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(54) Title: TLR4 LIGAND ISOLATED FROM HONEY

(57) Abstract: The invention concerns the isolation of an immunostimulatory molecule from honey and in particular manuka honey wherein said molecule has the molecular mass of 5.8 KDa and it induces the production of at least one of the following cytokines: TNF- α , IL-1 β or IL-6.

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TLR4 LIGAND ISOLATED FROM HONEY

The invention relates to a novel molecule that is isolated from honey and interacts with monocyte cell surface receptors to stimulate the release of inflammatory cytokines. The invention has application, particularly but not exclusively, in the wound healing industry.

Honey has been traditionally used in wound dressings for thousands of years. The treatment has regained popularity in recent times as an adjunct therapy to improve wound healing. A number of small clinical trials have been completed which indicated that topical application of honey to wounds clinically improved wound healing, and reduced healing times and scarring (1-3). Understanding the scientific basis of these effects could potentially lead to the development of novel therapeutic agents for the treatment of acute and chronic wounds. To date much of the research associated with honey and wound healing has concentrated on the effects of manuka honey. Manuka honey is produced from nectar collected from *Leptospermum scoparium*, which grows wild in New Zealand. It is a complex mixture of carbohydrates, fatty acids, proteins and amino acids, vitamins and minerals (4). Active manuka honey is renowned for its antibacterial activity, with batches assigned a Unique Manuka Factor (UMF) value corresponding to antibacterial activity (e.g. UMF 20 has equivalent antibacterial activity to 20% phenol w/v)(5).

Normal wound healing is a complex process in which damaged tissue is removed and gradually replaced by restorative tissue during an overlapping

series of events that include coagulation, inflammation, cell proliferation and tissue remodelling (6). The inflammatory phase of healing has an essential role in clearing the wound site of infectious agents and debris; this is facilitated by the activities of innate immune cells such as neutrophils and macrophages that migrate to the wound site in response to tissue damage (7). These cells aid the resolution of infection and removal of foreign material and cellular debris by phagocytosis (7). The individual role of neutrophils and macrophages have been investigated and previous studies indicate that macrophages have an essential role in wound resolution (8), since the absence of macrophages leads to poor debridement of the wound site and delayed repair (9; 10). In contrast, depletion of neutrophils leads to enhanced wound closure (11). In addition to their phagocytic role, macrophages release various growth factors and cytokines which are important in perpetuating the healing process (12). Recent studies indicate that production of IL-6 and TNF- α by macrophages, and other cells at the wound site are essential in the healing process (13; 14). We have previously shown that a variety of honey types can stimulate human monocytic cells to produce inflammatory cytokines (e.g. TNF- α , IL-6) important in resolution of infection and tissue repair (15). However, the components of honey responsible for this modulatory effect and the mechanism of action are yet to be determined. Previous studies have indicated that honey samples may be adulterated with micro-organisms including spore forming aerobic and anaerobic bacteria (16). The presence of micro-organisms or their cellular components could possibly explain the immune stimulatory activity of honey. Innate immune cells such as monocytes and macrophages produce inflammatory mediators in response to

the presence of microbes following engagement of microbial components with pattern recognition receptors expressed by the cells; these include Toll Like Receptors (TLRs) (17).

Somewhat surprisingly, our studies demonstrate that our observed effects of honey on cytokine production in myeloid cells are not a consequence of bacterial contamination of honey but are specifically associated with a 5.8 KDa moiety which stimulates inflammatory responses in monocytes by interaction with the monocyte TLR4 receptor.

Statements of Invention

Accordingly, our invention concerns the isolation of an immuno stimulatory molecule that is isolated from honey and has a molecular mass of 5.8 KDa and binds to the TLR4 receptor on monocytes to induce the production of at least one of the following inflammatory cytokines: TNF- α , IL-1 β or IL-6.

Moreover, in a preferred embodiment of the invention said immuno stimulatory molecule enhances the proliferation of human fibroblasts and so is particularly, but not exclusively, useful in wound healing.

Furthermore, we have discovered that the immuno stimulatory molecule of the invention does not possess anti-bacterial activity. Therefore, the ability of our immuno stimulatory molecule to induce the production of the said inflammatory cytokine cannot be attributed to any anti-bacterial agent.

In a preferred embodiment of the invention said honey is manuka honey which is endogenous to New Zealand and made from nectar collected from *Leptospermum Scoparium*.

In a preferred embodiment of the invention the molecular weight of the isolated molecule is determined by either microcon centrifugal filtration, gel filtration, dialysis or MALDI-ToF mass spectrometry. These methods, although well known to those skilled in the art, are described herein under the heading Materials and Methods.

