is relevant to therapeutic intervention in a

15   potentially wide range of degenerative and other human disorders may be concluded

from three key observations:

- First, it was found that cultured adult human astrocytes have a remarkable

preference to express elevated levels of TLR3 when activated in different ways,

notably including TLR3 engagement itself. Supplying pro-inflammatory cytokines,

20   TLR3 or TLR4 agonists or applying oxidative stress generally led to strong induction

of TLR3 expression on human astrocytes and much less so, if at all, of other TLR

family members.

- Secondly, a cDNA array-based analysis of the response by cultured human

adult astrocytes to TLR3 activation revealed that such activation (but not TLR4

25   activation) induces a wide range of well-known anti-inflammatory, neuroprotective,

angiogenic and chemotactic mediators. In functional assays it was verified that,

together, these TLR3-triggered factors promote survival of human neurons in brain

slice cultures, stimulate endothelial cell growth and inhibit astrocyte growth (gliosis).

Thus, TLR3-mediated signalling by astrocytes appears functional in promoting repair

30   and regeneration rather than initiating a pro-inflammatory host-defence response. A

similar response was observed in human synoviocytes.

- Thirdly, in search of a potential endogenous TLR3 agonist by screening gene

products produced by human brain tumours the inventors surprisingly identified a

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sequence of stathmin as candidate TLR3 agonist. The ability of this gene product to
activate astrocytes was mimicked by full-length recombinant human stathmin and
demonstrated to be TLR3-dependent by showing the failure of stathmin to activate
astrocytes from TLR3-deficient mice and ruling our TLR2- or TLR4-mediated effects.

5   Thus, stathmin and stathmin-like proteins activate TLR3-mediated signalling at least
in astrocytes, and in doing so activate the production of a range of anti-inflammatory
and tissue-repair mediators. The data also suggest that the response of TLR3 to
stathmin is at least to some extent self-amplifying since TLR3-mediated signaling
induces higher levels of TLR3 itself and promotes stathmin expression in different

10   neural cell types.
        Stathmin is the founding member of a family of four phosphoproteins that
share a conserved so-called stathmin-like alpha helical domain of 94 amino acids
containing as many as 24 negatively charged amino acids. Stathmins play key roles in
the regulation of microtubule formation and they are expressed at relatively high

15   levels by both neurons and glial cells in the CNS. The current invention discloses that
stathmin acts as an agonist for TLR 3, a TLR family member that so far has been
known to act as a receptor for double-stranded RNA only. When activated on human
fibroblast-like cells, in particular on astrocytes of the CNS, TLR3 mediated signalling
leads to production of a variety of mediators with neuroprotective, anti-inflammatory,

20   angiogenic and chemotactic functions. A similar response is disclosed for human
synoviocytes, fibroblast-like cells that are important in the pathogenesis of
rheumatoid arthritis. It is also known that TLR3 mediates anti-allergic responses in
human mast cells. The invention thus reveals that by activating the said repair-like
response mediated by TLR3 stathmin and stathmin-like proteins may have broad

25   therapeutic potential in CNS disorders, arthritis, allergies and other disorders
involving acute or chronic tissue degeneration.
        Stathmin is the generic element of a phosphoprotein family, including the
proteins SCG10 (Superior Cervical Ganglion 10 protein; stathmin-2), SCLIP (SCG10-
like protein; stathmin-3), and RB3 (stathmin-4) that are highly conserved amongst

30   vertebrates. For example, the human stathmin sequence of 149 amino acids differs
from the rat sequence by only a single amino acid, and from murine stathmin by only
two residues. Different members of the stathmin-related family of proteins contains
variable N-terminal sequences that are relevant to intracellular targeting, in

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particular with regard to interactions with membranes. The common feature of the family of stathmin-like proteins is the conserved stathmin-like domain, an unusually long alpha helical structure that interacts with tubulin. As a consequence, stathmin family members also share the functional activity of regulating microtubule dynamics.

5   It is tempting to speculate that the conserved very long C-terminal alpha helix of stathmin and stathmin-like proteins is the key structural feature that allows members of this structural family to interact with the ectodomain of TLR3. This continuous helix extends in the human stathmin sequence from residue 44 to residue 138. Thus, it extends over about 95 amino acids forming an only slightly bent rod-like

10   structure of about 140 Å in length. Especially since negatively charged amino acids are abundant in this stathmin-like domain –accounting for about 25% of all residues- this structure is intriguingly reminiscent of the extended rod-like structure of a double-stranded RNA helix, and appears similarly suitable as a ligand for the ectodomain of TLR3 as suggested by its crystal structure.

15   In humans stathmin is expressed at relatively high levels in the central nervous system, testis and thymus and its expression is particularly prominent during neurogenesis and elevated in response to damage (see below). In a variety of other organs stathmin is also expressed, but at levels that are at least 10-fold lower than in the above organs. It is remarkable that the expression levels of stathmin are

20   the highest in those vital organs that are generally recognised as immune-priviledged sites in the mammalian body and require a powerful local anti-inflammatory potential. The thymus is key in programming for central immunological tolerance for self antigens, and high expression of stathmin in this organ secures effective control of adaptive immunity against it. As a consequence of marked thymic expression of

25   stathmin in humans, it may be expected that robust immunological tolerance exists for the molecule. Administration of stathmin to humans is therefore unlikely to provoke cellular or humoural (antibody) immune responses which is of significant advantage when considering its potential therapeutic use.

The predominance of stathmin in the nervous system is even more pronounced

30   for the related stathmin-like proteins SCG10 (stathmin-like 2) and RB3 (stathmin-like 4) that are expressed in the human central nervous system at about 100-fold higher levels than in other organs. SCG10 in particular appears to be neuron-specific and tends to accumulate in membranes of growth cones of axons and dendrites.

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Several data indicate that stathmin levels in the central nervous system gradually decline with increasing age, and that trauma-induced elevated expression of stathmin becomes blunted. Also in rodents stathmin is widely expressed in the embryonic and neonatal brain while its levels of expression progressively decline during development.

5   In the adult rat brain, immunoreactivity is only retained in highly proliferative areas such as the adult subventricular zone (SVZ) which has a particularly important role in generating glial and neural progenitors. Also in response to demyelinating conditions cells in the SVZ re-acquire this gliogenic potential. In line with its likely function in development and/or migration of glial progenitor cells stathmin is down-

10   regulated during oligodendrocyte differentiation *in vitro* and re-expressed in oligodendrocytes in the brains of MS patients. This parallels its elevated expression in animal models of demyelination. Stathmin accumulates not only inside the oligodendrocyte cell body but also closely associated with myelin membranes. Apart from the apparent stathmin response to demyelination, increased expression of

15   stathmin or its family members has also been documented in rats after application of experimental cortical lesions and hypoglossal nerve injury. In rat cortical neuronal cultures, stathmin is induced in response to cycloheximide-induced stress, heat stress and the glutamate antagonist MK801. In cultured oligodendrocytes stathmin expression is induced by mediators including TNF-α and TGF-β. In rat retinal

20   ganglion cells, ciliary neurotrophic factor (CNTF) selectively induces RB3 after axotomy. Interestingly therefore, expression of stathmin and stathmin-like proteins is induced in these different neural cells at least in part by several factors such as TNF-α, TGF-β and CNTF that are produced by astrocytes in response to TLR3 activation (see below). These observations suggest the existence of a positive feedback

25   mechanism that promotes stathmin-TLR3 interactions in the CNS: TLR3 activation on astrocytes leads to secretion of several factors that promote stathmin expression in other neural cells, while at the same time (in this case primarily through IFN-β) it also promotes TLR3 expression itself.

The pattern of stathmin expression in vertebrates along with accumulating

30   functional data thus strongly suggest that the various members of the stathmin family play complementary roles in the development, maturation, and functional regulation in response to damage or trauma especially of the central nervous system. Inhibition of stathmin expression with antisense oligonucleotides prevents nerve

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growth factor-induced differentiation of rat PC12 cells into sympathetic-like neurons.
Reducing the expression of stathmin with an antisense oligonucleotide inhibits
migration of new neurons from the SVZ to the olfactory bulb via the rostral migratory
stream. In line with an important role of stathmin especially in the CNS, inactivation

5   of the stathmin gene in mice leads to axonopathy in aged (20-month old) mice. Also, it
has recently been shown that stathmin-deficient mice show deficits in long-term
potentiation in the lateral nucleus of the amygdala, resulting in decreased memory in
fear conditioning. Stathmin deficient mice additionally displayed defects in innate
fear that normally occurs in naturally adverse environments. Interestingly therefore,

10   stathmin appears to play an important role in the molecular mechanisms gouverning
innate and learned fear.

       Other than these effects, ablation of stathmin expression in mice does not
appear to produce gross defects. Probably, the presence of various stathmin family
members (in particular in the CNS) compensates for the loss of stathmin and limits

15   the functional deficits in mice when they fail to produce only one of the four stathmin-
like family members. Such redundancies do not exist in Drosophila in which only a
single stathmin-related gene is known to exist, designated the D-stathmin gene. D-
stathmin is structurally homologous to vertebrate stathmin and displays very similar
molecular and biochemical properties. Also, the intron/exon junctions of the

20   Drosophila stathmin gene are the same as those in vertebrates indicating common
ancestry and like for the RB3 gene in vertebrates, the Drosophila stathmin gene
encodes for several splice variants. During Drosophila embryogenesis, expression of
D-stathmin is mainly restricted to migrating germ cells and neurons of the central and
peripheral nervous system. D-stathmin expression in the early Drosophila embryo is

25   similar to that of stathmin in the mouse or in Xenopus. D-stathmin is highly and
uniformly expressed at the very beginning of embryogenesis before cellularisation,
when mitosis is very active and microtubules are highly dynamic. During
embryogenesis, after the end of the blastoderm stage, D-stathmin is only expressed in
germ cells and cells of the central and peripheral nervous systems. This pattern that

30   is very similar to that found for vertebrate stathmin. In Drosophila, inhibiting
stathmin expression by siRNA leads to dramatic defects only in the nervous system.
In the normal embryo, most of the axons of the ventral nerve cord are organized in a
repetitive pattern of commissures. In siRNA-treated embryos on the other hand,

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formation of the commissures fails to occur or is defective. Also the peripheral nervous system is affected. Thus, D-stathmin is crucially important at various stages of differentiation and maturation of the *Drosophila* nervous system, mostly in ways that are relevant to neurite and axon formation.

5   The transcriptional and/or translational regulation of stathmin expression is only partly documented. Based on a sequence analysis of the stathmin gene, putative binding sites exist for the transcription factors E2F (position -701 to -694; -28 to -21; -19 to -11 and 715 to 722), sp1 (-123 to -114; -97 to -88, 760-769, 789-798 and 871-880) and AP-2 (-231 to -223 and -207 to -198). The regulatory role of E2F has been

10   documented in more detail In response to genotoxic stress. E2F mediates down-regulation of stathmin expression in the NIH3T3 cell line (Polager and Ginsberg, J Biol Chem 278: 1443-1449 [2003]). In the human prostate carcinoma cell line PC-3-M, the E2F sites in the stathmin gene were found essential for overexpression of the gene product. Especially E2F therefore appears to be important for controlling levels of

15   stathmin although in different cell types, E2F-mediated regulation can apparently impact on expression levels in different ways.

        Limited information is available on stathmin expression during neurodegenerative disorders in humans. In contrast to markedly elevated levels of stathmin in inflammatory demyelinating lesions that typify multiple sclerosis, levels

20   of stathmin appear to be lower in Alzheimer's Disease-associated pathology as compared to normal brains. The difference in stathmin levels between Alzheimer's and normal brains, however, is relatively modest. Okzazaki and co-workers have reported decreased levels and altered subcellular distribution of stathmin and SCG10 only in association with tangles in Alzheimer's brains but not with plaques. Such a

25   mild decrease has also been noted by others. Also in a mouse model of amyotrophic lateral sclerosis, stathmin levels appear lower than normal. Together, these data suggest that high levels of stathmin are induced by inflammation as seen during multiple sclerosis, and low levels of stathmin are associated with a state in which the brain's ability to appropriately respond to trauma or damage is compromised.

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Fields of application

        The invention discloses that stathmin or stathmin-like proteins can act as agonists for TLR3-mediated signalling by astrocytes, and that this property can be

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used to activate anti-inflammatory and tissue-repair responses at a time that such is
required. With regard to the CNS, this is for example the case when the damaging
effects need to be counteracted of chronic or acute neurodegenerative disorders
including but not limited to those described herein. Activation of the TLR3-mediated

5   signalling pathway in astrocytes, either directly by ligation of TLR3 using stathmin or
stathmin-like proteins, or indirectly by another activation step involving one of the
down-stream TLR3-associated signalling molecules, will thus lead to production of a
range of astrocyte-derived neuroprotective, regenerative and anti-inflammatory
factors that are expected to have a beneficial effect in CNS disorders. In line with

10   previous reports, our data also confirm that TLR3-mediated signalling will lead to
induction of higher levels of expression of TLR3 itself on astrocytes. At the same time,
several of the factors that are secreted in response to TLR3 activation promote the
expression of stathmin and/or stathmin-like proteins. These observations support the
idea that stathmin/TLR3 interactions are self promoting.

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Identifying a TLR3 agonist

At the present time versions of double-stranded RNA are known for their
ability to activate TLR3-mediated signalling. Ways to identify additional compounds
that can activate TLR3-mediated signalling pathways involve screening assays that

20   monitor TLR3 signal transduction pathway in cells that express TLR either naturally,
or after transfection with an TLR3-encoding gene construct. Such methods have been
recently described in detail in the literature. In preferred embodiments, the readout
for the screening assay is based on the use of native genes or, alternatively,
transfected or otherwise artificially introduced reporter gene constructs which are

25   responsive to the TLR3 signal transduction pathway. Reporter genes and reporter
gene constructs particularly useful for the assays include, e.g., a reporter gene
operatively linked to a promoter sensitive to NF-κB or IRF-3. Reporter genes
operatively linked to the TLR3-sensitive promoter can include, without limitation, an
enzyme (e.g., luciferase, alkaline phosphatase, ß-galactosidase, chloramphenicol

30   acetyltransferase (CAT), etc.), a bioluminescence marker (e.g., green-fluorescent
protein (GFP, etc.), a surface-expressed molecule (e.g., fibroblast growth factor
receptor), and a secreted molecule (e.g., CXCL8, MIP-2α, MIP-1α, TNF-α). In assays
relying on enzyme activity readout, substrate can be supplied as part of the assay,

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and detection can involve measurement of chemiluminescence, fluorescence, colour development, incorporation of radioactive label, drug resistance, or other marker of enzyme activity. For assays relying on surface expression of a molecule, detection can be accomplished using flow cytometry (FACS) analysis or functional assays. Secreted

5   molecules can be assayed using enzyme-linked immunosorbent assay (ELISA) or bioassays. These and other suitable readout systems are well known in the art and are commercially available. Using such assays the functional TLR3 is, preferably *in vitro*, contacted with a test compound in order to identify a compound that activates the TLR3-mediated production of repair factors. Such a compound is a natural or

10   synthetic compound that is capable of inducing a TLR3-mediated response when contacted with the reporter cell. Such an test compound that selectively binds to TLR3 and induce a TLR3 signal transduction pathway includes but is not limited to nucleic acids, including oligonucleotides and polynucleotides; oligopeptides; polypeptides; lipids, including lipopolysaccharides; carbohydrates, including

15   oligosaccharides and polysaccharides; and small molecules.

Libraries of compounds that can be used as test compounds are available from various commercial suppliers, and they can be made to order using techniques well known in the art, including combinatorial chemistry techniques. Especially in combination with high throughput screening methods, such methods including in

20   particular automated multichannel methods of screening, large libraries of test compounds can be screened according to the methods of the invention. Large libraries can include hundreds, thousands, tens of thousands, hundreds of thousands, and even millions of compounds.

Thus in preferred embodiments, the methods for screening test compounds can

25   be performed on a large scale and with high throughput by incorporating, e.g., an array-based assay system and at least one automated or semi-automated step. For example, the assays can be set up using multiple-well plates in which cells are dispensed in individual wells and reagents are added in a systematic manner using a multiwell delivery device suited to the geometry of the multiwell plate. Manual and

30   robotic multiwell delivery devices suitable for use in a high throughput screening assay are well known by those skilled in the art. Each well or array element can be mapped in a one-to-one manner to a particular test condition, such as the test compound. Readouts can also be performed in this multiwell array, preferably using a

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multiwell plate reader device or the like. Examples of such devices are well known in the art and are available through commercial sources. Sample and reagent handling can be automated to further enhance the throughput capacity of the screening assay, such that dozens, hundreds, thousands, or even millions of parallel assays can be

5   performed in a day or in a week. Fully robotic systems are known in the art for applications such as generation and analysis of combinatorial libraries of synthetic compounds. Alternatively, the binding of a test compound to the TLR3 polypeptide can also be determined directly. For example, a radiolabelled test substance can be incubated with the TLR3 polypeptide so that binding of the test substance to the

10   polypeptide can be monitored. Typically, the radiolabelled test substance can be incubated with cell membranes containing the polypeptide until equilibrium is reached. The membranes can then be separated from a non-bound test substance and dissolved in scintillation fluid to allow the radioactive content to be determined by scintillation counting. Non-specific binding of the test substance may also be

15   determined by repeating the experiments in the presence of a saturating concentration of a non-radioactive ligand. Preferably, a binding curve is constructed by repeating the experiment with various concentrations of the test substance.

As used herein, preferred TLR3-agonists are selected from the group consisting of:

20   - stathmin, TLR3-activating derivatives thereof and precursors thereof,

         - stathmin-like proteins, TLR3-activating derivatives thereof and precursors thereof, and

         - proteinaceous compounds comprising a polypeptide chain having an amino acid sequence similarity of at least 60% with an amino acid sequence selected

25   from the group consisting of the amino acid sequences of stathmin (SEQ ID NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3 (SEQ ID NO: 4).

The above compounds all exhibit the ability to activate TLR3. The structure of TLR3 was described by Choe et al. in 2005 (Science, 2005 Vol. 309 (5734):581-5). They

30   found that the human TLR3 ectodomain structure at 2.1 angstroms reveals a large horseshoe-shaped solenoid assembled from 23 leucine-rich repeats (LRRs). Asparagines conserved in the 24-residue LRR motif were found to contribute extensive hydrogen-bonding networks for solenoid stabilization. TLR3 was further

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found to be largely masked by carbohydrate, but one face was glycosylation-free, which suggested a potential role in ligand binding and oligomerization. Highly conserved surface residues and a TLR3-specific LRR insertion were found to form a homodimer interface in the crystal, and two patches of positively charged residues

5   and a second insertion were thought to provide an appropriate binding site for for instance double-stranded RNA. Without wishing to be bound by theory, it is believed that the alpha helix of stathmin, which contains a large amount of negatively charged glutamine residues, is equally capable of binding in this region as double stranded RNA and poly I:C molecules.

10   Therefore in order to assess whether a stathmin derivative, a stathmin-like protein, or a derivative thereof, or in fact any compound is capable of activating TLR3 and therefore capable of eliciting a TLR3-mediated response, one may test the ability of the molecule to bind to the ligand binding site described by Choe *et al.* (*supra*), i.e. to bind the two patches of positively charged residues together. The skilled person will

15   be aware of the various possibilities of testing this capability *in silico*, for instance by testing the interaction between the proteins using 3D-visualization of protein/ligand interaction or virtual reality models presently used in drug design (see for instance Morphy and Rankovic. 2005. J. Med. Chem. 48(21):6523-43; Raitio *et al.* 2005. Review. Curr. Med. Chem. 12:1217-1237; Salo *et al.* 2004. J. Med. Chem. 47:3048-

20   3057; Salo, *et al.* 2005. J Med Chem. 48:7166-71; Tervo *et al.* 2003. J. Comp. Aid. Mol. Des. 17:797-810; Warren *et al.* 2006. J. Med. Chem. 49(20):5912-31 the contents of which are incorporated herein by reference).

Use of the TLR3-agonist

25   In addition to using the TLR3-agonists, such as stathmin and/or stathmin-like proteins directly, the present invention also envisages the *in vivo* promotion of the TLR3-agonists, such as stathmin and/or stathmin-like proteins. To promote the beneficial consequences of interactions between the agonist compound, such as stathmin or stathmin-like proteins, with TLR3 on astrocytes, various strategies can

30   be envisaged. First, the agonist can be administered in such as way that they gain access to various tissues and can activate TLR3 expressed on the surface or intracellularly by target cells such as fibroblast-like cells (astrocytes, synoviocytes), epithelial cells, mast cells, macrophages or macrophage-like cells and dendritic cells

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or dendritic cells-like cells. Under normal conditions, access of peripherally administered proteins to the CNS is very limited due to the existence of a blood-brain barrier which severely restricts passage of blood-borne substances into the brain and spinal cord parenchyma. Yet, it should be noted that in inflammatory CNS disorders

5   activation and consequently, partial disruption of this barrier has been well documented. The activated and partially disrupted state of the blood-brain barrier under those conditions allow for "leakage" of blood-derived proteins of molecular weights far exceeding that of for instance the stathmin protein through the endothelial barrier into CNS tissue. Thus, especially in anatomical areas of ongoing

10   inflammation in the CNS (areas where TLR3 expression is also increased and where therapeutic intervention should be directed at), access by peripheral proteins is strongly enhanced. Secondly, substances can be administered that induce higher levels of expression of the agonist such as stathmin or stathmin-like proteins notably in cells of the CNS, or that promote surface exposure or even secretion of the agonist

15   by resident cells, thus promoting local activation of TLR3-mediated signalling. Thirdly, substances can be administered that promote formation and/or stabilise the functional structural element of the agonist for interactions with TLR3, which in the case of stathmin most likely includes the extended alpha helical structure between residues 44 and 138. Fourthly, substances can be administered that induce higher

20   levels of expression of TLR3 on target cells such as fibroblast-like cells (astrocytes, synoviocytes), epithelial cells or mast cells. The present invention discloses that this is likely to contribute to beneficial responses. In fact, IFN-β is already an example of one such substance that has this effect and is known to act in a clinically beneficial way during multiple sclerosis. Fifthly, substances can be administered that activate

25   selective TLR3-mediated signalling pathways in target cells such as fibroblast-like cells (astrocytes, synoviocytes), epithelial cells, mast cells or dendritic cells.

Stathmin, stathmin-like proteins or any of the substances that promote stathmin expression, stabilisation of its helix, TLR3 expression or TLR3-mediated signalling used in aspects of the present invention may be used in non-complexed,

30   non-bound form, i.e. in free form, not covalently linked to another (bio)molecule, but may also be used in complexed form designed for example to facilitate transport to a target tissue such as the CNS, or to enhance its biological half-life. It is also possible

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to use a combination of stathmin and stathmin-like proteins in aspects of the present invention.

In principle, stathmin and stathmin-like proteins of all possible sources are suitable for use in aspects and embodiments of the present invention. For instance, a

5   suitable stathmin and stathmin-like proteins may be obtained from plants, from animals or from micro-organisms, such as yeasts. They may be produced in those sources naturally as a metabolite or may be produced therein as recombinant proteins. The stathmin and stathmin-like proteins may be isolated from these organisms or can be used in a less pure form, i.e. as an enriched fraction, or in the

10   case of microorganisms such as (recombinant) yeasts by taking the complete organism(s) or fractions thereof. Further, stathmin and stathmin-like proteins may be isolated from other suitable sources, such as from milk, egg, soy, yeast, bacteria, algae, plants, meat, brain, etc. or may be synthetically prepared, for use in a pharmaceutical composition according to the invention or for instance in a

15   nutraceutical composition (i.e. a food item or food supplement).

Stathmin and stathmin-like proteins may be derived from the above sources by methods known to the skilled person for instance by extraction with (organic) solvents, chromatographic separation, precipitation, crystallization and/or enzymatic of chemical hydrolysis. The isolation of stathmin or stathmin-like proteins may for

20   instance occur by using stathmin-binding proteins.

Stathmin as used in compositions of the present invention can be purified from natural or recombinant sources, or can be purchased commercially. Recombinant stathmin may for instance be produced and purified as described by Curmi *et al.* (1994) *Biochem. J.* **300**:331-338. Alternatively, one may use a procedure wherein

25   recombinant stathmin is produced in a producer strain such as E. coli using methods well known in the art. For instance, full length cDNAs comprising the complete or partial coding sequence of stathmin may be prepared synthetically and ligated into a suitable expression system to yield a polynucleotide construct comprising the complete or partail coding sequence of stathmin under the control of a suitable

30   promotor for expression in a producer strain. Appropriate polynucleotide constructs are purified using standard recombinant DNA techniques as described in, for example, Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.). The polypeptides encoded by

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the polynucleotides are expressed in any expression system, including, for example, bacterial, yeast, insect, amphibian and mammalian systems. Suitable vectors and host cells are described in U.S. Pat. No. 5,654,173.

Expression systems in bacteria include those described in Chang et al., Nature

5   (1978) 275:615, Goeddel et al., Nature (1979) 281:544, Goeddel et al., Nucleic Acids Res. (1980) 8:4057; EP 0 036,776, U.S. Pat. No. 4,551,433, DeBoer et al., Proc. Natl. Acad. Sci. (USA) (1983) 80:21-25, and Siebenlist et al., Cell (1980) 20:269.

When production occurs in bacterial cells, the recombinant stathmin may be purified from the producer strain by sonication of the stathmin-expressing

10   recombinant bacteria in extraction buffer, supplementing the low speed supernatant of the sonicated culture with 100 mM NaCl, and heating the mixture at 100 °C for 3 min, followed by ultracentrifugation. Next, anion exchange chromatography using DEAE-Sepharose CL-6B and gel filtration using Superose 12 will lead to a pure preparation of stathmin that can be concentrated in aqueous buffers and stored in a

15   refrigerated environment.

Salts of peptides or functional equivalents are prepared by known methods, which typically involve the mixing of the peptide or peptoid with either a pharmaceutically acceptable acid to form an acid addition salt, or with a pharmaceutically acceptable base to form a base addition salt. Whether an acid or a

20   base is pharmaceutically acceptable can be easily decided by a person skilled in the art after taking the specific intended use of the compound into consideration. For instance, not all acids and bases that are acceptable for in vitro diagnostic compositions can be used for therapeutic compositions. Depending on the intended use, pharmaceutically acceptable acids include organic and inorganic acids such as

25   formic acid, acetic acid, propionic acid, lactic acid, glycolic acid, oxalic acid, pyruvic acid, succinic acid, maleic acid, malonic acid, cinnamic acid, sulfuric acid, hydrochloric acid, hydrobromic acid, nitric acid, perchloric acid, phosphoric acid, and thiocyanic acid, which form ammonium salts with free amino groups of peptides and functional equivalents. Pharmaceutically acceptable bases, which form carboxylate salts with

30   free carboxylic groups of peptides and functional equivalents, include ethylamine, methylamine, dimethylamine, triethylamine, isopropylamine, diisopropylamine, and other mono-, di- and trialkylamines, as well as arylamines. Moreover, also pharmaceutically acceptable solvates are encompassed.

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Stathmin, stathmin-like proteins or any of the substances that promote stathmin expression, stabilisation of its helix, TLR3 expression or TLR3-mediated signalling may be formulated with standard pharmaceutically acceptable carriers and/or excipients as is routine in the pharmaceutical art. These compounds may be

5   administered by enteral or parenteral routes such as via oral, buccal, anal, pulmonary, intra-nasal, intravenous, intra-arterial, intramuscular, intraperitoneal, transdermal, topical, subcutaneous, sublingual, intralesional, or delivered to the interstitial space of a tissue or other appropriate administration routes.

The skilled person is aware that therapeutic proteins/peptides are mostly

10   administered as parenteral (injectable) preparations as a result of their poor oral bioavailability which is due to degradation by proteolytic enzymes, poor membrane permeability and large molecular size. However, the oral route would be preferred to the parenteral administration because it is more convenient for self-administration, non-invasive and more patient friendly. Consequently, efforts have intensified over

15   the past two decades to maximize the extent of absorption of protein and peptide drugs in order to achieve optimum bioavailability via the oral route. A suitable oral delivery system retains the drug and maintains its integrity until it gets to the region of maximum absorption where the protein/peptide is released. The skilled person may perform various pharmacokinetic studies to evaluate the effect of the various routes of

20   administration. Generally, the requirements for therapeutic proteins with respect to evaluating the pharmacokinetics of the product are the same as for conventional products, but specific considerations are needed related to the inherent characteristics of proteins. The pharmacokinetics (absorption, distribution and elimination) are preferably characterised during single-dose and steady-state conditions in relevant

25   populations. However, the pharmacokinetic requirements may differ depending on the type of protein.

A stathmin or stathmin-like protein, or a derivative or a precursor thereof, may be provided to a subject in need thereof for prophylactic or therapeutic reasons in any administration form. For instance in the form of a food product, a food

30   supplement, or a pharmaceutical preparation, all such administration forms being capable of initiating the TLR3-mediated repair response, whereby in particular the exertion of the neuroprotective effects of TLR3-activation are considered. The pharmaceutical preparation may be in the form of a capsule, tablet, lozenge, dragee,

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pill, droplet, suppository, powder, spray, vaccine, ointment, paste, cream, inhalant, patch, aerosol, as a drip, an infusion, injection, and the like. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection

5   may also be prepared. Yet in another alternative embodiment, the stathmin or stathmin-like proteins or compositions of the invention may be administered from a controlled or sustained release matrix inserted in the body of the subject.

Further to the stathmin or stathmin-like protein, or a derivative or a precursor thereof, the pharmaceutical composition of the invention may comprise a suitable

10   pharmaceutically acceptable carrier, and optionally additional pharmaceutically acceptable excipients

For therapeutic treatment, stathmin or stathmin-like proteins may be obtained or produced as described above and applied to the subject in need thereof. The stathmin or stathmin-like protein may be administered to a subject by any suitable

15   route, preferably in the form of a pharmaceutical composition adapted to such a route and in a dosage that is effective for the intended treatment. Therapeutically effective dosages of the stathmin or stathmin-like protein required for treating the disorder, for instance for prevention and/or treatment of exaggerated inflammation in the body of a human or animal subject, can easily be determined by the skilled person, for instance

20   by using animal models.

As stated above, the term "therapeutically effective amount" as used herein refers to an amount of a therapeutic, viz. a stathmin or stathmin-like protein, to reduce or prevent the tissue deterioration process or induce the TLR3-mediated repair response, or to exhibit a detectable therapeutic or prophylactic effect. The effect can

25   be detected by, for example, measurement of clinical signs resulting from tissue degradation such as motor functions or behaviour, direct assessment of tissue degeneration by histological examination, or by monitoring levels of biomarkers that are associated with tissue degeneration in biological fluids or tissue samples or by any other suitable method of assessing the progress or severity of inflammation which

30   methods are known per se to the skilled person. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the condition, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance.

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However, the effective amount for a given situation can be determined by routine experimentation and is within the judgment of the clinician or experimenter. Methods that permit the clinician to establish initial dosages are known in the art. The dosages to be administered must be safe and efficacious.

5   Dosages for achieving the therapeutic effects of the medicament, pharmaceutical composition, food item or food supplement described herein may easily be determined by the skilled person. The dose of a compound to be administered may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be

10   treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient.

In developing a proper dosage regime, the skilled person may be guided by studies available on the therapeutic effect of poly I:C in animal models. For example,

15   Fitzgerald et al. (J. Immunol 2006 Vol.177: 7505-7509) have recently described such a study for the treatment of a degenerative inflammatory disease in a standard animal model for multiple sclerosis (experimental autoimmune encephalomyelitis or EAE) using poly I:C. The chronic paralytic disorder EAE is caused by T-cell reactivity against self brain antigens, which is elicited in rodents or primates by immunization

20   with such brain antigens. Usually, the disease becomes apparent 2 weeks after immunization, and takes a chronic relapsing-remitting course thereafter. Treatment of mice with the TLR3 agonist poly I:C 5, 7 and 9 days after experimental induction of the disease almost completely blocks subsequent disease development. This effect is mediated largely by anti-inflammatory mediators that are induced by TLR3

25   activation, and includes IFN-beta.

Such approaches may also be used in investigating proper therapeutic dosage regimes for stathmin and stathmin-like proteins. For instance, stathmin and/or one or more stathmin-like proteins may be dissolved in phosphate-buffered saline which solution is administered by intraperitoneal injection at a dose of 100 microgram per

30   mouse for each injection. Injection is then for instance performed three times, every second day, starting 5 days after immunization of the animal to induce disease, in the same way as described by Fitzgerald et al. (supra) for poly I:C. The dose of poly I:C

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used in the experiments described by Fitzgerald *et al.* (*supra*) was found to be about 5 mg/kg of body weight.

In humans intraperitoneal injection is less acceptable, but other systemic routes of administration such as intramuscular, subcutaneous, oral or intravenous administration are very suitable alternatives. When taking surface to body weight ratio's between mouse and human into account, the mg/kg dose that would be equivalent in humans is 12.6 times less than that in mice, i.e., about 0.4 mg/kg of human subject. *In vitro* data obtained so far indicate that stathmin at the same weight doses would be at least as effective as poly I:C in inducing anti-inflammatory mediators in various cell types. Therapeutically effective doses in humans can thus be estimated at around this level of 0.4 mg/kg body weight, based on comparable studies performed in mice.

For purposes of the present invention, an effective daily dose will be from about 0.01 µg/kg to 1 g/kg and preferably from about 0.5 µg/kg to about 400 mg of therapeutic protein/kg body weight in the individual to which it is administered. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the compound, the age, weight and conditions of the subject to be treated, the type and severity of the disease and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

In a pharmaceutical composition as described above, a stathmin or stathmin-like protein, or a derivative or a precursor thereof, is used in an amount of from 0.01 to 99.9 % by (dry) weight based on the total weight of the composition, preferably from 0.01 to 10 wt.%, and more preferably from 0.01 to 5 wt.%.

The present invention further relates to a method for the preparation of a pharmaceutical composition for the prevention and/or treatment of a neurodegenerative disorder selected from the group consisting of stroke, hypovolemic shock, traumatic shock, reperfusion injury, multiple sclerosis, AIDS-associated dementia, neuron toxicity, Alzheimer's disease (AD), head trauma, acute spiral cord injury, Huntington's disease (HD), Parkinson's Disease (PD) and related synucleinopathies, dystonia, Tourette Syndrome, Frontotemporal Dementias (FTDs) and related tauopathies, prion disorders such as Fatal Familial Insomnia (FFI) and other disorders related to Creutzfeldt-Jakob disease, amyotrophic lateral sclerosis (ALS), trinucleotide repeat diseases, Motor Neurone disease, progressive

supranuclear palsy, REM Sleep Behavior Disorder (RBD) and cancer in a subject, comprising processing or incorporating a stathmin or stathmin-like protein, or a derivative or a precursor thereof, as an active substance, together with a pharmaceutically acceptable carrier in a pharmaceutical composition.

5   The preparation of a pharmaceutical composition may very suitably occur by mixing all separate ingredients such as fillers, binders, lubricants and optionally other excipients together with a stathmin or stathmin-like protein, or a derivative or a precursor thereof, and processing the mixture obtained to a pharmaceutical preparation.

10   A pharmaceutical composition according to the present invention may, in addition to comprising one or more stathmin or stathmin-like proteins, comprise one or more other pharmaceutically active compounds. Very suitable additional pharmaceutically active compounds are for instance additional anti-inflammatory compounds, antiviral agents, antibodies against infectious prions, and other

15   therapeutic compounds generally used for the treatment of degenerative inflammatory disorders, in particular neurodegenerative and/or neuro-inflammatory diseases. A preferred pharmaceutical composition comprising additional pharmaceutically active compounds takes the form of a combined preparation.

The present invention in particular relates to a combined preparation of a

20   stathmin or stathmin-like protein, or a derivative or a precursor thereof, and a medicament which is neuro-toxic, or is recognized as damaging nervous tissue. The advantage of such a combined preparation is that the harm to the nervous tissue as imparted by said medicament (i.e. by a pharmaceutical compound or by an excipient comprised in a pharmaceutical composition comprising said compound) is

25   considerably decreased as a result of the presence of the stathmin or stathmin-like proteins in said preparation. A combined preparation may have the form of at least two separate preparations at least one preparation comprising a stathmin or stathmin-like protein and at least another preparation comprising the neuro-damaging medicament, which two separate preparations are packaged together and

30   intended for simultaneous, separate or sequential use. Preferably, however, a combined preparation according to the present invention comprises a stathmin or stathmin-like protein and a neuro-damaging medicament together in the form of a single preparation.

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The present invention thus contemplates the prevention or inhibition of drug-
or drug excipient induced neuro-toxicity or nervous tissue damage in general terms,
preferably arising from, but not limited to exaggerated inflammation associated with
acute or chronic use of drugs. Examples of drugs and excipients that have been

5   associated with neuro-toxicity are analgesic drugs (for example opioids), anti-
depressants, drugs to manage pain, psychoses or personality disorders, drugs to
manage sleeping disorders, cytostatic drugs used in the management of cancer. These
examples are medicaments which can cause neuro-toxic effects, often in conjunction
with some level of inflammation.

10   In another aspect, the present invention relates to the use of a stathmin or
stathmin-like protein, or a derivative or a precursor thereof, and a medicament which
causes inflammation, or is recognized as enhancing inflammation, for the
manufacture of a combined preparation for simultaneous, separate or sequential use
in therapy.