Gel filtration and dialysis experiments revealed that the molecule had a mass of between 5-6 KDa. However, MALDI-ToF mass spectrometry revealed the molecular weight of the molecule was 5-8 KDa.

In a preferred embodiment of the invention said molecule is heat sensitive.

According to a further aspect of the invention there is therefore provided a dietary supplement comprising the aforementioned molecule of the invention.

According to a further aspect of the invention there is provided a food product augmented with the molecule or dietary supplement of the invention.

According to a yet further aspect of the invention there is provided a wound

healing product comprising the molecule of the invention.

In this aspect of the invention the wound healing product may comprise a cream lotion or spray. Alternatively, the wound healing product may comprise a dressing which has applied thereon or is impregnated therewith the molecule of the invention.

The invention will now be described with reference to the following Table and Figures wherein:

Table 1 shows characterisation of manuka honey samples. Batches of manuka honey were subjected to analysis of antibacterial activity, bacterial and spore content and LPS quantitation. The bioactivity of the batches was assessed; MM6 cells were incubated with 1% (w/v) honey solutions and cytokine production determined by ELISA. Data represent mean cytokine production $\pm$ 1SD (n=3). Control 1 (C1) represents MM6 cells incubated with 1 ng/ml LPS as described in methods. Control 2 (C2) represents MM6 cells incubated with syrup control; data represent mean cytokine production $\pm$ 1SD (n=3).

Table 2 shows antibacterial activity of honey fractions against reference strain of staphylococcus aureus E540. (+) indicates growth and (–) indicates no growth or antibacterial activity.

Figure 1 shows blocking TLR4 inhibits honey stimulated TNF- α production in human monocytes. MM6 cells were preincubated with anti-TLR4 antibody or isotype control IgG_{2a} antibody prior to incubation overnight with 1% (w/v) honey samples or LPS (100ng/ml). TNF- α production was determined by ELISA. Results are expressed as mean $\pm$ 1SD of three independent experiments ($\dagger$ $p < 0.0001$, $\ddagger p < 0.00001$);

Figure 2 shows Honey stimulated production of TNF- α is mediated via the TLR4 receptor. Murine myeloid cells expressing wildtype or non-functional TLR4 (wildtype or BMDMs and 32Dc13 TLR2 or TLR4 KO mice cells respectively) were incubated in the presence of 1% (w/v) honey or LPS (100ng/ml) for 4 hours. TNF- α production was determined by ELISA. Results are expressed as mean $\pm$ 1SD of three independent experiments;

Figure 3 shows effect of heat treatment and fractionation of manuka honey on cytokine stimulatory activity. Manuka honey samples were heat treated or fractionated according to molecular weight using microcon filtration. MM6 cells were incubated with fractionated, heat treated 1% (w/v) honey, untreated 1% (w/v) honey or LPS (100ng/ml) for 4 hours. TNF- α production was determined by ELISA. Results are expressed as mean $\pm$ 1SD of three independent experiments ($\ddagger p < 0.00001$);

Figure 4 shows MALDI-ToF MS analysis of fractionated manuka honey. MALDI-ToF MS detection of components present in A) >30KDa fraction of manuka honey and B) 5.8 KDa purified component;

Figure 5 shows a 5.8KDa component isolated from manuka honey stimulates TNF- α in human monocytes. MM6 cells were incubated with purified fractions of manuka honey containing a 5.8KDa component or LPS (100ng/ml) for 4 hours. Figure 5a. MM6 cells were preincubated with or without anti-TLR4 antibody or isotype control IgG_{2a} antibody prior to incubation with purified fractions of manuka honey containing a 5.8KDa component or LPS (100ng/ml) as a positive control overnight. Figure 5b. TNF- α production was determined by ELISA. Results are expressed as mean $\pm$ 1SD of three independent experiments; and

Figure 6 shows blocking TLR4 inhibits TNF- α production in human monocytes stimulated by the 5.8KDa component of manuka honey. MM6 cells were preincubated with anti-TLR4 antibody or isotype control IgG_{2a} antibody prior to incubation with purified fractions of manuka honey containing a 5.8KDa component or LPS (100ng/ml) for 4 hours. Figure 6a. Monocytes were preincubated with or without anti-TLR4 antibody or isotype control IgG_{2a} antibody prior to incubation overnight with 1% (w/v) honey fractions or LPS (100ng/ml). Figure 6b. TNF- α production was determined by ELISA. Results are expressed as mean $\pm$ 1SD of three independent experiments ($\ddagger$ p<0.00001, $\dagger$ p<0.005).