15   In general terms, the stathmin-inducible TLR3-mediated response can be
defined as 'wound healing", which is a complex process involving integrated steps of
cell proliferation, migration, differentiation and production of extracellular matrix
components. Based on the present understanding of the biological functions of the
mediators induced by TLR3 activation in astrocytes and synoviocytes (as elaborated

20   above), these mediators are supportive for the process of wound healing. Several of
the factors produced by TLR3-activated astrocytes and synoviocytes have well-known
repair functions in various organs and tissues other than the CNS or the joint.
Therefore, application of compounds that selectively activate TLR3-mediated
responses can be envisaged also for inducing wound healing outside these organs. The

25   term "inducing" as used herein, refers to the activation, stimulation, enhancement,
initiation and/or maintenance of cellular mechanisms or processes necessary for the
formation of a tissue or a portion thereof, repair process or tissue development as
described herein. This can be achieved in a subject by contacting a wound with an
effective amount of stathmin or a stathmin-like protein or another substance that can

30   activate TLR3-mediated responses. A subject may be any mammal, preferably human.
The contacting may be topically or systemically. Examples of topical administration
include, for example, contacting the wound with a lotion, salve, gel, cream, paste,
spray, suspension, dispersion, hydrogel, ointment, or oil comprising stathmin, a

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stathmin-like protein or another TLR3 agonist. Systemic administration includes, for example, intravenous, intraperitoneal, intranasal, intramuscular injections of a composition containing stathmin, a stathmin-like protein or another TLR3 agonist. Administration of such a TLR3 activating compound may produce beneficial

5   effects following traumatic injuries or in conditions including arthritis, osteoporosis and other musculo-skeletal disorders, burns, ulcers and other skin lesions, peripheral nerve disease and cardiovascular diseases including ischemia and atherosclerosis. Other potential tissues which can be treated by the methods and compositions of the invention include epidermal, eye, uro-genital, gastrointestinal, cardiovascular, muscle

10   and connective tissues.

       The invention may also be applied in a wide variety of other disorders where an anti-inflammatory, anti-fibrotic or regenerative response is beneficial. These disorders may include but not be limited to fibrotic disorders of various organs, allergies and inflammatory degenerative disorders of various organs including joints,

15   gastro-intestinal tract, pancreas, skin, mucosal tissue and lungs.

       EXAMPLES

       Example 1. TLR3 activation inhibits astrocyte growth, stimulate endothelial cell

20   **growth and promotes survival of neurons in organotypic brain slice cultures.**

       Given the emerging differences in both expression and functionality of several different TLR family members between rodents and humans, data from rodent models for CNS disorders cannot be directly extrapolated to the human situation without additional supportive evidence. To circumvent this problem we chose to study TLR

25   functions in cultured human astrocytes obtained from post-mortem brain samples shortly after death of a donor. Such astrocytes display many morphological and functional properties (especially in the regulation of inflammatory pathways) that are indistinguishable from human astrocytes *in vivo*. Cell biological features of these cultured human adult astrocytes are thus considered sufficiently representative for

30   their behaviour *in vivo* in an adult human brain to provide a useful conceptual framework to build on.

       Materials and methods used

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Human brain samples

Human brain material was obtained via the rapid autopsy system of the Netherlands Brain Bank which supplies postmortem specimens from clinically well documented and neuropathologically confirmed cases and controls. Autopsies are

5   performed on donors from whom written informed consent has been obtained either from the donor or direct next of kin. Samples were controlled for age, sex, seasonal alterations, circadian variation, clock time of death and medication. Postmortem factors include postmortem delay, storage time and laterality. Samples were also controlled for quality by monitoring the agonal state by pH. For all experiments, brain

10 material from controls was used except for the experiment of Fig. 5A and 5B that was derived of a case of Parkinson's disease.

Isolation and culture of human adult astrocytes and endothelial cells

Adult human astrocytes from post-mortem subcortical white matter samples

15 were isolated and cultured as previously described (Bsibsi *et al.*, J Neuropath Exp Neurol 61:1013-1021 [2002]). As determined by specific staining for cellular markers for astrocytes (glial fibrillary acidic protein; GFAP) and microglia (CD68) astrocyte cultures were essentially homogeneous; as a rule all cells expressed GFAP and none of them expressed CD68. When indicated astrocytes were stimulated with 50 μg/mL poly

20 I:C (Amersham Pharmacia Biotech, Piscataway, NJ) or 200 ng/mL LPS (Sigma-Aldrich, Steinheim, Germany) to activate TLR3 and TLR4, respectively. These doses were selected on the basis of maximum effects in astrocytes. It should be noted that contaminants in the commercial LPS preparation could possibly lead to some level of TLR2 activation in addition to TLR4 by the LPS preparation used but not of TLR3.

25 When stimulated with cytokines, astrocytes were supplied with TNF-α, IFN-γ, IL-1β (each at 500 U/mL), IL-4 (at 250 U/mL), IL-6 (at 200 U/mL), IL-12 (at 50 ng/mL), IL-10 (at 20 ng/mL) and TGF-ß (at 12.5 ng/mL) (PeproTech, Rocky Hill, NY). Control cDNA array profiling experiments included treatment with 50 μg/mL cycloheximide for 30 min prior to and during poly I:C treatment, or with 200 U/mL IFN-ß (Avonex).

30 Endothelial cells (UVEC) were isolated from umbilical cords and cultured on gelatin-coated dishes in M199 supplemented with 20nM HEPES, 10 % human serum, 10 % heat-inactivated NBCS, 150 mg/mL ECGF, 5 U/mL heparin, 100 IU/mL penicillin and 100 mg/mL streptomycin.

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Proliferation of human astrocytes and human endothelial cells was determined by conversion of WST-1 by viable cells (Roche Applied Science, Indianapolis, IN) for 3 h, and quantified by determining optical density at 490 nm.

5   *Real-time PCR*

Total cellular RNA was isolated using RNA-Bee™ as previously described (Bsibsi *et al.*, [2002] *supra*). Subsequently, RNA was reverse transcribed into cDNA and levels of TLR1-10 and β-actin as a reference were determined by quantitative real-time PCR. The following fluorogenic molecular beacons and primers (Biolegio,

10 Nijmegen, The Netherlands) were used:

β-actin: sense primers: 5' GGTCATCACCATTGGCAATGA 3';

anti-sense primer 5' ACGTCACACTTCATGATGGAGTTG 3'; beacon

cgtgccGCACTCTTCCAGCCTTCCTTCCTGggcacg;

TLR1: sense primer 5' GAAGAAAGTGAATTTTTAGTTGATAGGTCA 3'; anti-sense

15 primer

5' ACAGTGATAAGATGTCAGAAGTCCAAAG 3'; beacon:

cgtgccATCCACGTTCCTAAAGACCTATCCCAGAggcacg; TLR2: sense primer 5'

GAAATGTGAAAATCACCGATGAAAG 3'; anti-sense primer: 5'

TCCACTTTACCTGGATCTATAACTCTGTC 3'; beacon

20 CgtgccTTTGATGACTGTACCCTTAATGGAGTTggcacg; TLR3: sense primer 5 '

CAGTACATCGAGTTCTTGGTTTCAAA 3'; anti-sense primer:

5'GAGAAATGTTCCCAGACCCAATC 3'; beacon:

CgtgccCAGACAGACAGAACAGTTTGAATATGCAGCggcacg; TLR4: sense primer 5 '

TAAAGAATTTAGAAGAAGGGGTGCC 3'; anti-sense primer: 5'

25 CAACAATCACCTTTCGGCTTTTA 3'; beacon:

CgtgccGAGACTTTATTCCCGGTGTGGCCAggcacg.

The β-actin beacon contained 5' fluorescein VIC, and the 3'quencher Dabcyl. The TLR1-4 beacons contained 5' fluorescein FAM, and the 3'quencher Dabcyl. Thermal cycling consisted of 95 °C for 5 min and 40 cycles of 95 °C for 30 s, 56 °C for 20 s, 56 °C

30 for 20 s and 72 °C for 30 s. PCR was performed using an ABI PRISM® 7700 sequence detection system (Applied Biosystems, Foster City, CA). Data were analyzed using sequence detector version 1.7 software.

44

Organotypic brain slice cultures

Organotypic human brain slice cultures were set up and analyzed as previously described (Verwer *et al.*, *FASEB J.* **16**:54-60 [2002]). Briefly, tissue blocks were collected from different post-mortem cortical samples and 200-μm slices were

5   produced. These slices were cultured for 1 week in Poly I:C- or LPS-conditioned astrocyte media that were obtained upon culturing astrocytes in the presence of either 50 μg/mL poly I:C or 200 ng/mL LPS. As a control untreated astrocyte-conditioned media from the same donor was used. All astrocyte-conditioned media were harvested after 48 h and stored at -20 °C. Viability of neurons in the slice cultures was evaluated

10   using calcein-AM and ethidium homodimer-1 (Molecular Probes, Eugene, OR). A Zeiss 410 Invert confocal laser scanning microscope was used to inspect the slices and quantify staining.

cDNA array profiling

15   Analysis of the mRNA profile of astrocytes was performed by hybrid selection of ^{32}P-labeled cDNA on Clontech human cytokine/chemokine Atlas® arrays as previous described (Meeuwsen *et al.*, *Glia* **43**:243-253 [2003]). Hybridization signals for each of the 268 cytokine, chemokine and growth factor (receptor) genes were calculated as the mean of duplicate measurements, corrected for background intensity

20   and quantified using software provided by the manufacturer. Relative expression was calculated by dividing these hybridization signals by the mean signal for all nine housekeeping reference genes on the corresponding array.

Expression signals are expressed as the ratio of these relative levels of expression over those found in the untreated control cultures ± standard error of the

25   mean. Gene profiling was performed using astrocytes from four different control donors.

cDNA bank screening

Cos-7 cells (25,000 cells per well in 96-well plates) were transfected for 5 h

30   with 768 pools of cDNA derived from human brain tumours (5 pooled tumours including meningioma, oligodendroglioma, astrocytoma grades II and IV, and medulloblastoma; NCI CGAP Brn35; Invitrogen) using lipofectamine, after which cells were thoroughly washed. Transfection with green-fluorescent protein-encoding

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cDNA was used as an internal control for transfection efficiencies. After continued
culture of the transfected cells for 48 h, 75 µL of each culture supernatant was
harvested and added to cultured human astrocytes seeded at 1,000 cells per well in 75
µL. After 48 h of culture, supernatants were harvested and evaluated for CXCL8

5   release using ELISA. Stimulation of astrocytes with poly I:C served as positive
controls for CXCL8 release in all cases, non-transfected Cos 7 culture supernatants as
negative controls.

       Additional control experiments using murine astrocytes or transfected
HEK293 cells were performed essentially as described previously by several other

10   research groups.

Statistical methods

       All data in Fig. 2 are expressed as means ± standard deviations. Groups were
compared by one-way analysis of variance (ANOVA), and p values were calculated by

15   post-hoc multiple comparison tests. To evaluate statistical significance in neuronal
viability in organotypic brain slice cultures the non-parametric Kruskal-Wallis test
was applied to the absolute counts of viable or dead neurons in each microscopic field.
P values < 0.05 were considered statistically significant.

20   **Results**

Preferential TLR3 induction in human astrocytes in response to cytokines or TLR
agonists

       The TLR expression profile of cultured adult human astrocytes derived from
six different donors was examined by RT-PCR. Taking into account some donor-to-

25   donor variation post-confluent astrocytes predominantly express TLR4 accompanied
by lower levels of TLR2 and TLR3 (Fig. 1A). Expression levels of TLR1 were generally
very low while in all six cultures analysed expression of TLR5-10 was essentially
undetectable by RT-PCR. TLR expression levels were next compared to that of cells
from the same donor activated with a range of cytokines (Fig. 1B). Following

30   stimulation with TNF-α, IL-1β, IFN-γ, IL-12, IL-4 or IL-6 a strikingly selective
induction of TLR3 of up to 360 fold was observed. The other TLR were not induced
and in several cases were even suppressed which was particularly apparent for TLR2.
No induction of TLR3 was seen after treatment of astrocytes with the anti-

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inflammatory cytokines TGF-β and IL-10. Induction of elevated levels of TLR3
expression was also observed after stimulation of astrocytes with poly I:C (a synthetic
mimic of double-stranded RNA) or with LPS to activate TLR3 and TLR4, respectively
(Fig. 2A). Upon stimulation with poly I:C or LPS up to 400-fold induction of TLR3,

5   and in some cases also of TLR2 was observed while as a rule, expression of the other
TLR did not change. Induction of TLR2 was an indirect effect of TLR activation by
poly I:C or LPS since it could be inhibited by cycloheximide, a protein synthesis
inhibitor, whereas the induction of TLR3 was insensitive to this treatment (Fig. 2B).
Also upon exposure to H_2O_2 to induce oxidative stress selective induction of TLR3 was

10   observed (data not shown).

These data extend previously published data on the restricted TLR profile of
adult human astrocytes and reveal marked expression of TLR4 on cultured cells and a
strikingly strong and selective induction of TLR3 upon activation by pro-
inflammatory cytokines, TLR agonists or oxidative stress. This is fully in line with our

15   previous observation that within the inflammatory environment of late stage multiple
sclerosis lesions TLR3 and TLR4 are prominently present on gliotic astrocytes
whereas they are undetectable by immunohistochemistry in healthy brains.
Interestingly, astrocytes express TLR3 and TLR4 only on their cell surface which
contrasts exclusive intracellular localization of these TLR in other cell types such as

20   dendritic cells or microglia. These data, as summarised in Figs. 3A and 3B, are largely
consistent with recent reports by other groups confirming TLR3 expression on foetal
human and murine astrocytes.

Gene profiling of human adult astrocytes stimulated with poly I:C and LPS

25   The dominant expression of TLR3 and TLR4 on activated astrocytes prompted
us to examine the functional response mediated by these two TLR family members by
gene profiling. Expression signals were evaluated by cDNA array analysis for 268
cytokines, chemokines, growth factors and their receptors that are key to the variety
of astrocyte functions. Validation of these cDNA arrays in examining astrocyte

30   responses has previously been documented by us. Astrocytes were cultured in the
presence of poly I:C, LPS or both agonists together and after 48 h, gene expression
profiles were compared to those in untreated parallel cultures. Figure 9 summarises
the averaged data from four separate experiments, each time using cells derived from

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a different donor. The results reveal that poly I:C but not LPS induced a range of mediators of growth, differentiation and neuroprotection along with several chemokines, anti-inflammatory cytokines and cytokine receptors. In line with previous reports (Doyle *et al.*, *Immunity* 17:251-263 [2002], Doyle *et al.*, *J. Immunol.*

5   170:3565-3571 [2003]), mediators of type I interferon production were also induced. Only three other gene products were found to be down-regulated by at least a factor of 2. These included VEGF receptor 2 (ratio of expression 0.22), ERBB4 receptor protein-tyrosine kinase (0.43) and *Smo* (0.48). The p40 subunit of the powerful pro-inflammatory mediators IL-12 and IL-23 was down-regulated by a factor of almost 2.

10   Several of the gene products induced by poly I:C are well-known mediators of neuroprotection. Brain-derived neurotrophic factor for example regulates dendrite formation in cortical neurons and protects neurons from infectious damage and apoptosis. Neurotrophin-4 promotes neuronal survival, differentiation and maturation (Poo, Nat Rev Neurosci 2: 24-32 [2002]). Similarly, glial growth factors 1 and 2 and

15   pleiotrophin support survival and differentiation of glial cells and neurons and the ephrin type-B receptor 1 guides migration and promotes connectivity of new neurons. Another mediator triggered by poly I:C is TGF-ß2 which reduces virus-induced demyelination in mice, protects neurons from cell death and inhibits proliferation of astrocytes. The neuroprotective mediators ciliary neurotrophic factor and leukemia

20   inhibitory factor that are induced by poly I:C are also well known for their ability to promote remyelination. This particular function is likely to be aided by the concomitant poly I:C-mediated downregulation with a factor of 1.96 of *Jag1*. Along with these neuroprotective and repair mediators, several growth and differentiation factors are also induced including GM-CSF, embryonic growth/differentiation factor 1

25   and vascular endothelial growth factor (VEGF)-C. The latter is a potent angiogenic molecule like CXCL8 that is also induced by poly I:C.

        While so far most attention has been given to the ability of different TLRs to trigger pro-inflammatory cytokines, poly I:C activation of astrocytes turns out to primarily trigger potent anti-inflammatory cytokines. One of the most prominent

30   responses to TLR3 activation in astrocytes (as well as in synoviocytes) is over 10-fold induction of tumour necrosis factor-stimulated gene 6 (TSG-6) which is a powerful anti-inflammatory product as assessed in several models of acute and chronic tissue damage, especially of arthritis. The anti-inflammatory character of the TLR3-

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mediated response is further supported by 7.5-fold induction of IL-9, 2.3-fold induction
of IL-10 and 3.2-fold induction of IL-11, all three of which are well-known anti-
inflammatory cytokines. None of these three cytokines are induced by LPS. Both poly
I:C and LPS also induced TNF-α. This mediator is often regarded as a typical pro-

5   inflammatory mediator but especially in the human CNS its functional role varies
and can be anti-inflammatory as well. Concomitant with induction of these mediators
poly I:C leads to almost two-fold downregulation of the p40 subunit of IL-12 and IL-23
further emphasising the overall anti-inflammatory character of the astrocytic TLR3-
mediated response. Within the time span of the experiment type I interferons were

10   not markedly induced by poly I:C but induction was seen of upstream regulators of
IFN type I synthesis such as interferon consensus sequence-binding protein and
interferon regulatory factor-1. This suggest that type I IFN production by astrocytes
in response to TLR3 is likely to occur, but relatively late in the response. It is of
interest to note the generally central role of IFN-β in TLR3 functions. Not only is

15   IFN-β a major product released upon TLR3 signalling in several experimental cellular
systems, it also induces elevated expression of TLR3 itself. At the same time, IFN-β is
a therapeutic agent with proven beneficial effects in the neurodegenerative disorder
multiple sclerosis. This in itself appears to support the notion that in the CNS, where
astrocytes are the most abundant cells and preferentially express TLR3, a self-

20   amplifying loop between TLR3 expression, TLR3 activation and IFN-β production
forms part of a beneficial mechanism.

        Among the gene products strongly induced by poly I:C as well as LPS are several
chemokines including CXCL8, CXCL2 and CCL3. Poly I:C but not LPS also leads to
induction of CCL4, CXCL6, CCL5, CXCL9 and CCL2 albeit to a lesser extent.

25   Generally, the predominant functional association of chemokines is with
inflammation and recruitment of leukocytes. Yet, it should be noted that for all the
above chemokines that are triggered by TLR3 both astrocytes and neurons express
functional receptors. This emphasises a more particular relevance of these
chemokines for regulating neural cell migration and tissue remodelling within the

30   CNS. To underpin this notion, CXCL8 and CXCL2 are also strongly induced in
astrocytes by IFN-β. Together with cycloheximide treatment the impact of IFN-β was
examined by cDNA arrays as a control to exclude possible indirect effects on

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astrocytes by TLR3-triggered mediators (data not shown). Interestingly, IFN-ß is also a therapeutic agent in multiple sclerosis. Its beneficial effects on this condition would be difficult to reconcile with IFN-β-induced CXCL8 and CXCL2 predominantly acting as inflammation-promoting signals. Also for this reason, we favour the notion that the

5   above TLR3-induced chemokines are likely to primarily regulate neural cell migration within the CNS itself rather than promote inflammation.