Figure 7. The 5.8KDa component stimulated production of TNF- α is mediated via the TLR4 receptor. BMDMs from wild type, TLR2 or TLR4 KO mice were incubated in the presence of >30KDa fraction, the isolated 5.8KDa component or LPS (100ng/ml) for 4 hours. TNF- α production was determined by ELISA. Results are expressed as mean $\pm$ 1SD of three independent experiments (*<0.01, ‡p<0.0001).

Figure 8. Effect of honey on cellular proliferation. Proliferation of human fibroblasts derived from normal skin or chronic wound site. Cellular proliferation was assessed by MTS assay in cells incubated with 1% unfractionated fresh or freeze dried honey or >30KDa freeze dried fraction. Figure represents mean + 1SD, n=4.

Materials and Methods

Cell culture

The effect of honey or honey components on inflammatory cytokine production was assessed in primary human monocytes, MonoMac6 (MM6), 32Dc13 cells or murine bone marrow derived macrophages (BMDMs) from wild type C57BL/6 mice or TLR2 (18) or TLR4 (19) knock out (KO) mice. Monocytes were obtained from peripheral blood from healthy volunteers. Polymorphonuclear cells were isolated by density gradient centrifugation and monocytes were enriched using the mini-MACS monocyte negative selection kit (Miltenyi Biotec, Surrey, United Kingdom) according to the manufacturer's instructions. Monocytes were cultured in RPMI 1640 medium (Sigma-Aldrich Co Ltd., Dorset,

UK), supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% 2mM L-glutamine, 1% non-essential amino acids, 1% penicillin (50 IU/ml) / streptomycin (100 µg/ml) and 1% sodium pyruvate (Invitrogen) at 37°C in 5% CO₂-humidified atmosphere. The human monocytic cell line, MM6 (18), and the murine myeloid cell line, 32Dc13 originally derived from C3H/ HeJ mice (19) were obtained from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). MM6 cells were maintained in RPMI 1640 medium (Sigma-Aldrich Co Ltd., Dorset, UK). Media was supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% 2mM L-glutamine, 1% non-essential amino acids, 1% penicillin (50 IU/ml) / streptomycin (100 µg/ml) and 1% sodium pyruvate (Invitrogen) at 37°C in 5% CO₂-humidified atmosphere. Cells were subcultured every 3 days at a density of 0.4×10^6 cells/ml. 32Dc13 cells were cultured in RPMI medium supplemented with 10% FBS and 10% WEHI conditioned medium (WEHI-CM) as a source of murine IL-3, an essential growth factor for these cells as previously described (20). The 32Dc13 cell line does not express functional TLR4 receptor (21). This cell line was used to assess the role of TLR4 in cellular responses to manuka honey. BMDMs, which express functional TLR4 were used as a control. BMDMs were isolated from 6-8 week old mice and cultured as previously described (22).

Further, in order to assess the role of TLRs in cellular responses to manuka honey, TLR2 and TLR4 knockout mice were examined.

Preparation of honey supplemented media

Fifteen different batches of New Zealand manuka honey were used throughout the study. The honey samples were from known floral sources and assessed for their anti-microbial activity by a *S. aureus* (ATCC 25923) inhibition assay and UMF values assigned accordingly (5). A control sugar syrup (artificial honey) was prepared as previously described (15). Honey solutions were made up to 1% (w/v) in supplemented medium and rendered sterile by filtration (0.45µM).

Bacterial content of honey samples

All honey samples were assessed for the presence of viable bacteria and spores under both aerobic and anaerobic conditions. Honey samples were either spread directly onto blood agar or following enrichment for 5 days in either cooked meat broth or Hartley's digest broth before culture on blood agar.

LPS (endotoxin) content of honeys

All honey samples were assessed for LPS content using the kinetic LAL assay (KQCL), purchased from Biowhittaker Ltd, Wokingham, UK. The assay was performed according to the manufacturer's instruction.

Cellular viability of cells incubated with honey

MM6 cells at a density of 1×10^6 cells/ml were incubated with 1% (w/v) of each honey solution for 0-24h. The cells were washed in PBS (x3) and resuspended in 1ml of fresh media. Assessment of cellular viability was determined using trypan blue or the MTS bio-reduction assay as previously described (15; 23).

Cell viability remained above 90% for all samples tested at all time points assessed.

Measurement of TNF- α , IL-1 β or IL-6 release from human monocytes or murine myeloid cells

In order to determine the effect of honey or honey fractions on cytokine release, 1×10^6 cells/ml were incubated with 1% (w/v) honey, individual honey fractions or heat treated honey for 4h (TNF- α production) or 24 h (IL1- β and IL-6 production) at 37 °C in 5% CO₂ atmosphere. Following incubation, supernatants were collected and stored at -80°C. TNF- α , IL-1 β or IL-6 in cell culture supernatants were quantified by ELISA in accordance with the manufacturer's instructions (R&D systems).

Determining the effect of Polymyxin B on honey stimulated cytokine release

In order to assess the role of LPS in honey mediated cytokine release, MM6 cells were incubated with polymyxin B, a chelator of LPS prior to honey treatment. MM6 cells were incubated with polymyxin B (10 μ g/ml) for 1 hour before addition of 1% honey samples or LPS (100ng/ml) for 4 or 12 hours. Following incubation, supernatants were collected and stored at -80°C. TNF- α , IL-1 β or IL-6 in cell culture supernatants were quantified by ELISA in accordance with the manufacturer's instructions (R&D systems).

Separation of honey components by microcon centrifugal filtration, gel filtration and dialysis

In order to begin to identify the active components of manuka honey, samples were fractionated according to molecular weight. Initially manuka honey was fractionated by three different methods in order to determine the approximate mass of the component(s) responsible for cytokine stimulation. Honey samples were first subjected to dialysis using membranes with MWCO of 3, 8 and 14KDa (D-tubeTM Dialyzers, Novagen, Nottingham, UK). Alternatively, honey samples were sequentially fractionated using microcon centrifugal filters (Millipore, Massachusetts, USA), to provide fractions with apparent molecular weights of <3, 3-10, 10-30 and >30KDa (24). Honey was also fractionated by gel filtration using sephadex G-25 column (GE Healthcare, Amersham UK). Component elution was carried out with standard RPMI medium at a flow rate of 0.2ml / min. Fractions equivalent to 1% total honey (w/v) were assessed for their ability to stimulate TNF- α , IL-1 β or IL-6 synthesis in MM6 cells (see above). Fractions were freeze dried for MALDI-ToF analysis and further fractionation using reverse phase solid extraction SPE (see below).

MALDI-ToF mass spectrometry

Freeze dried honey fractions were reconstituted in water and mixed in a 1:10, sample: matrix ratio with Sinapinic acid matrix solution (10mg/ml sinapinic acid in 70/30 0.1% aqueous trifluoroacetic acid (TFA)/acetonitrile (ACN)). 1 μ l of the resulting mixture was applied to a MALDI plate and allowed to air dry. The plate was then inserted into a Voyager DE-STR MALDI-ToF mass spectrometer (Applied Biosystems, Warrington, UK) and spectra acquired between 1000 to 150000 amu in positive ionisation linear mode using an acceleration voltage of

25kV, a grid voltage of 90% of the acceleration voltage, a delay time of 750nsec and 100 laser shots per spectrum.

Miniaturised reverse solid phase extraction SPE fractionation

The sample was dissolved in 10µl of 0.1% TFA. A C18 reverse phase ziptip (Millipore, UK) was conditioned with 5 washes of 50/50 methanol/0.1% TFA followed by 5 washes with 0.1% TFA before sample application. The sample was applied to the ziptip and allowed to wash over the packing 10 times, the sample solution therefore contained only the components not bound to the ziptip. The ziptip was washed 5 times with 0.1% TFA before elution using 80/20 CAN /0.1% TFA. Both fractions were freeze dried prior to bioassay.

Monosaccharide composition analysis

The isolated 5.8KDa component was assessed for the presence of monosaccharide.

Briefly, the internal standard (Arabitol) was added to each sample and lyophilised. The sample was then subjected to methanolysis in 1N methanolic/HCl (80°C for 16 hours under nitrogen) followed by derivatisation and analysis by GC-MS.

Amino acid composition analysis

The isolated 5.8 KDa component was assessed for the presence of amino acids. Briefly, the internal standard (Norleucine) was added to each sample and hydrolysed in 6N HCl for 4 hours at 145oC. The samples were derivatised and

analysed by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) coupled with UV detection. The data was then compared to that obtained from analysis of a standard mixture containing 50nmoles of each amino acid and 50nmoles of the internal standard.