       As is apparent from Figure 9, LPS induces a much more limited response in astrocytes and lacks the ability to activate many of the neuroprotective mediators and anti-inflammatory cytokines induced by poly I:C. Also the combination of LPS and

10   poly I:C mimics the poly I:C-mediated stimulation of astrocytes only to a very limited extent.

TLR3- but not TLR4-triggered mediators inhibit astrocyte growth, stimulate endothelial cell growth and promote neuronal survival

15   Given the multi-faceted response to TLR3 or TLR4-activation, it is difficult to assess the functional relevance of the induced mediators at face value alone. Therefore, functional assays were performed with astrocyte culture media harvested at different times following stimulation with poly I:C or LPS. Such poly I:C- and LPS-conditioned media were examined for their effects on the growth of purified astrocytes

20   and endothelial cells. In addition, their impact was tested on neuronal viability in organotypic human brain slice cultures.

       Poly I:C-conditioned astrocyte medium, but not LPS-conditioned medium inhibited astrocyte proliferation over a 3-day culture period by about 35% (p< 0.003; Fig. 4A). Since such inhibition was not observed when culture media were harvested

25   after only 2 h, this effect cannot be attributed to residual poly I:C in the conditioned astrocyte medium but reflects the combined action of mediators secreted by the cells. Possibly, TGF-ß2 and insulin growth factor-binding protein 6 (cf. Figure 9) play a role in the anti-proliferative effect given their previously documented ability to inhibit proliferation of different cell types. Astrocyte growth is a major feature of gliosis, a

30   common response to injury in the CNS that can be regarded as a mechanism to rapidly seal off healthy tissue from a damaging insult. Yet, it results in dysfunctional scar tissue and attenuating gliosis is known to allow for improved functional recovery in animal models of CNS trauma. It should be noted that this appears not to be

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unique to the CNS since fibrosis (rapid and abundant growth of fibroblast-like cells to form scar tissue in response to injury) is associated with impaired recovery after trauma or damage to other tissues as well including skin, liver and lungs. Very often, reducing the extent of scar formation is considered beneficial and may allow for

5 improved functional recovery. Thus, anti-fibrotic drugs or agents hold significant potential as therapeutic substances in a wide variety of human disorders. Given the similarity between the responses of astrocytes and synoviocytes to TLR3 activation it appears likely that the anti-fibrotic effects of the respond extends to other cell types and tissues as well suggesting that stathmin or stathmin-like proteins could serve as

10 anti-fibrotic substances for a wide variety of applications.

Consistent with the presence in poly I:C-conditioned media of the well-known angiogenic mediators VEGF-C and CXCL8 (cf. Figure 9), these media significantly promoted growth of human endothelial cells over a 3-day period (p< 0.003; Fig. 4B). These growth-promoting effects were similar to those of recombinant fibroblast

15 growth factor-2 that was used as a positive control. Clearly, angiogenesis in the CNS can be considered a beneficial response to injury. Especially in the CNS, optimal blood supply in order to secure sufficient influx of nutrients and oxygen is of key importance.

Finally, poly I:C-and LPS-conditioned media were tested for their effects on

20 neuronal survival in organotypic human brain slice cultures. Such cultures were maintained for 1 week and neuronal survival was evaluated with calcein-AM and ethidium homodimer-1. This revealed significantly improved survival of neurons in slice cultures supplied with poly I:C-conditioned medium but not LPS-conditioned medium (p< 0.03; Fig. 5). Culturing organotypic human brain slices in control medium

25 with freshly added poly I:C promoted neuronal survival equally well (Fig. 5C). This suggests that also in the context of such slices local astrocytes can be activated by poly I:C to produce neuroprotective mediators during culture, and that any TLR3 activation on other neural cell types does not interfere with this protection.

The major conclusion from the above data is that astroglial TLR3 mediates a

30 response that is distinct from TLR4 and involves production of a variety of neuroprotective factors, angiogenic factors, chemokines and anti-inflammatory cytokines. Consistent with the well-known properties of many of these TLR3-mediated products the combined collection of mediators produced by astrocytes in

culture upon TLR3 activation inhibit astrocyte growth, stimulate endothelial cell growth and, importantly, promote survival of neurons in organotypic brain slice cultures. None of these effects were observed using the collection of TLR4-triggered mediators from astrocyte culture supernatants. Such a beneficial and largely

5   neuroprotective quality of the TLR3-mediated response in astrocytes appears to be in line with the observation that astroglial TLR3 expression in MS lesions is particularly prominent at late stage lesions, and induced at least in cultured astrocytes by pro-inflammatory mediators that are known to be present in an active lesion. Thus, it appears more likely that TLR3 on astrocytes (but not microglia) plays a role in

10   promoting repair as a follow-up to inflammation rather intensifying ongoing inflammatory reactions. Also it is striking that especially IFN-β, known for its beneficial effects in MS, plays a key role in signalling by TLR3 much more so than by other TLR. IFN-β release and subsequent IFN-β-induced TLR3 expression represents an important self-amplifying element of the TLR3-mediated response in many cell

15   types. In astrocytes the production of IFN-β in response to TLR3 activation is probably a slow response as it did not reach significant levels during the 48-h culture period monitored in the present study. We did, however, observe induction of factors that may be expected to eventually induce type I interferon production.

20   *Stathmin acts as novel TLR3 agonists*

The above data thus indicate that abundant TLR3 expression is induced on human astrocytes as a response to inflammatory mediators, and that TLR3-mediated signalling activates a powerful anti-inflammatory response, and one that promotes neuroprotection, repair and regenerative processes. In sharp contrast to the selective

25   and strong induction of TLR3 on the surface of activated astrocytes, the only known agonist for TLR3, viz. double-stranded RNA, is remarkably scarce as an extracellular substance in the CNS. Since also for other reasons a role of double-stranded RNA as the only TLR3 agonist can be rationalised in the present context only with difficulty we decided to examine the possibility that additional TLR3 agonist molecules could be

30   present in the human CNS itself. The existence of such an agonist would be consistent with the possibility of a TLR3-mediated endogenous repair mechanism that could be activated following an inflammatory episode or other forms of tissue damage or trauma.

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A cDNA bank derived from 5 pooled tumours including meningioma,
oligodendroglioma, astrocytoma grades II and IV and medulloblastoma was used as a
source of sequences potentially encoding an endogenous protein agonist for TLR3. The
cDNAs were cloned into plasmid vectors promoting expression and secretion of the

5   cDNA-encoded product by mammalian cells, and these plasmids were transfected into
COS-7 cells and evaluated in a two-step approach. First, pools of 50-100 cDNA-
containing plasmids were transfected together and such pools (768 in total) were
screened for their ability to instruct COS-7 cells to produce and secrete proteins that
trigger a response in human astrocytes consistent with TLR3 activation. To monitor

10   such an astrocyte response in this first stage, secretion of CXCL8 was evaluated since
this is a sensitive marker for TLR3 activation in these and other cells (cf. Figs. 9 and
10). In murine astrocytes, IL-6 is used as a marker for TLR3-mediated responses
since there is no murine CXCL8 ortholog. It should be kept in mind, however, that
CXCL8 or IL-6 release is not specific for TLR3 activation: a variety of other stimuli

15   and signalling pathways can also mediate CXCL8 or IL-6 secretion by astrocytes.
When CXCL8 secretion by human astrocytes in response to the products produced by
COS-7 cells was observed, the individual plasmids contained in that pool were tested
individually on their ability to encode for a product that induces CXCL8 secretion. In
this way, two candidate sequences were identified that -when transcribed by COS-7

20   cells- led to the production of proteins that triggered marked CXCL8 secretion in
astrocytes (Fig. 6). One of the two candidate cDNAs (clone C11) was identified as a
product irrelevant to the current invention since it was found to act through TLR2 in
follow-up experiments. The other CXCL8-inducing product (clone F5) was identified
as an amino acid sequence identical to residues 44-149 of human stathmin (Figure 11

25   and SEQ ID NO.1).
       To verify that the partial stathmin sequence encoded by the selected cDNA is
indeed the CXCL8 inducing factor derived from the corresponding COS-7 transfected
cells, the CXCL8 inducing effect was evaluated of purified recombinant human
stathmin from a commercial source. This experiment (Fig. 7A) confirmed that at doses

30   between 5 and 100 μg/mL, also recombinant human stathmin was capable of inducing
marked CXCL8 release by cultured human astrocytes from all four donors tested.
Control experiments using TLR3-directed small interfering RNA to block expression
of TLR3 in astrocytes revealed that the stathmin-induced response in human

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astrocytes was largely –in one case completely- inhibited by such selective interference with TLR3 expression (Fig. 7B). Additional control experiments (Figs. 7C and 7D) using HEK293 cells stably transfected with individual TLR-encoding plasmids verified that the recombinant stathmin used for these experiments did not

5   contain levels of endotoxins that allowed for activation of TLR2 or TLR4 under these conditions. In a crucial experiment it was observed that astrocytes from TLR3-deficient mice failed to respond to recombinant stathmin, like they failed to respond to poly I:C, whereas their ability to respond to activation via either TLR2 by zymosan, tigggering an only very mild response, or TLR4 by LPS was retained (Fig. 7E). These

10 observations provide crucial evidence that the stathmin-induced response is fully TLR3-dependent.

The extended unphosphorylated alpha helix of the conserved stathmin-like domain is likely to be the structural element binding to the TLR3 ectodomain

15   Combining the above novel data with information previously reported strongly suggests that the structural part of stathmin that is essential for ligation to TLR3 is the 95-amino acid long alpha helical structure between residues 44 and 138. Importantly, the TLR3-activating protein product encoded by clone F5 identified in the cDNA bank screen contains the helix-forming residues 44-149, but not the N-

20 terminal portion 1-43 of stathmin. No less than 25% of the residues contained in this helix are negatively charged amino acids, predominantly glutamic acid residues. These render the (conserved) stathmin-like helical domain structurally reminiscent of the phosphate-lined helical structure of double-stranded RNA. Recent modelling of the ectodomain of TLR3 is consistent with the idea that an extended rod-like

25 structure containing abundant negative charges is a crucial structural requirement for TLR3 ligation and dimerisation that are essential for signalling.

The N-terminal part of stathmin is a target of phosphorylation that is known to regulate its interaction with tubulin. Four serine residues at positions 16, 25, 38 and 63 are known to become phosphorylated by different kinases. Only residue 63 is

30 contained in the functional F5 sequence 44-149 but additional data suggest that it is highly unlikely that phosphorylation at this only remaining site is a requirement for TLR3 agonist activity. By structural as well as functional analyses of a truncated variant protein containing resides 40-140, it has previously been demonstrated that

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phosphorylation of serine at position 63 almost completely disrupts the alpha helical

structure of stathmin. As explained above, it is this helical structure that is likely to

be essential for TLR3 ligation implying that a functional form of stathmin would

include an unphosphorylated Ser-63 rather than a phosphorylated form of this

5   residue. The studies by Steinmetz and colleagues have also clarified that as purified

recombinant proteins, stathmin or truncated forms of stathmin containing the helix-

forming domain are structurally ordered into such an alpha helix only for about 40-

60% when examined in buffers similar to culture media. This renders it likely that

under experimental conditions used by us only at best about half the protein

10   molecules may be expected to be functional in binding to the TLR3 ectodomain. This

negatively affects the apparent dose-response relationship between stathmin and

TLR3-activation. As is illustrated in Figure 13, the stathmin-like proteins stathmin,

SCG-10, SCLIP and RB3 all share marked sequence homologies in the region that

defines the alpha helix in the conserved stathmin-like domain, including the high

15   number of negatively charged amino acids. This is consistent with the notion that all

four family members are highly likely to also share their ability to act as TLR3

agonists. A sequence comparison between stathmin, SCG-10, SCLIP and RB3 is

provided below. As can be seen in the following alignments, stathmin (STM-1) and

stathmin-2 (SCG10 or STM-2), in a window of 134 overlapping amino acids, exhibit

20   74.6% sequence identity (double points indicate identical amino acid residues),

corresponding to 93% homology (single points indicate homologous amino acid

residues).

```
                10        20        30        40        50        60
25   STM-1  DIQVKELEKRASGQAFELILSPRSKESVPEFPLSPPKKKDLSLEEIQKKLEAAEERRKSH
                :..::....::::::::::::..: :   :       :. ::::::::::::::::::::::.
       STM-2  DMEVKQINKRASGQAFELILKPPSPISEAPRTLASPKKKDLSLEEIQKKLEAAEERRKSQ
                40        50        60        70        80        90

30                70        80        90        100       110       120
       STM-1  EAEVLKQLAEKREHEKEVLQKAIEENNNFSKMAEEKLTHKMEANKENREAQMAAKLERLR
                ::.:::::::::::.::::::::::.::::::::::::: ::: ::::::::...:: .:::.
       STM-2  EAQVLKQLAEKREHEREVLQKALEENNNFSKMAEEKLILKMEQIKENREANLAAIIERLQ
                100       110       120       130       140       150

35                130
       STM-1  EKDKHIEEVRKNKE
                ::..:  :::..:::
       STM-2  EKERHAAEVRRNKE
40              160       170
```

55

Moreover, stathmin (STM-1) and stathmin-3 (SCLIP or STM-3), in a window of 139

overlapping amino acids, exhibit 67.6% sequence identity, corresponding to 92%

homology.

```
5                  10        20        30        40        50        60
       STM-1   DIQVKELEKRASGQAFELILSPRSKESVPEFPL--SPPKKKDLSLEEIQKKLEAAEERRK
               :..::.:.::::::.::.::.   :   :  :: :.   :::::::: ::::.::.:::::::
       STM-3   DMEVKQLDKRASGQSFEVILKSPSDLS-PESPMLSSPPKKKDTSLEELQKRLEAAEERRK
                      40        50        60        70        80        90
10
                  70        80        90        100       110       120
       STM-1   SHEAEVLKQLAEKREHEKEVLQKAIEENNNFSKMAEEKLTHKMEANKENREAQMAAKLER
               ..::.:::::::.::::.:::.::.:::::::::...:::::..::: .:: :::..:: ::
       STM-3   TQEAQVLKQLAERREHEREVLHKALEENNNFSRQAEEKLNYKMELSKEIREAHLAALRER
15                 100       110       120       130       140       150

                  130       140
       STM-1   LREKDKHIEEVRKNKESKD
               ::::. :  :::.:::...
20     STM-3   LREKELHAAEVRRNKEQRE
```

Also, stathmin (STM-1) and stathmin-4 (RB3 or STM-4), in a window of 140

overlapping amino acids, exhibit 72.1% sequence identity, corresponding to 95%

25   homology.

```
                  10        20        30        40        50        60
       STM-1   SDIQVKELEKRASGQAFELILSPRSKESVPEFPLSPPKKKDLSLEEIQKKLEAAEERRKS
               ::..: ::.: .:::.::.::.: :  ..::::  :  :....: ::::::::::::::::
30     STM-4   SDMEVIELNKCTSGQSFEVILKPPSFDGVPEFNASLPRRRDPSLEEIQKKLEAAEERRKY
                      50        60        70        80        90        100

                  70        80        90        100       110       120
       STM-1   HEAEVLKQLAEKREHEKEVLQKAIEENNNFSKMAEEKLTHKMEANKENREAQMAAKLERL
               .:::.::.:::::::::.::.:::::::::: :::.:::..:::.:::::::::..:: ::::
35     STM-4   QEAELLKHLAEKREHEREVIQKAIEENNNFIKMAKEKLAQKMESNKENREAHLAAMLERL
                      110       120       130       140       150       160

                  130       140
       STM-1   REKDKHIEEVRKNKESKDPA
               .:::::: :::::::::: :. :
40     STM-4   QEKDKHAEEVRKNKELKEEA
                      170       180

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```

__Example 2__. Comparison between the stathmin-induced response by astrocytes

isolated from either wild-type mice, or TLR3-deficient mice.

50   __Method__

Astrocytes were isolated from either newborn wild-type (Biozzi ABH) mice that

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express TLR3 or newborn TLR3-deficient mice that do not (Alexopoulou et al. (2001) *Nature* 413:732-8). Astrocytes were cultured in poly L-lysine coated 96 well-flat-bottom plates at 1 x 10⁴ cells per well in 100 µl DMEM/HAM-F10 medium containing 10 % FCS and antibiotic supplements for 24 h to obtain post-confluent cultures.

5   Recombinant human stathmin was next added to astrocytes, and parallel control stimuli were performed with either poly I:C, an established TLR3 agonist, or ultrapure lipopolysaccharides, established TLR4 agonists. After addition of these stimuli astrocytes were incubated for another 48 h. Astrocyte culture supernatants were subsequently collected and using a commercial ELISA kit levels of IL-6 were

10   determined, as a marker for TLR-mediated activation of murine astrocytes.

Result

As illustrated in Fig. 14 a dose-dependent IL-6 induction by stathmin is visible in cultures of wild-type astrocytes, but completely absent from cultures of astrocytes

15   isolated from TLR3-deficient animals. This response profile is identical to that against polyI:C, a previously established TLR3 agonist. In either astrocyte culture the strong IL-6 response to ultrapure LPS, a TLR4 agonist, is very similar demonstrating normal viability and TLR4-dependent responses in both cultures. These results provide conclusive evidence that the astrocyte response to stathmin, like the response to poly

20   I:C, is critically dependent on TLR3 expression. It also shows that the ability of recombinant human stathmin to activate astrocytes is not limited to human astrocytes and extends at least also to mouse astrocytes.

Example 3. cDNA array profiling of the cytokine, chemokine and growth factor
25   **response of human astrocytes to either stathmin or poly I:C.**

Method

Astrocytes were isolated from post mortem human brain tissue samples and cultured under conditions as described elsewhere in this application. One part of the

30   culture was stimulated with 50 µg/ml stathmin, the second with 50 µg/ml poly I:C and a third was left untreated to served as a refence control. Following another 48 h RNA was isolated from each culture and ³²P-labeled cDNA was prepared by reverse transcription. Subsequent hybrid selection of these ³²P-labeled cDNA samples was

performed on Clontech Atlas® arrays containing selective probes for 268 cytokines,

chemokines and growth factors, and various receptors for these mediators. All

hybridization signals were calculated as the mean of duplicate measurements,

corrected for background intensity and quantified using software provided by the

5   manufacturer. Relative signal strength was next calculated by dividing these

averaged hybridization signals by the mean signal for all nine housekeeping reference

genes that were also present on each array as a reference. The extent to which a TLR

agonist affects expression signals for every gene can thus be expressed as the ratio of

the relative hybridization signals found in a TLR3-agonist-treated culture of

10   astrocytes over those found in the untreated control cultures.

Result

       As illustrated in Figs. 15A and 15B both stathmin and poly I:C led to a

comprehensive and marked change in expression of genes encoding various immune

15   mediators and growth factors. Essentially all of the relevant changes involve

enhancement of gene expression and not suppression, as already summarized in Fig.

9. This is evident from the position of a large number of gene markers in the upper

left half of panels A and B that deviate significantly from the diagonal (dotted lines in

Fig. 15), while no such markers are evident in the lower right half of the panels. A

20   comparison between gene expression signals in astrocytes stimulated with either

stathmin or poly I:C (Fig. 15C) clearly reveals that essentially all of these signals are

very similar indeed. Calculation of the Pearson correlation coefficient (r) that

expresses the extent of similarity between the two data sets of 268 expression signals

produced the value of 0.951, which implies a very strong similarity. The probablility of

25   this being a coincidence is negligable ($p < 10^{-35}$). The conclusion from this result is that

the gene response to stimulation with either agonist is identical. Since this implies an

equal similarity in signalling pathways that are activated in response to either

stimulation, this provides additional strong evidence for the notion that stathmin, like

poly I:C, is a TLR3 agonist.

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Example 4. The requirement of an alpha helix for the TLR3 agonist function of

stathmin.

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Method

Purified recombinant stathmin was exposed to two different conditions that
differentially affect the extent of helix formation within the protein chain. One part of
the protein preparation was exposed to 5% trifluoroacetic acid (TFA) in water for 1 h
5   at 37°C, strongly acidic conditions that tend to disrupt an alpha helix. In parallel,
another part was exposed for 1 h at 37°C to 5% of the helix-promoting solvent
trifluoroethanol (TFE) in phosphate-buffered saline (PBS). After exposure to these
different conditions, both protein samples were extensively dialysed for 48 h against
several changes of PBS. Next, the samples were analyzed by near-UV circular
10   dichroism spectroscopy that allows calculation of helical content on the basis of
elipticity in the absorption of circularly polarized light. In both cases, another part of
the dialyzed sample was tested in parallel for induction of TLR3-mediated release of
CXCL8 (IL-8) in human astrocytes as described elsewhere in this application.

15   **Result**

Upon CD spectroscopy the TFA-treated sample produced a spectrum indicative
of 12% alpha helical content, while the TFE-treated sample was found to contain 37%
alpha helix (Fig. 16A). As a reference control sample, stathmin was also dissolved in a
solution of 50% TFE in PBS and immediately monitored by CD spectroscopy without
20   dialysis. Under these conditions, the stathmin protein chains adopts an essentially
complete helical conformation (helical content = 98%; see Fig. 16A). Upon testing the
two pre-treated and dialyzed samples of stathmin on their ability to activate
astrocytes a marked difference was observed between either the TFA- or TFE-treated
sample, the latter being about 3-fold more active. Since no differences exist between
25   these samples in either the purity or concentration of protein, not in their final buffer
composition following extensive dialysis, we attribute the higher activity of the TFE-
treated sample to the markedly higher helical content of stathmin in that sample. The
3-fold higher activity on weight basis of the TFE-treated sample as compared to the
TFA-treated samples corresponds well with its 3-fold higher alpha helical content. We
30   therefore conclude that the alpha-helical conformation of stathmin is crucial to its
ability to serve as an agonist for TLR3. This notion is fully consistent with the idea
that by assuming an alpha-helical conformation, lined with as many as 24%
negatively charged amino acids (cf. Figs. 12 and 13), stathmin effectively produces a

structural mimic of the negatively charged double helix that also characterizes poly I:C and has been demonstrated to be the key feature for its interaction with TLR3.

Example 5. Activation by stathmin of human peripheral blood cells.

Method

Peripheral blood mononuclear cells (PBMC) were isolated from two healthy blood donors and from part of the cells, $CD14^+$ monocytes were isolated by positive selection using magnetic beads coated with an antibody against CD14. The purified $CD14^+$ monocytes were subsequently cultured for 5 days in the presence of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF). These growth factors induce differentiation of monocytes into pre-dendritic cells (pre-DC) within this time frame. At this stage part of the pre-DC culture was stimulated with a mixture of IL-1β and TNF-α for another 2 days in culture, thus stimulating final maturation of the cells into plasmacytoid dendritic cells (DC).

Using this differentiation method, separate populations of PBMC, pre-DC and DC were generated and after washing of the cells to remove all previously used stimuli, cells were subsequently re-cultured in fresh medium and tested for their response to either poly I:C, stathmin or LPS. Accumulation of CXCL8 in the culture medium over a 24-h period was used as a measure for cellular activation.

Result

As illustrated in Fig. 17, the addition of stathmin at varying doses to PBMC, pre-DC or DC led to a substantial increase in CXCL8 accumulation in the culture medium in all three cases, and for cells isolated from both donors. Stathmin-induced levels of CXCL8 levels were consistently similar to those induced by LPS, illustrating the relative strength of this effect. These data therefore demonstrate that stathmin is able to activate CXCL8 production in peripheral blood-derived human cells and both immature and mature DC.

Example 6. The response profile of immature human dendritic cells to stathmin, poly I:C and LPS.

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Method

To examine the effects of stathmin on the maturation and cytokine production of immature human plasmacytoid dendritic cells (pre-DC), such pre-DC were generated from samples of peripheral blood mononuclear cells by maturation of purified CD14+ monocytes using GM-CSF and IL-4. The cells were subsequently exposed to 50 µg/ml stathmin and after 48 h, culture supernatants were collected and the accumulation of 40 different cytokines ands chemokines was evaluated by antibody-array profiling using a commercially available antibody array.