Blocking of pattern recognition receptors TLR 2 and 4 mediated responses

In order to assess the role of pattern recognition receptors (PRRs) in cellular responses to manuka honey, cytokine production was assessed in the presence of anti-TLR2 and anti-TLR4 antibodies. TLR2 and TLR4 receptors were blocked on the surface of MM6 cells or primary human monocytes prior to incubation with 1% (w/v) honey or fraction solutions. Monocytes or MM6 cells (1×10^6 /ml) were incubated with 10 µg/ml of anti-TLR2 (TLR2.1, TCS cell works, Buckinghamshire, UK) or 20 µg/ml anti-TLR4 (HTA 125, Insight Biotechnology Ltd, Wembley, UK) respectively for one hour prior to addition of honey or fraction samples, to give a final concentration of 1% (w/v) honey. Cells were then incubated overnight and assayed for TNF-α or IL-6 as described above. To control for non-specific binding an IgG_{2a} isotype control antibody (Insight Biotechnology Ltd, Wembley, UK) was used. Experimental conditions were previously optimised for maximum inhibition of specific ligand stimulated cytokine responses for TLR2 and TLR4, Lipoteichoic acid (LTA) and LPS respectively.

Results

Characterisation of manuka honey

Human monocytic cells, MM6 were incubated with different batches of 1% (w/v) manuka honey in order to profile their effect on cytokine production. In accordance with our previous studies (15), manuka honey stimulated the production of inflammatory cytokines TNF- α , IL-1 β or IL-6 (Table 1) in this cell line and in human peripheral blood monocytes (data not shown). Each batch of manuka honey was assessed for bacterial contamination and the presence of spores under both aerobic and anaerobic conditions. All samples were negative for vegetative bacterial growth. However, from just over a third of honey samples (6/15), aerobic and / or anaerobic spores were recovered, with the majority identified as *Bacillus* species. Further, when LPS content was assessed, only very low levels of LPS (<0.27ng/ml) were detected in each batch of honey (Table 1). In order to assess the stimulatory activity of equivalent LPS concentrations, MM6 cells were stimulated with 1ng/ml of LPS and cytokine production was assessed. Stimulation with this concentration of LPS resulted in low levels of cytokine production; 10-, 4- and 3-fold less synthesis of IL-1 β , IL-6 and TNF- α , respectively, when compared to cells treated with 1% (w/v) honey (Table 1). As a further control, sugar syrup was shown not to stimulate significant cytokine production under identical culture conditions (Table 1). Although stimulation of inflammatory responses by the honey samples slightly varied from batch to batch, there was no correlation of stimulation with bacterial spore or LPS concentration. Further, manuka samples were assessed for antibacterial activity and assigned a UMF value accordingly. The antibacterial activity of the samples ranged from equivalent to 5 – 24.4% (v/v) phenol but was not associated with cytokine production (Table 1).

Honey stimulated cytokine production is mediated via interactions with TLR4 but not TLR2

Innate immune cells respond to the presence of microbes, debris and foreign material via pattern recognition receptors, which include the Toll like receptors (TLRs). Of the TLRs identified to date, TLR2 and TLR4 are amongst the best characterised with regard to their specificity and downstream signalling pathways. TLR2 recognises lipoproteins / lipopeptides and peptidoglycan (25-27), whereas TLR4 recognises LPS from Gram negative bacteria (21), however numerous other exogenous and endogenous ligands have been identified for these receptors (28). In order to investigate whether manuka honey stimulates myeloid cells via TLR2 or 4, MM6 cells were pre-incubated with antibodies directed against the ligand binding domain of TLR2 or TLR4. Treatment of cells with anti-TLR2 antibody did not affect cytokine production by cells stimulated with honey (data not shown). However, anti-TLR4 blocking antibody significantly inhibited honey induced TNF- α production by ~70% ($p < 0.0001$) (Figure 1). This data suggests that the active component(s) of honey signal through TLR4, but not TLR2. This was further confirmed in murine cells deficient for TLR4 and BMDMs isolated from TLR2 and TLR4 KO mice. Following stimulation with 1% (w/v) honey, wildtype and TLR2 KO murine macrophages produced TNF- α in a similar manner to that observed in human monocytic cells. However BMDMs derived from TLR4KO mice and 32Dc13 cells lacking the TLR4 receptor did not produce TNF- α in response to honey incubation (Figure 2).

Fractionation of manuka honey indicates that it contains an active component of approximately 5.8KDa

In order to determine the component(s) of honey responsible for inducing cytokine production in myeloid cells, gel fractionation and dialysis was initially performed. Initial data indicated that a component of around 5-6 KDa present in honey was able to stimulate TNF- α production. These methodologies proved problematic for downstream applications, namely associated with dilution / contamination and loss of active components respectively. Further studies utilising microcon centrifugal filtration allowed concentration of the active fractions and control of dilution. Utilising this method the majority of cytokine stimulatory activity was found to be associated with fractions with an apparent molecular weight greater than 30KDa, although some activity was present in the <3KDa fraction (Figure 3). To further characterise the chemistry of these components, honey and its fractions were heat treated prior to incubation with MM6 cells. As shown in Figure 3, heat treatment of unfractionated honey caused a significant ($P < 0.0001$) reduction in the ability of honey to stimulate IL-1 β , IL-6 or TNF- α production in MM6 cells. A similar effect was observed in the fractionated honey (data not shown). Taken together, the data indicates that the active components are heat sensitive, and provides further evidence that components other than LPS (a heat stable molecule) are responsible for the activity associated with manuka honey. Therefore, further analysis was performed by MALDI-ToF mass spectrometry.

MALDI-ToF analysis was restricted to the >30KDa fraction, since the majority of cytokine stimulatory activity was associated with these components. This strategy demonstrated the presence of a small number of high molecular weight components (Figure 4A). More surprisingly a number of less than 30KDa molecular weight moieties were also observed in this fraction, possibly as a consequence of binding of these components to larger molecules (Figure 4A). There was a variation in the peaks present across the samples, and their relative proportions. Since gel filtration and dialysis indicated that the active component had a mass of around 5-6 KDa, this peak was further purified utilising miniaturised reverse phase SPE separation (Figure 4B) with purity confirmed by MALDI-ToF analysis and its ability to stimulate cytokine production assessed (Figure 5). This component was found to stimulate TNF- α production in monocytes (Figure 5) whereas a fraction containing the remaining components determined to be present in the >30KDa fraction did not stimulate the production of this cytokine (data not shown). In experiments assessing the activity of the isolated component, a number of samples isolated from different batches of honey were used to ensure that the data was representative of the different batches. There was some variation in activity associated with different batches; however all batches stimulated similar levels of cytokine induction.

The isolated component was examined for the presence of amino acids and monosaccharides. Analysis revealed the absence of amino acids from the isolated component, indicating that the component is not a protein. Monosaccharide analysis revealed the presence of monosaccharides. Since

unfractionated honey stimulated TNF- α production via TLR4, we assessed the role of the 5.8KDa component in stimulating production of this cytokine via this receptor. As shown in Figure 6a, TNF- α production stimulated by the 5.8KDa component was abrogated in MM6 cells by pre-treatment with anti-TLR4 ($p < 0.00001$).

This data was confirmed in primary human monocytes, as illustrated in Figure 6b. TNF- α production stimulated by the >30 KDa fraction or the purified 5.8KDa component was significantly inhibited in primary human monocytes following pre-treatment with anti-TLR4 antibody ($p < 0.0005$ and $p < 0.005$ respectively). In addition when BMDMs from wild type and TLR4 knockout mice were incubated in the presence of the isolated component, TNF- α production was significantly depressed ($p < 0.00005$) in TLR4 KO compared to wildtype or TLR2 KO BMDMs (Figure 7). Taken together, the data suggests that manuka honey contains a heat sensitive 5.8KDa component that stimulates cytokine production via TLR4 and that this activity is not associated with bacterial endotoxin.

Whilst the wound healing properties of honey have been documented, there is no evidence regarding the scientific basis of the observed benefits associated with honey treatment. We have therefore fractionated manuka honey by several methods in order to identify a single protein which acts via the TLR4 receptor to stimulate cytokine production and so bring about the inflammatory phase of wound healing.

In accordance with conventional teaching, initially we investigated the possibility that honey stimulated cytokine induction may be initiated by the presence of microbes or their components present as contaminants in honey. The low water activity and acidic nature of honey make it generally unsuitable medium for bacterial growth (31), nevertheless a number of reports have described bacterial and fungal contamination of honey (16; 32). This suggests contaminated honey may act as a potential source of infection. Indeed honey has been identified as a source of botulism in infants (33). The origin of these infectious agents in honey has been discussed in a previous review (16), and include the gut of honey bees and raw nectar. In our present study, no vegetative bacteria were cultured from any of the batches of manuka honey used. However bacterial spores were isolated from around a third of the samples, the majority of spores isolated in this study were *Bacillus* species, which correlates well with previous studies of bacterial contamination (16). The presence of bacterial spores raises the possibility that honey samples may be contaminated with bacterial components including LPS, which may be responsible for the cytokine inducing activity of the honey. We assessed LPS concentrations in our honey samples and detected very low levels of LPS (<0.3 ng/ml). Since LPS induces inflammatory cytokine production in monocytic cells, the stimulatory activity of the honey samples and a LPS dose response were assessed in MM6 cells. When MM6 cells were stimulated with LPS at concentrations of between 10-1000pg/ml (several fold higher than detected in the honey samples), cytokine production was considerably lower than that observed for manuka honey (Table 1). Statistical analysis revealed that the cytokine stimulatory activity of individual