Result

As summarized in Figure 18 stathmin triggers marked CXCL8 release in pre-DC (as already illustrated in Fig. 17B and 17E) but only few additional cytokines. Of the 40 different cytokine and chemokine mediators analysed by the antibody-based array, only 4 were found the be induced at a marked level (induction ratio > 1.5) by stathmin. These included, in addition of CXCL8, MCP-1, IL-10 and the p40 subunit of IL-12. Almost all other cytokines and chemokines tended to accumulate in the stathmin-treated cultures at lowel levels than in the control culture. The presence of stable and large amounts of both GM-CSF and IL-4 is not surprising since these growth factors are added to the culture medium for growth of pre-DC prior the the anlaysis. These two cytokines thus serve as a suitable internal control for the analytical method used.

The only two marked cytokine responses induced in pre-DC by stathmin both strongly support the notion that the overall effect of stathmin is anti-inflammatory. Of all cytokines on the array, only IL-10 and the p40 subunit of IL-12 are induced by stathmin whereas all other cytokines tend to be suppressed. Both mediators represent powerful anti-inflammatory mediators. IL-10 is well-known as an anti-inflammatory cytokine that plays a central role in controlling inflammation and promoting tissue repair. While the p40 subunit of IL-12 can be part of the strongly pro-inflammatory cytokines IL-12 and IL-23, in the absence of its counterpart to form these cytokines (p70 for IL-12 and p19 for IL-23) p40 on its own actually represents a strong inhibitor for both IL-12 and IL-23. In contrast to enhancing p40 secretion, stathmin reduces levels of the p70 subunit of IL-12 two-fold. This combination will result in accumulation of the anti-inflammatory p40 subunit without its counterpart to form

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IL-12. Thus, this effect in pre-dendritic cells represents another stathmin-induced mechanism to inhibit inflammatory processes.

As another aspect of the result as summarized in Figure 18, the data set obtained clearly illustrates that the TLR3-mediated activating effect of stathmin is not limited to human fibroblast-like cells that express TLR3 on the cell surface. Human pre-DC, which express TLR3 only inside intracellular endosomal vesicles, clearly also respond to stathmin and again, this lead to release of potent anti-inflammatory mediators.

Example 7. Activation by stathmin of human microglial cells.

Method

Using either gray matter samples or white matter samples of post mortem adult human brains collected within 9 h after death, several cultures of microglia were produced as previously described. Microglia were subsequently stimulated with different TLR agonists and after 24 h accumulation of CXCL8 in the culture medium was quantified using a commercial ELISA assay, as a measure of TLR-mediated activation.

Result

As illustrated in Fig. 19, microglia from various donors, and from different anatomical areas of the human brain consistently show a response to all three TLR agonists, including stathmin, as evidenced by enhanced CXCL8 acccumulation in the culture medium. Again, this emphasizes that also intracellular TLR3, the only form of TLR3 in microglia, can be activated by stathmin.

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Claims

1.   A method for the treatment and/or prophylaxis of a degenerative inflammatory process in a tissue of a subject, said method comprising increasing the activity of Toll-like receptor 3 (TLR3) in cells of said tissue.

5   2.   Method according to claim 1, wherein said method comprising administering to said subject:
            - a therapeutically effective amount of at least one TLR3-agonist;
            - a therapeutically effective amount of at least one compound that promotes *in vivo* expression of a TLR3-agonist;
10   - a therapeutically effective amount of at least one compound that promotes TLR3 expression, and/or
            - a therapeutically effective amount of at least one compound that promotes TLR3-mediated signalling.

15   3.   Method according to claim 1 or 2, wherein said subject is a mammal, preferably a human.

4.   Method according to any one of claims 1-3, wherein said tissue is nervous tissue, preferably brain tissue and/or synovial tissue.
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5.   Method according to any one of the preceding claims, wherein said cells are glial cells, preferably astrocytes, and/or synovial cells, preferably synoviocytes.

6.   Method according to any one of the preceding claims, wherein said
25   degenerative inflammatory process is the result of a chronic or acute neurodegenerative disorder.

7.   Method according to claim 6, wherein said neurodegenerative disorder is selected from the group consisting of stroke, hypovolemic shock, traumatic shock,
30   reperfusion injury, multiple sclerosis, AIDS-associated dementia, neuron toxicity,

Alzheimer's disease (AD), head trauma, acute spiral cord injury, Huntington's disease (HD), Parkinson's Disease (PD) and related synucleinopathies, dystonia, Tourette Syndrome, Frontotemporal Dementias (FTDs) and related tauopathies, prion disorders such as Fatal Familial Insomnia (FFI) and other disorders related to

5   Creutzfeldt-Jakob disease, amyotrophic lateral sclerosis (ALS), trinucleotide repeat diseases, Motor Neurone disease, progressive supranuclear palsy, REM Sleep Behavior Disorder (RBD), and cancer.

8.   Method according to any one of claims 5-7, wherein said treatment is

10   accompanied by stimulation of a neuroprotective response in cells of said nervous tissue and/or wherein said treatment is accompanied by stimulation of a repair functions in cells of said synovial tissue.

9.   Method according to any one of claims 2-8, wherein said at least one TLR3-

15   agonist is selected from the group consisting of:

- stathmin, TLR3-activating derivatives thereof and precursors thereof;
- stathmin-like proteins, TLR3-activating derivatives thereof and precursors thereof, and
- proteinaceous compounds comprising a polypeptide chain having an amino

20   acid sequence similarity of at least 60% with an amino acid sequence selected from the group consisting of the amino acid sequences of stathmin (SEQ ID NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3 (SEQ ID NO: 4).

25   10.   Method according to claim 9, wherein said amino acid sequence similarity relates to the stathmin-like alpha helix domain corresponding to residues 44 to 138 of SEQ ID NO: 1.

11.   Method according to claim 9, wherein said stathmin-like protein is SCG10,

30   SCLIP and/or RB3.

12.   Method according to any one of claims 9-11, wherein said method further comprising the use of said TLR3-agonist in combination with compounds that promote

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stabilisation of the stathmin-like alpha helix domain corresponding to residues 44 to 138 of SEQ ID NO: 1.

13.   A pharmaceutical composition for the treatment and/or prophylaxis of a degenerative inflammatory process in a tissue of a subject, wherein said composition comprises a therapeutically effective amount of at least one compound capable of increasing the activity of Toll-like receptor 3 (TLR3) in cells of said tissue; and b) a pharmaceutically acceptable carrier.

14.   Composition according to claim 13, wherein said at least one compound is:
        - a TLR3-agonist;
        - a compound that promotes *in vivo* expression of a TLR3-agonist;
        - a compound that promotes TLR3 expression, and/or
        - a compound that promotes TLR3-mediated signalling.

15.   Composition according to claim 13 or 14, wherein said subject is a mammal, preferably a human.

16.   Composition according to any one of claims 13-15, wherein said tissue is nervous tissue, preferably brain tissue and/or synovial tissue.

17.   Composition according to any one of claims 13-16, wherein said cells are glial cells, preferably astrocytes, and/or synovial cells, preferably synoviocytes.

18.   Composition according to any one of claims 13-17, wherein said degenerative inflammatory process is the result of a chronic or acute neurodegenerative disorder.

19.   Composition according to claim 18, wherein said neurodegenerative disorder is selected from the group consisting of stroke, hypovolemic shock, traumatic shock, reperfusion injury, multiple sclerosis, AIDS-associated dementia, neuron toxicity, Alzheimer's disease (AD), head trauma, acute spiral cord injury, Huntington's disease (HD), Parkinson's Disease (PD) and related synucleinopathies, dystonia, Tourette Syndrome, Frontotemporal Dementias (FTDs) and related tauopathies, prion

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disorders such as Fatal Familial Insomnia (FFI) and other disorders related to Creutzfeldt-Jakob disease, amyotrophic lateral sclerosis (ALS), trinucleotide repeat diseases, Motor Neurone disease, progressive supranuclear palsy, REM Sleep Behavior Disorder (RBD) and cancer.

20.   Composition according to any one of claims 17-19, wherein said compound is capable of stimulating a neuroprotective response in cells of said nervous tissue and/or wherein said compound is capable of stimulating repair functions in cells of said synovial tissue.

21.   Composition according to any one of claims 14-20, wherein said at least one TLR3-agonist is selected from the group consisting of:
- stathmin, TLR3-activating derivatives thereof and precursors thereof,
- stathmin-like proteins, TLR3-activating derivatives thereof and precursors thereof, and
- proteinaceous compounds comprising a polypeptide chain having an amino acid sequence similarity of at least 60% with an amino acid sequence selected from the group consisting of the amino acid sequences of stathmin (SEQ ID NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3 (SEQ ID NO: 4).

22.   Composition according to claim 21, wherein said amino acid sequence similarity relates to the stathmin-like alpha helix domain corresponding to residues 44 to 138 of SEQ ID NO: 1.

23.   Composition according to claim 21, wherein said stathmin-like protein is SCG10, SCLIP and/or RB3.

24.   Composition according to any one of claims 21-23, wherein said composition further comprising compounds that promote stabilisation of the stathmin-like alpha helix domain corresponding to residues 44 to 138 of SEQ ID NO: 1.

25.   A TLR3-agonist selected from the group consisting of:

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- stathmin, TLR3-activating derivatives thereof and precursors thereof,

- stathmin-like proteins, TLR3-activating derivatives thereof and precursors
  thereof, and

- proteinaceous compounds comprising a polypeptide chain having an amino
  acid sequence similarity of at least 60% with an amino acid sequence selected
  from the group consisting of the amino acid sequences of stathmin (SEQ ID
  NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3 (SEQ ID
  NO: 4),

for use as a medicament.

26.   The use of a TLR3-agonist selected from the group consisting of:

- stathmin, TLR3-activating derivatives thereof and precursors thereof,

- stathmin-like proteins, TLR3-activating derivatives thereof and precursors
  thereof, and

- proteinaceous compounds comprising a polypeptide chain having an amino
  acid sequence similarity of at least 60% with an amino acid sequence selected
  from the group consisting of the amino acid sequences of stathmin (SEQ ID
  NO: 1), SCG-10 (SEQ ID NO:2), SCLIP (SEQ ID NO: 3) and RB3 (SEQ ID
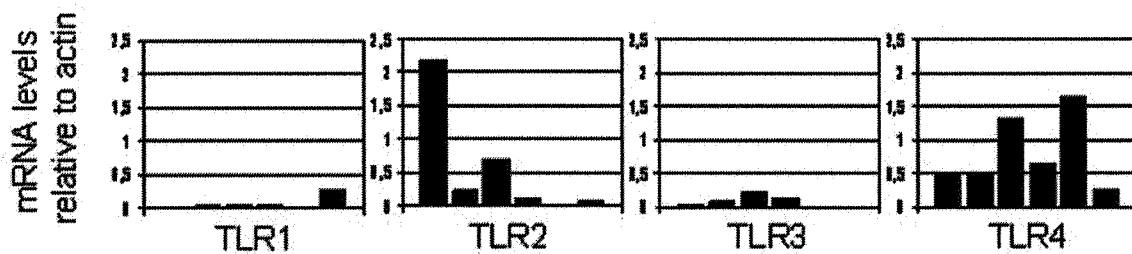 NO: 4),

for the preparation of a medicament for the treatment and/or prophylaxis of a
degenerative inflammatory process in a tissue of a subject.

27.   The method of any one of claims 1- 12, which is for preventing or retarding the
onset of a neurodegenerative disorder in a subject, in the absence of any disorder,
disease, or injury.

28.   The composition of any one of claims 13- 24, which is for preventing or
retarding the onset of a neurodegenerative disorder in a subject, in the absence of any
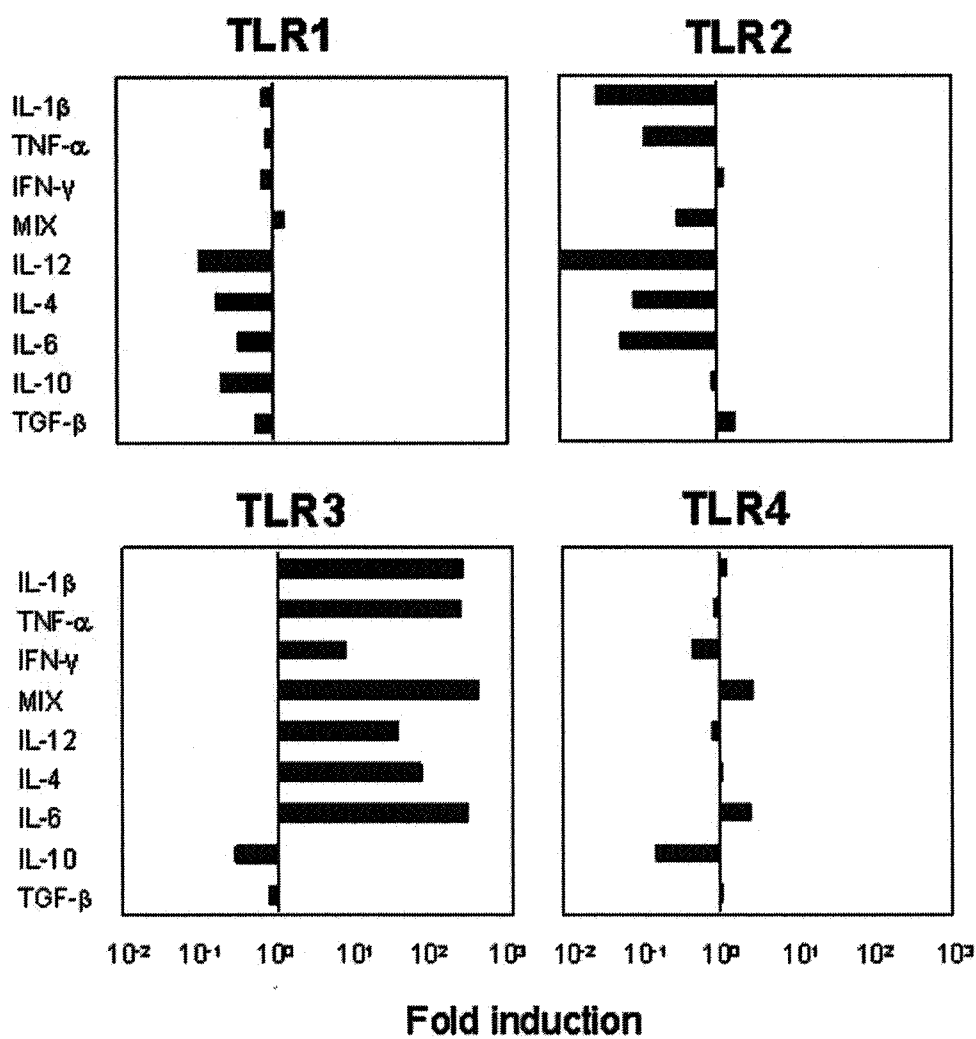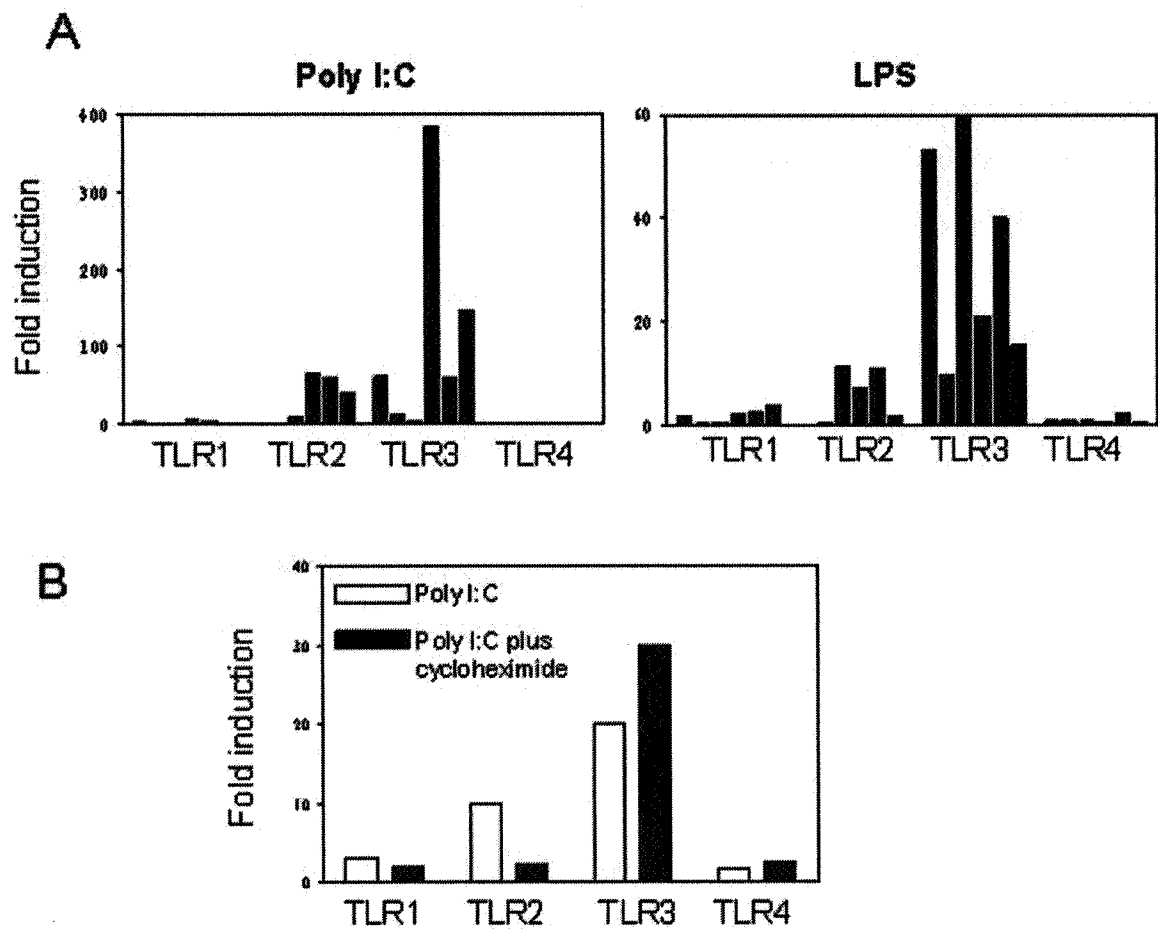disorder, disease, or injury.
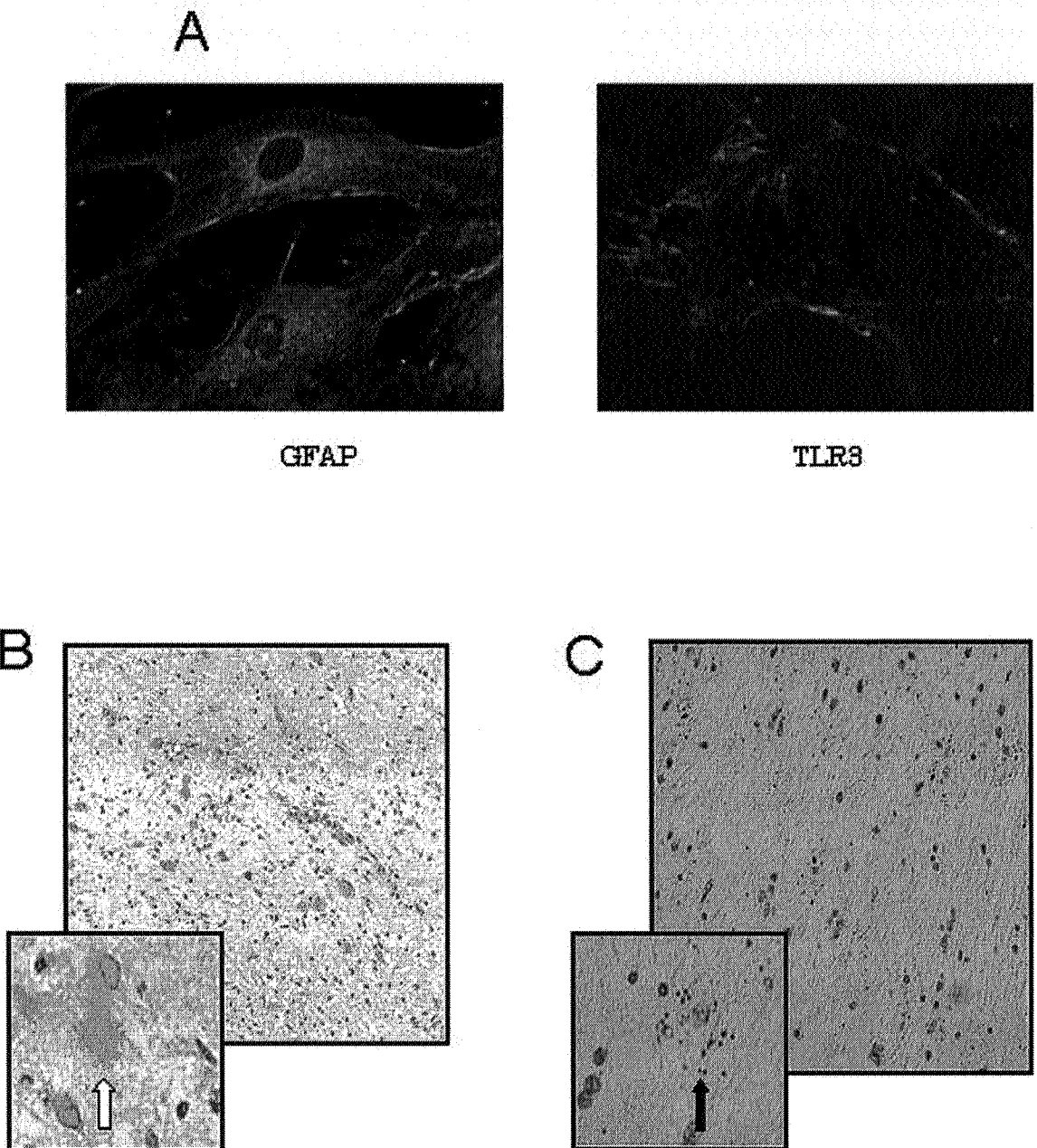
Figure 1

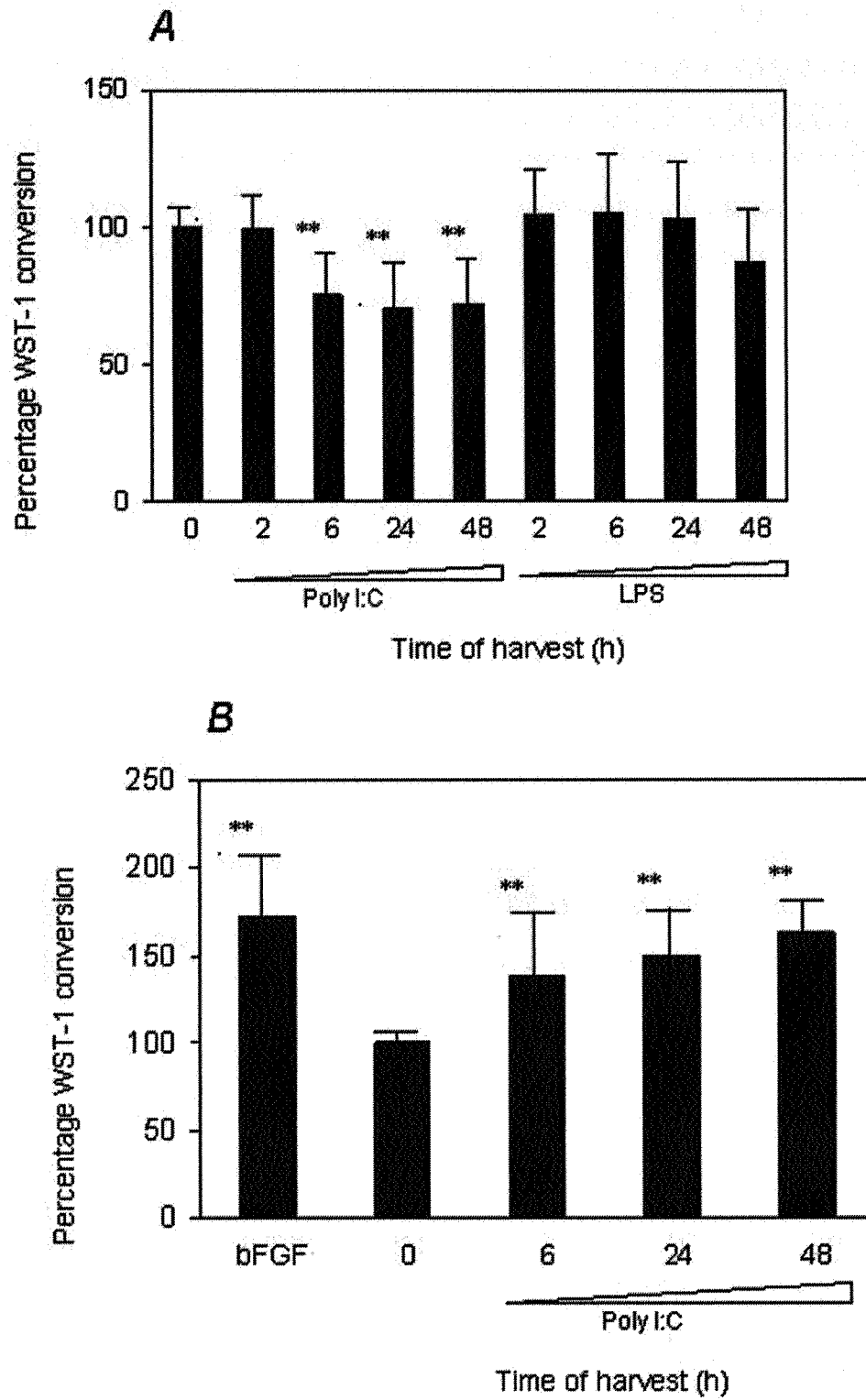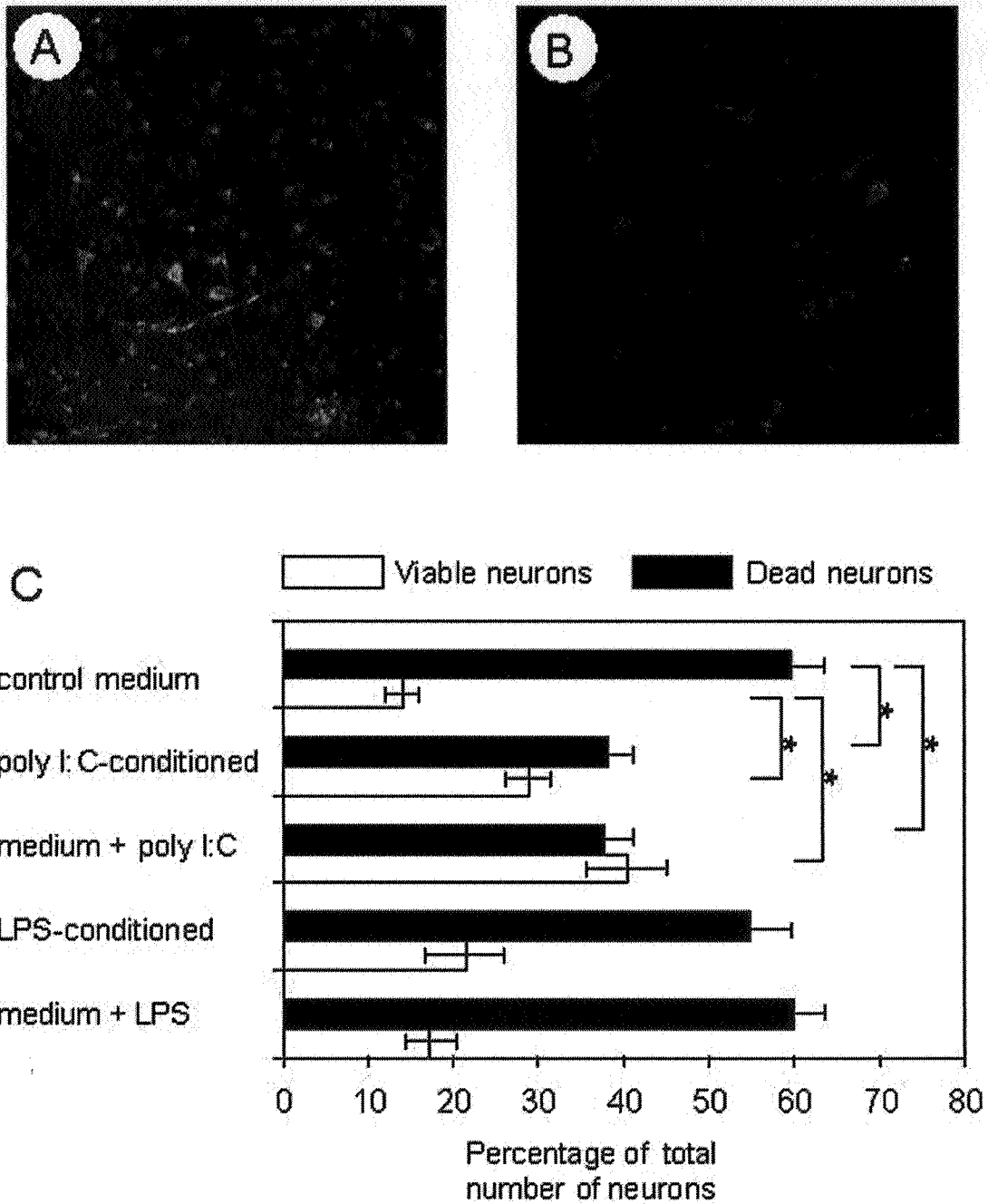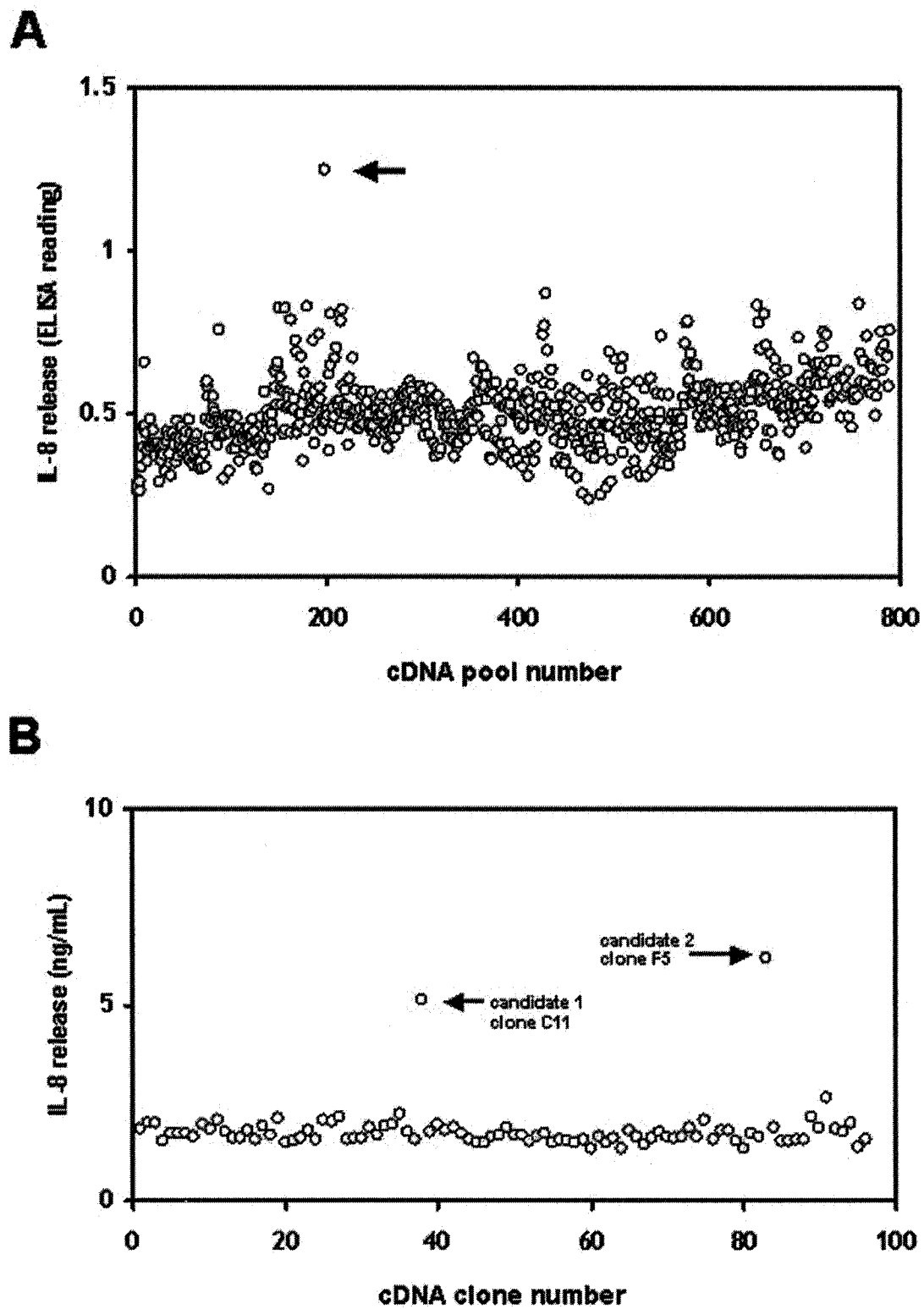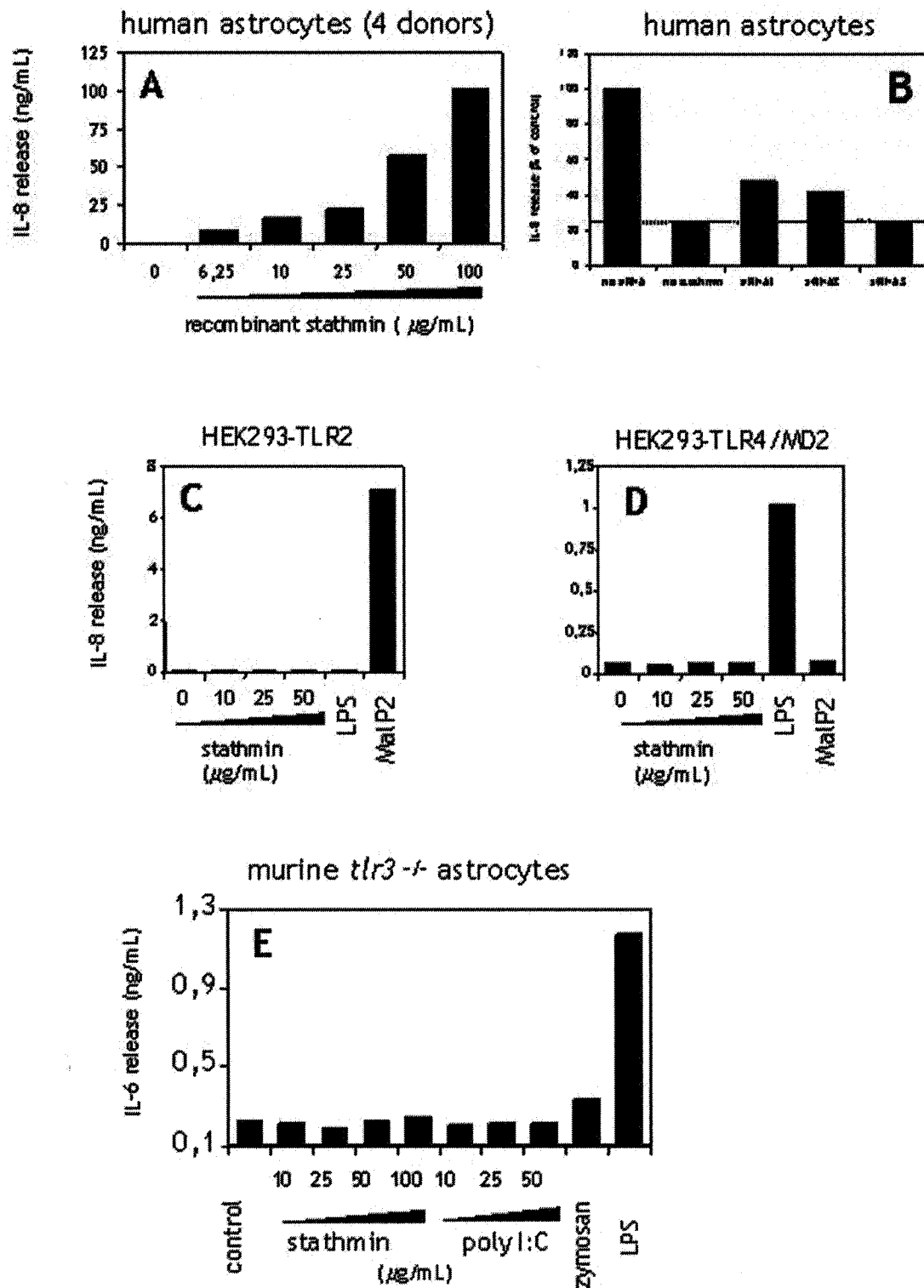**Figure 2**

Figure 3

GFAP

TLR3

Figure 4

A

B

Figure 5

Figure 6

Figure 7.

Figure 8. Expression of TLR3 in various human tissues and cell types

cell type	analysis	localization	ref
Dendritic cells	protein/mRNA	intracellular	Matsumoto *et al.*, 2003
Natural killer cells	protein/mRNA	surface	Hart *et al.*, 2005
Fibroblasts	protein/mRNA	surface	Matsumoto *et al.*, 2002
Astrocytes	protein/mRNA	surface	Bsibsi *et al.*, 2002
Microglia	protein/mRNA	intracellular	Bsibsi *et al.*, 2002
Oligodendrocytes	mRNA	ND	Bsibsi *et al.*, 2002
Neurons	protein	surface	Jackson *et al*, 2006
Keratinocytes	mRNA	ND	Mempel *et al.*, 2003
Intestinal epithelial cells	mRNA	intracellular	Furrie *et al.*, 2005
Corneal epithelial cells	mRNA/protein	surface	Ueta *et al.*, 2005
Bronchial epithelial cells	protein/RNA	surface	Hewson *et al.*, 2005
Endometrial epithelial cells	mRNA	ND	Young *et al..* 2004
Uterine epithelial cells	mRNA	ND	Schaefer *et al.*, 2005
Endothelial cells (HUVEC)	mRNA	ND	Tissari *et al.*, 2005
Mast cells	mRNA/protein	surface	Kulka *et al.*, 2004
Cancer cells	protein	surface	Salaun *et al.* 2006

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Figure 9. Gene products induced in astrocytes by TLR3 or TLR4 agonists.

Gene product (accession code)	stimulus		
	poly I:C	LPS	Poly I:C plus LPS
Growth differentiation and neuroprotection			
L03840 fibroblast growth factor receptor 4 (FGF4R)	23.24 ± 19.14	*N.D*	*N.D.*
L40636 ephrin type-B receptor 1 (EPHT2)	13.31 ± 10.19	12.68 ± 8.32	*N.D.*
M31165 tumor necrosis factor-inducible protein-6 (TSG-6)	10.88 ± 10.87	*N.D.*	7.11 ± 4.11
A26792 ciliary neuronotrophic factor isoforms B & C (CNTF)	4.86 ± 2.39	4.88 ± 2.84	*N.D.*
M86528 neurotrophin-4 (NT-4)	3.91 ± 1.06	*N.D.*	*N.D.*
X17648 GM-CSF receptor alpha	3.78 ± 0.59	2.28 ± 0.49	5.88 ± 3.22
M31213 ret proto-oncogene (RET)	3.63 ± 1.94	1.49 ± 0.56	*N.D.*
X13967 leukemia inhibitory factor (LIF)	3.47 ± 1.42	2.65 ± 0.72	2.38 ± 0.38
M11220 GM-CSF	3.44 ± 0.87	3.15 ± 1.54	*N.D.*
U43142 vascular endothelial growth factor C (VEGF-C)	3.34 ± 0.68	*N.D.*	5.37 ± 1.33
M62302 embryonic growth/differentiation factor 1 (GDF-1)	2.84 ± 0.97	*N.D*	*N.D.*
L12261 glial growth factor (neuregulin 1, GGF1)	2.73 ± 0.62	*N.D.*	*N.D.*
M57399 pleiotrophin (neurite growth promoting factor 1, PTN)	2.61 ± 0.59	1.09 ± 0.10	1.88 ± 0.27
M62402 insulin-like growth factor binding protein 6 (IGFBP6)	2.64 ± 0.35	1.76 ± 0.17	1.05 ± 0.44
X06234 migration inhibitory factor-related protein 8 (MRP8)	2.57 ± 0.87	1.64 ± 0.56	*N.D.*
P23560 brain-derived neurotrophic factor (BDNF)	2.48 ± 1.01	1.01 ± 0.23	0.38 ± 0.01
M19154 transforming growth factor beta2 (TGF-beta2)	2.46 ± 0.31	1.67 ± 0.37	*N.D.*
L12260 glial growth factor 2 (neuregulin; GGF2)	2.20 ± 0.45	1.62 ± 0.10	*N.D.*
J03634 erythroid differentiation protein (EDF)	2.11 ± 0.53	1.15 ± 0.26	1.87 ± 0.34
D14012 hepatocyte growth factor activator (HGF activator)	1.25 ± 1.05	11.76 ± 10.37	*N.D.*
D13365 growth inhibitory factor, metallothionein-III (MT-III)	1.33 ± 0.14	1.42 ± 0.17	2.99 ± 0.99
Intracellular signalling			
M91196 interferon consensus sequence-binding protein (ICSBP)	4.82 ± 2.57	1.98 ± 0.52	1.84 ± 0.17
X02492 inteferon alpha-inducible protein (G1P3)	3.34 ± 1.13	*N.D.*	1.98 ± 0.12
M76673 FMLP-related receptor I (FMLPII)	2.30 ± 0.14	2.51 ± 1.04	*N.D.*
X14454 interferon regulatory factor 1(IRF1)	2.93 ± 0.55	1.79 ± 0.23	1.35 ± 0.09
L17075 activin A receptor type II-like 1 (ACVRL1)	2.13 ± 1.10	1.12 ± 0.18	*N.D.*

Figure 9 (continued)

Gene product (accession code)	stimulus		
	poly I:C	LPS	Poly I:C plus LPS
Chemokines and cytokines			
Y00787 interleukin-8 (CXCL8)	24.56 ± 10.28	10.98 ± 6.91	78.20 ± 50.9
X53799 macrophage inflammatory protein 2 alpha (CXCL2)	15.41 ± 6.6	18.70 ± 4.88	33.58 ± 27.6
M23452 macrophage inflammatory protein 1 alpha (CCL3)	14.94 ± 2.53	9.44 ± 0.40	*N.D.*
X17543 interleukin-9 (IL-9)	7.57 ± 5.82	1.54 ± 0.13	*N.D.*
J04130 macrophage inflammatory protein 1 beta (CCL4)	7.25 ± 4.47	1.37 ± 0.26	*N.D.*
X01394 tumor necrosis factor (TNF-α)	6.91 ± 2.96	8.93 ± 2.42	*N.D.*
Y08770 granulocyte chemotactic protein 2 (CXCL6)	5.07 ± 2.45	1.97 ± 0.53	3.46 ± 1.86
M57765 interleukin-11 (IL-11)	3.23 ± 1.93	1.48 ± 0.19	5.52 ± 2.88
X04602 interleukin-6 (IL-6)	3.18 ± 0.85	1.83 ± 0.32	3.16 ± 1.38
M21121 RANTES (CCL5)	2.83 ± 0.56	1.36 ± 0.02	1.66 ± 0.31
L08187 cytokine receptor EBI3 (EBI3)	2.81 ± 0.79	*N.D.*	*N.D.*
X72755 interferon-gamma-induced monokine, (MIG CXCL9)	2.44 ± 0.61	3.73 ± 0.48	0.49 ± 0.35
X04688 interleukin-5 (IL-5)	2.45 ± 0.81	1.14 ± 0.41	0.59 ± 0.45
M57627 interleukin-10 (IL-10)	2.34 ± 0.51	1.09 ± 0.26	1.90 ± 0.26
M24545 monocyte chemotactic protein 1 (CCL2)	2.11 ± 0.53	1.61 ± 0.31	1.73
Chemokine and cytokine receptors			
M60459 erythropoietin receptor (EPOR)	3.74 ± 1.80	2.39 ± 0.03	1.59 ± 0.36
D10924 stromal cell derived factor 1 receptor (CXCR4)	3.21 ± 1.57	*N.D.*	*N.D.*
M59818 granulocyte colony stimulating factor receptor (CD114)	3.06 ± 0.56	*N.D.*	4.22 ± 2.68
M26062 interleukin-2 receptor beta subunit (CD122)	2.66 ± 0.15	1.42 ± 0.04	1.52 ± 1.16
X60592 CD40	2.66 ± 0.50	*N.D.*	*N.D.*
M84747 interleukin-9 receptor (IL-9R)	2.54 ± 0.40	1.58 ± 0.15	1.41 ± 0.40
M29696 interleukin-7 receptor alpha subunit (IL-7R-alpha)	2.49 ± 0.37	1.14 ± 0.52	3.34 ± 1.56
X77722 interferon-alpha/beta receptor b subunit (IFN-α/βR)	2.47 ± 0.43	*N.D.*	2.25 ± 0.59
X59770 interleukin-1 receptor type II (IL-1R-beta)	2.37 ± 0.36	1.50 ± 0.10	2.72 ± 0.93
U05875 interferon-gamma receptor beta subunit (IFN-γR beta)	2.19 ± 0.33	1.16 ± 0.07	1.62 ± 0.27
Apoptosis			
D38122 fas antigen ligand (FASL)	5.15 ± 3.73	5.51 ± 3.72	*N.D.*
AF016268 death receptor 5 (DR5)	3.54 ± 1.54	4.03 ± 2.06	0.78 ± 0.04

Figure 10. Gene products induced in human synoviocytes by the TLR3 agonist poly I:C

Gene product	Ratio of expression [1]
CXCL8 (IL-8)[2]	**20,90**
follicle stimulating hormone receptor (FSHR)	17,05
CXCL2 (MIP2-alpha)	**14,72**
tumor necrosis factor-inducible protein TSG-6	**14,10**
CXCL6 (granulocyte chemotactic protein 2, GCP 2)	**13,81**
CCL3 (MIP1-alpha)	**13,30**
granulocyte-macrophage colony stimulating factor (GM-CSF)	**12,42**
interleukin-11 (IL-11)	**9,60**
interleukin 10 receptor (IL-10R)	9,35
granulocyte colony-stimulating factor (G-CSF)	7,78
interleukin-1 receptor antagonist protein (IL-1RA)	7,70
interleukin-5 (IL-5)	**7,46**
heparin-binding EGF-like growth factor (HBEGF)	6,92
tumor necrosis factor (TNF-alpha)	**6,53**
TNF-related apoptosis inducing ligand (TRAIL)	6,18
endothelin 2 (ET2)	5,89
pleiotrophin (PTN)	**5,61**
glial cell line-derived neurotropic factor (GDNF)	5,56
endothelin 3 (ET3)	5,49
brain-derived neurotrophic factor (BDNF)/NT-3 growth factors receptor	5,29
granulocyte-macrophage colony-stimulating factor receptor alpha (GM-CSFR-alpha)	**4,62**
leukocyte interferon-inducible peptide (LIP)	4,53
manic fringe	4,21
leukemia inhibitory factor (LIF)	**3,85**
neurotrophin-4 (NT-4)	**3,84**
erythroid differentiation protein (EDF)	**3,69**
interleukin-7 receptor alpha subunit	**3,41**
oncostatin M (OSM)	3,28
interleukin-12 alpha subunit (IL-12alpha)	3,22
interferon-alpha/beta receptor b subunit	**3,16**
CCL5 (RANTES)	**3,07**
erythropoietin receptor (EPOR)	**3,07**
angiopoietin 1	3,04
interleukin-6 (IL-6)	**2,76**
bone-derived growth factor 1 (BPGF1)	2,75
Wnt-5A	2,69
interleukin-6 receptor beta subunit (IL-6R-beta)	2,62
interleukin-1 alpha (IL-1 alpha)	2,60
interleukin-10 (IL-10)	**2,57**
interferon gamma antagonist (IFN-gamma antagonist)	2,54
calcitonin receptor (CTR)	2,47
growth inhibitory factor, metallothionein-III (MT-III)	**2,45**
interferon-alpha/beta receptor alpha subunit	2,38
migration inhibitory factor-related protein 8 (MRP8)	**2,36**
CXCL9 (interferon-gamma-induced monokine, MIG)	**2,32**
CD40	**2,25**
fibroblast growth factor 5 (FGF5)	2,23
CD123 (interleukin-3 receptor alpha subunit)	2,22
interleukin-14 (IL-14)	2,21
CCL2 (monocyte chemotactic protein 1, MCP-1)	**2,20**
interleukin-1 receptor type II (IL-1R-beta)	**2,16**
CCL4 (MIP1-beta)	**2,13**
interleukin-12 receptor (IL-12R)	2,12
amphiregulin (AR)	2,11
interferon-gamma (IFN-gamma) receptor beta subunit	2,03
T-cell-secreted protein I-309, small inducible cytokine A1 (SCYA1)	2,01

1 Ratio of expression is given relative to untreated parallel control synoviocytes, only results are given for those products that displayed an induction level of at least a factor of 2.

2 Entries in bold indicate gene products induced by poly I:C in synoviocytes that are also induced at least 2-fold in astrocytes; these comprise about half the products listed in Figure 9.

Figure 11

Nucleotide sequence of F5 (plasmid sequence in italics and underlined):

*GGCTGGTCCGGTCCGGAATTCCCGGGGAATCGTCGACCCACGCGTCCG*CCCACGCGTCCGGGATCT
TTCCCTGGAGGAAATTCAGAAGAAATTAGAAGCTGCAGAAGAAAGACGCAAGTCCCATGAAGCTGA
GGTCTTGAAGCAGCTGGCTGAGAAACGAGAGCACGAGAAAGAAGTGCTTCAGAAGGCAATAGAAGA
GAACAACAACTTCAGTAAATGGCAGAAGAGAAACTGACCCACAAAATGGAAGCTAATAAAGAGAAC
CGAGAGGCACAAATGGCTGCCAAACTGGAACGTTTGCGAGAGAAGGATAAGCACATTGAAGAAGTG
CGGAAGAACAAAGAATCCAAAGACCCTGCTGACGAGACTGAAGCTGACTAATTTGTTCTGAGAACT
GACTTTCTCCCCATCCCCTTCCTAAATATCCAAAGACTGTACTGGCCAGTGTCATTTTATTTTTTC
CCTCCTGACAAATATTTTAGAAGCTAATGTAGGACTGTATAGGTAGATCCAGATCCAGACTGTAAG
ATGTTGTTTTAGGGGCTAAAGGGGAGAAACTGAAAGTGTTTTACTCTTTTTCTAAAGTGTTGGTCT
TTCTAATGTAGCTATTTTTCTTGTTGCATCTTTTCTACTTCAGTACACTTGGTGTACTGGGTTAAT
GGCTAGTACTGTATTGGCTCTGTGAAAACATATTTGTGAAAGAGTATGTGTGGCTTCTTTTGAAC
TGTTAGATGCTGAATATCTGTTCACTTTTCAATCCCAATTCTGTCCCAATCTTACCAGATGCTACT
GGACTTGAATGGTTAATAAAACTGCACAGTGCTAAAAAAAAAAAAAAA

Protein sequence