honey batches did not correlate with LPS levels ($r^2 = 0.0169$, 0.1991 and 0.1826 for IL- 1β , IL-6 and TNF- α respectively) or to bacterial spore content. In order to eliminate LPS as an agent responsible for initiating inflammatory responses, it is conventional to assess the effect of heat treatment or inhibition of responses by polymyxin B (28). Previously, effects which are non-inhibitable by polymyxin B or are abrogated by heat treatment have been deemed non-LPS associated. To this purpose we heat treated honey by boiling for an hour and utilised the LPS chelator, polymyxin B. Heat treatment caused a significant ($P < 0.0001$) reduction in the ability of honey to stimulate IL- 1β , IL-6 or TNF- α production in MM6 cells. In addition, the cytokine stimulatory effect of honey was also assessed in the presence of polymyxin B. Under these conditions, no significant reduction of stimulatory activity was observed (data not shown).

The induction of inflammatory responses in innate immune cells is classically initiated by activation of pattern recognition receptors following engagement of pathogen associated molecular patterns commonly expressed by a variety of microbes (17). A number of pattern recognition receptors have been identified to date, including the TLR family (35). The specific ligands recognised by these receptors and the signalling pathways involved in receptor mediated cellular responses are currently being intensively investigated. Recent evidence suggests a role for TLRs in initiating tissue repair and regeneration (14). Tissue injury results in the release of intracellular components such as heat shock proteins and the production of extracellular matrix breakdown products such as hyaluronan fragments, both of which act as endogenous ligands of TLRs and

stimulate inflammatory responses (36; 37). Further, mice deficient in TLR2 or TLR4 have impaired tissue repair and regeneration (38; 39). Such evidence indicates that in addition to a role in removal of microbes, pattern recognition receptors appear to have a role in tissue repair. Given the important role of TLR signalling in wound healing, this study assessed the effect of blocking two key PRRs in cellular responses to honey (TLR2 and 4). Blocking of the TLR4 but not the TLR2 receptor significantly inhibited honey stimulated TNF- α production in human monocytes. To support the role of TLR4 in this response, we further examined honey stimulated cytokine production in murine myeloid cells either expressing wild type functional (BMDM) or non-functional (32Dc13) TLR4 and TLR2KO and TLR4KO mice. Honey stimulated TNF- α production was observed in BMDMs but not in 32Dc13 or TLR4KO cells suggesting a role for TLR4 in mediating honey stimulation. Previous studies have demonstrated that whilst 32Dc13 cells do not express a functional TLR4, they are capable of secreting TNF- α , in response to various stimuli (40). Whilst the majority of experiments were carried out using human and murine cells lines or primary murine cells, the major findings were confirmed in primary human monocytes isolated from peripheral blood. Cumulatively, these data indicate that honey stimulates monocytic cells via a TLR4 dependent mechanism.

Utilising a range of separation techniques we endeavoured to isolate active components present in manuka honey responsible for stimulating cytokine production via TLR4. In our study, initial experiments using gel filtration and dialysis indicated that cytokine stimulatory activity was associated with

components of around 5-6KDa. These methodologies proved problematic for downstream applications, namely associated with dilution / sterility of sample and loss of active components respectively. Therefore microcon centrifugal filtration was employed to produce a series of fractions of various apparent molecular weights from <3KDa through to >30KDa. The use of microcon filtration units allowed the control of concentration of the final isolated fractions for assessment of bioactivity. The >30KDa fraction was subjected to further analysis by MALDI-ToF mass spectrometry, since the majority of cytokine stimulatory activity was associated with this fraction. MALDI-ToF analysis revealed the presence of a small number of high molecular weight components and also a number of less than 30KDa molecular weight components. The presence of these smaller molecules was surprising since the microcon filters are supposed to have a molecular weight cut off (MWCO) of 30KDa. It is possible that these molecules escaped separation as a consequence of binding to larger molecules or forming aggregates. Further purification demonstrated a component of 5.8KDa present was able to stimulate cytokine production in human monocytes. This fraction was negative for LPS, heat sensitive and cytokine production could be inhibited by blocking TLR4. Therefore it would appear that whilst the component may associate with other larger molecules, this association is not required for the activity of the component, since activity is not impaired in preparations of the purified component. The finding that the isolated component has a molecular weight of 5.8kDa further supports the suggestion that this component is not LPS, since LPS molecules tend to have a molecular weight greater than 10KDa and form aggregates up to 100KDa (41).