```
Clone F5        1                           ...(plasmid sequence)...  DLSLEEI     7
                                                                      |||||||
Stathmin        1 massdiqvkelekrasgqafelilsprskesvpefplsppkkkdlsleei  50


Clone F5        8 QKKLEAAEERRKSHEAEVLKQLAEKREHEKEVLQKAIEENNNFSKMAEEK  57
                  |||||||||||||||||||||||||||||||||||||||||||||||||
Stathmin       51 qkkleaaeerrksheaevlkqlaekrehekevlqkaieennnfskmaeek 100


Clone F5       58 LTHKMEANKENREAQMAAKLERLREKDKHIEEVRKNKESKDPADETEAD  106
                  ||||||||||||||||||||||||||||||||||||||||||||||||
Stathmin      101 lthkmeankenreaqmaaklerlrekdkhieevrknkeskdpadetead 149
```

Figure 12

SEQ ID NO. 1   Stathmin (stathmin-1)

```
  1 massdiqvke lekrasgqaf elilsprske svpefplspp kkkdlsleei qkkleaaeer
 61 rksheaevlk qlaekrehek evlqkaieen nnfskmaeek lthkmeanke nreaqmaakl
121 erlrekdkhi eevrknkesk dpadetead
```

SEQ ID NO. 2   SCG10 (Superior Cervical Ganglion10; stathmin-2)

```
  1 maktamayke kmkelsmlsl icscfypepr niniytyddm evkqinkras gqafelilkp
 61 pspiseaprt laspkkkdls leeiqkklea aeerrksqea qvlkqlaekr eherevlqka
121 leennnfskm aeeklilkme qikenreanl aaiierlqek erhaaevrrn kelqvelsg
```

SEQ ID NO. 3   SCLIP (SCG10-like protein; stathmin-3)

```
  1 mastisayke kmkelsvlsl icscfytqph pntvyqygdm evkqldkras gqsfevilks
 61 psdlspespm lssppkkkdt sleelqkrle aaeerrktqe aqvlkqlaer reherevlhk
121 aleennnfsr qaeeklnykm elskeireah laalrerlre kelhaaevrr nkeqreemsg
```

SEQ ID NO. 4   RB3 (stathmin-4)

```
  1 mtlaaykekm kelplvslfc scfladplnk ssykyegwcg rqcrrkdesq rkdsadwrer
 61 raqadtvdln wcvisdmevi elnkctsgqs fevilkppsf dgvpefnasl prrrdpslee
121 iqkkleaaee rrkyqeaell khlaekrehe reviqkaiee nnnfikmake klaqkmesnk
181 enreahlaam lerlqekdkh aeevrknkel keeasr
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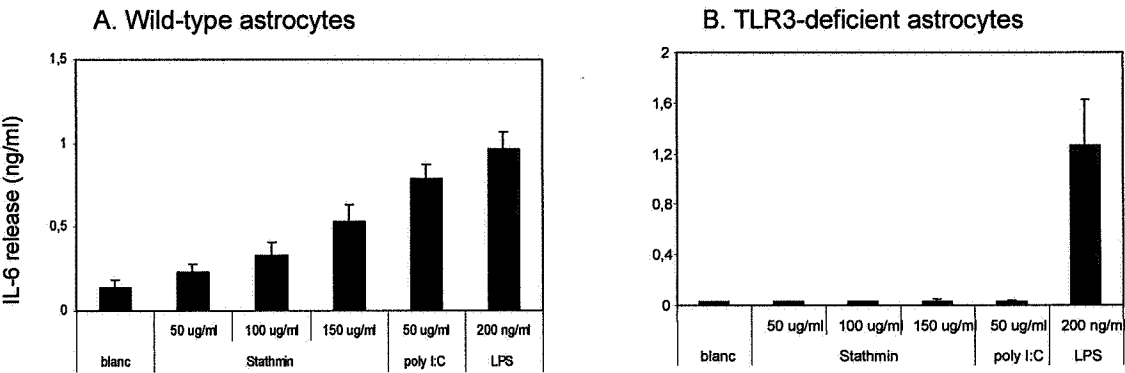
Figure 13

```
stathmin        mas-----------------------------------------------s
SCG10           maktamaykekmkelsmlslicscfypeprniniyty-----------d
SCLIP           mastisaykekmkelsvlslicscfytqphpntvyqy-----------g
RB3             m--tlaaykekmkelplvslfcscfladplnkssykyeadtvdlnwcvis
```

```
stathmin        diqvkelekrasgqafelilsprskes-vpefplsppkkk dlsleeiqkk
SCG10           dmevkqinkrasgqafelilkppspis-eaprtlaspkkk dlsleeiqkk
SCLIP           dmevkqldkrasgqsfevilkspsdlspespmlsspkkk  dtsleelqkr
RB3             dmevielnkctsgqsfevilkppsfdg-vpefnaslprrr dpsleeiqkk
```

```
stathmin        leaaeerrksheaevlkqlaekrehekevlqkaieennnfskmaeeklth
SCG10           leaaeerrksqeaqvlkqlaekreherevlqkaleennnfskmaeeklil
SCLIP           leaaeerrktqeaqvlkqlaerreherevlhkaleennnfsrqaeeklny
RB3             leaaeerrkyqeaellkhlaekreherevlqkaieennnfikmakeklaq
```

```
stathmin        kmeankenreaqmaaklerlrekdkhieevrknke skdpadetead
SCG10           kmeqikenreanlaaiierlqekerhaaevrrnke lqvelsg----
SCLIP           kmelskeireahlaalrerlrekelhaaevrrnke qreemsg----
RB3             kmesnkenreahlaamlerlqekdkhaeevrknke lkeeasr----
```
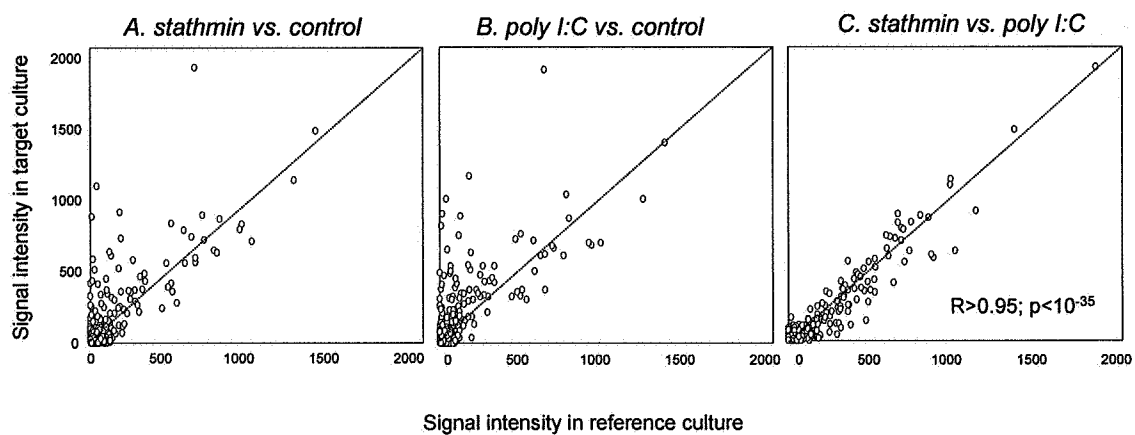
Figure 14

A. Wild-type astrocytes

B. TLR3-deficient astrocytes

Figure 15

A. stathmin vs. control   B. poly I:C vs. control   C. stathmin vs. poly I:C

Signal intensity in reference culture
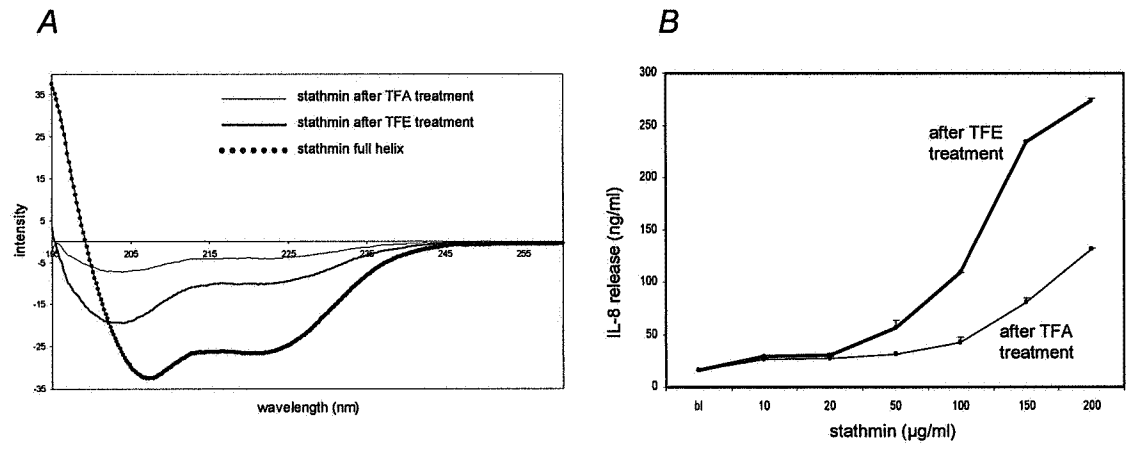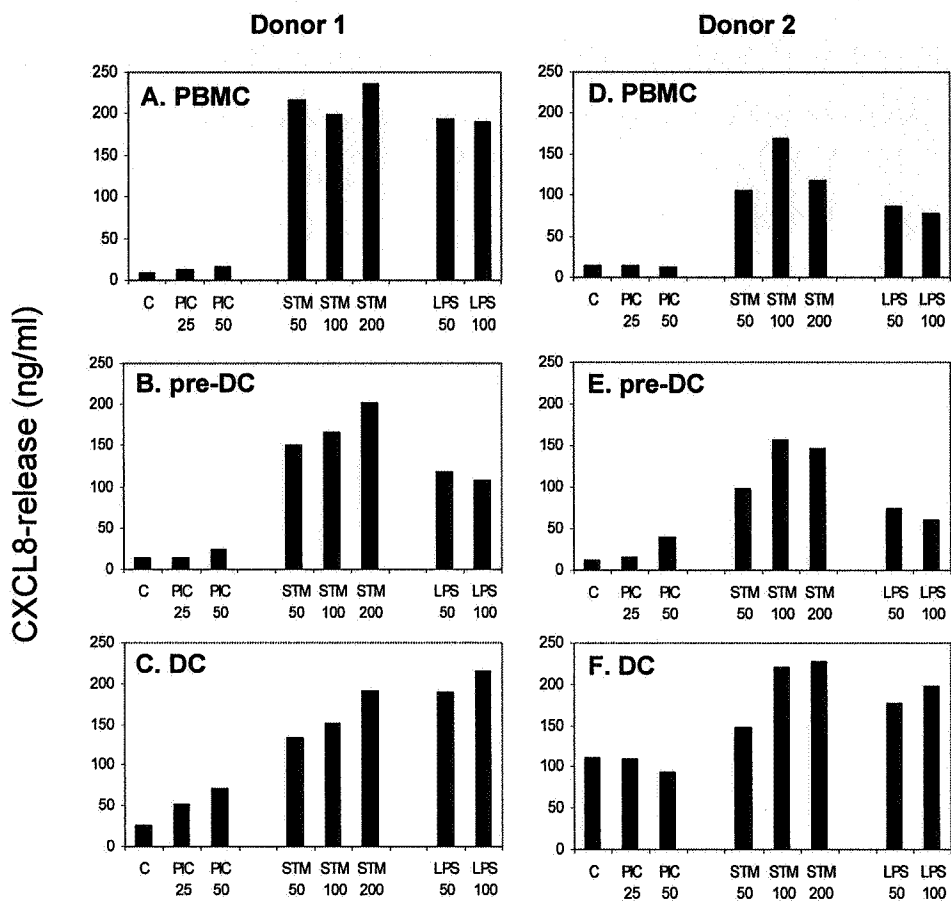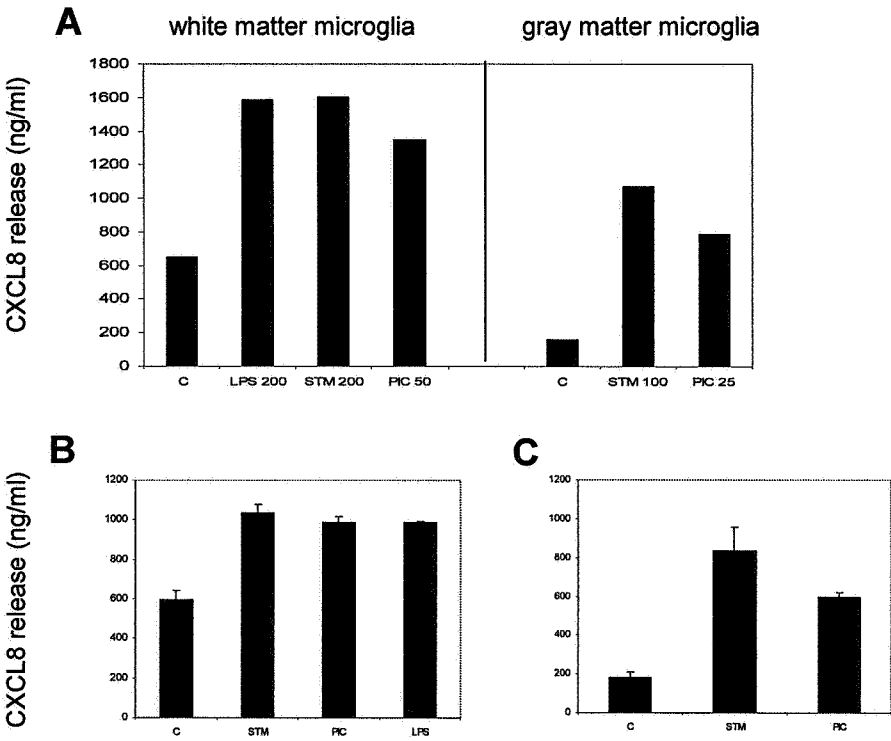
Figure 16

A

B

Figure 17

Figure 18. Accumulation after 48 h of cytokines and chemokines in a culture of stathmin-treated human pre-dendritic cells as compared to untreated control cells *.

Gene product	signal	ratio to control culture
GM-CSF	157,598	1,01
IL-8	**82,904**	**2,66**
IL-4	35,708	0,85
TIMP-2	29,311	0,72
ICAM-1	20,175	1,24
eotaxin2	20,060	0,87
MCP-1	**12,318**	**1,66**
IL-6	9,046	0,87
IL-6sR	8,074	0,81
MIP-1β	7,833	1,27
s TNF RII	7,380	0,50
IL-10	**5,360**	**1,59**
RANTES	4,712	1,16
IL-16	3,971	0,80
IL-1α	3,635	0,69
PDGF-BB	3,303	0,82
s TNF R1	2,967	0,42
I-309	2,877	0,61
IL-12p40	**2,674**	**1,77**
TNF-α	2,051	0,81
IL-3	1,631	0,43
IP-10	1,590	0,69
MIP-1α	1,584	0,76
TNF-β	1,456	0,39
MIP-1δ	1,402	0,55
IL-2	1,208	0,40
MCP-2	1,176	0,41
M-CSF	923	0,64
IFN-γ	787	0,68
IL-11	640	0,69
MIG	594	0,54
eotaxin	586	0,55
IL-12p70	560	0,48
IL-7	420	0,87
IL-15	398	0,41
IL-1β	382	0,22
TGF-β1	249	0,11
IL-17	76	0,08
G-CSF	-122	-0,15
IL-13	-267	-0,85

*Cytokine accumulation in the culture was quantified by using a commercial antibody-array. Cytokines are listed in decreasing order of quantity. Entries in bold indicate cytokines enhanced by at least a factor 1.5.

Figure 19

A white matter microglia   gray matter microglia

B

C

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/17 A61P25/00 A61P29/00

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)
A61K C07K A61P

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, MEDLINE, EMBASE, BIOSIS, COMPENDEX, INSPEC, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M ET AL: "The rationale for combined chemo/immunotherapy using a Toll-like receptor 3 (TLR3) agonist and tumour-derived exosomes in advanced ovarian cancer" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 23, no. 17-18, 18 March 2005 (2005-03-18), pages 2374-2378, XP004777559 ISSN: 0264-410X *p. 2376, 5.1.2 Synthetic double stranded RNA * -/--	1-3,6,7, 13-20

| X | Further documents are listed in the continuation of Box C. | | X | See patent family annex. |

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

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

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

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 June 2007	28/06/2007

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INTERNATIONAL SEARCH REPORT

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 1 288 226 A (NEDERLANDSE ORGANISATIE VOORTOEGEPAST-NATUURWETENSCHAPPELIJK ONDERZOEK) 5 March 2003 (2003-03-05) abstract; claims 1-40 paragraph [0008] - paragraph [0010] paragraph [0015] - paragraph [0016] paragraph [0020] paragraph [0023] -----	1-8, 13-20 1-28
Y	FARINA C ET AL: "Preferential expression and function of Toll-like receptor 3 in human astrocytes" JOURNAL OF NEUROIMMUNOLOGY, ELSEVIER SCIENCE PUBLISHERS BV, XX, vol. 159, no. 1-2, February 2005 (2005-02), pages 12-19, XP004709544 ISSN: 0165-5728 abstract page 17, column 2, last paragraph; figures 1,2; table 1 -----	1-28
Y	LIU, AIXIAO ET AL: "Expression of stathmin, a developmentally controlled cytoskeleton-regulating molecule, in demyelinating disorders" JOURNAL OF NEUROSCIENCE , 25(3), 737-747 CODEN: JNRSDS; ISSN: 0270-6474, 2005, XP002400765 * p. 740, col 2 - p. 741, col. 2 : Stathmin expression ... * * Fig. 3 * abstract -----	1-12
Y	JIN, L.-W. ET AL: "Neurofibrillary tangle-associated alteration of stathmin in Alzheimer 's disease" NEUROBIOLOGY OF AGING , 17(3), 331-341 CODEN: NEAGDO; ISSN: 0197-4580, 1996, XP002400766 page 336, column 1, paragraph 2 - page 337, column 2, paragraph 1; figure 5 -----	1-12
Y	OKAZAKI T ET AL: "SCG10, a neuron-specific growth-associated protein in Alzheimer's disease" NEUROBIOLOGY OF AGING, vol. 16, no. 6, 1995, pages 883-894, XP002400767 ISSN: 0197-4580 * p. 889, col. 2- p. 890, col. 2 : SCG10 Protein Accumulation ... *figures 2,3 -----	1-12

-/--

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	BSIBSI MALIKA ET AL: "Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators." GLIA. MAY 2006, vol. 53, no. 7, 15 February 2006 (2006-02-15), pages 688-695, XP002400768 ISSN: 0894-1491 * p. 694, col. 1, last * abstract -----	1-28

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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