Initial bio-analysis of the active components of the manuka honey by combined treatments and mass spectrometric means suggest that the heat labile active component may interact with a larger molecular weight moiety in honey and its lack of retention on the microSPE ziptip system suggests that the component is unlikely to be a protein or peptide (since these tips are commonly used for purification of such compounds). This is supported by the absence of amino acids when analysed by RP-HPLC. Monosaccharide component analysis revealed the presence of monosaccharides. The active components molecular weight rules out many of the known active components of manuka honey such as amino acids, vitamins and minerals.

In further studies we have assessed the antimicrobial activity of the active component of honey and we have discovered that when manuka honey was separated by microron filtration, as previously described, antimicrobial activity was found to be associated with the <3KDa fraction and not in the >30KDa fraction from which the immune stimulatory component was isolated (see Table 1). This Indicates that antimicrobial activity is distinct from immune stimulatory activity.

Further investigations revealed that the process of freeze drying significantly reduced antimicrobial activity of honey fractions (by approximately 50%). This is in contrast to the previously described immuno stimulatory component; the immuno stimulatory activity of the isolated component is not significantly reduced by freeze drying (see Table1).

Moreover, we have also discovered that whilst honey is antibacterial, it is more potent in inhibiting the growth of Gram positive organisms than Gram negative organisms. However, pre-incubation of monocytes with 1% (w/v) honey results in enhanced intracellular killing of Gram negative microbes as determined by the gentamicin protection assay. Pre-incubation of cells with honey enhanced killing by 20-50%, indicating an enhancement of host immune responses.

Finally, we have also discovered that incubation of human fibroblasts from normal skin or chronic wounds in the presence of the immuno stimulatory component of honey enhances cellular proliferation, as determined by MTS assays by between 21-40%. Please see Figure 8.

Summary

In summary, we report for the first time the isolation of a 5.8 KDa component responsible for cytokine induction in human monocytes and the mechanism via which this component stimulates innate immune cells. The component isolated from manuka honey stimulates the production of inflammatory cytokines via TLR4. These findings reveal mechanisms and components involved in honey stimulation of cytokine induction and lead to the development of novel therapeutics to improve wound healing for patients with both acute and chronic wounds.

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Table 1. Characterisation of manuka honey samples.

Batches of manuka honey were subjected to analysis of antibacterial activity, bacterial and spore content and LPS quantitation. The bioactivity of the batches was assessed; MM6 cells were incubated with 1% (w/v) honey solutions and cytokine production determined by ELISA. Control 1 (C1) represents MM6 cells incubated with 1 ng/ml LPS as described in methods. Control 2 (C2) represents MM6 cells incubated with syrup control. Data represent mean cytokine production $\pm$ 1SD (n=3).

Honey Samples	C1	C2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Antibacterial activity- UMF	n/a	n/a	5	6	12	13	14	14	14	17	18	19	21	22	23	24	24
Bacterial Growth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aerobic spores	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Anaerobic spores	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Lipopolysaccharide* (pg/ml)	1000	10	130	165	98	65	142	46	157	39	37	153	92	84	212	268	188
Cytokine Production (pg/ml)																	
IL-1 β	20	22	250	221	198	204	203	164	239	209	141	274	189	151	175	179	175
	($\pm$ 10)	($\pm$ 1)	($\pm$ 10)	($\pm$ 15)	($\pm$ 20)	($\pm$ 20)	($\pm$ 10)	($\pm$ 17)	($\pm$ 15)	($\pm$ 19)	($\pm$ 10)	($\pm$ 26)	($\pm$ 17)	($\pm$ 18)	($\pm$ 15)	($\pm$ 18)	($\pm$ 27)
IL-6	238	40	700	761	722	1201	767	859	1383	770	775	1397	778	790	796	725	709
	($\pm$ 19)	($\pm$ 2)	($\pm$ 24)	($\pm$ 19)	($\pm$ 16)	($\pm$ 16)	($\pm$ 10)	($\pm$ 16)	($\pm$ 19)	($\pm$ 77)	($\pm$ 10)	($\pm$ 21)	($\pm$ 16)	($\pm$ 22)	($\pm$ 18)	($\pm$ 22)	($\pm$ 20)
TNF- α	150	50	565	535	558	496	542	348	670	316	319	562	538	539	405	382	384
	($\pm$ 8)	($\pm$ 7)	($\pm$ 24)	($\pm$ 13)	($\pm$ 69)	($\pm$ 69)	($\pm$ 10)	($\pm$ 60)	($\pm$ 11)	($\pm$ 51)	($\pm$ 56)	($\pm$ 12)	($\pm$ 92)	($\pm$ 12)	($\pm$ 75)	($\pm$ 95)	($\pm$ 12)
	)	)	)	)	)	)	)	)	)	)	)	)	)	)	)	)	)

Freshly prepared honey	20%	10%	8%	6%	4%	2%
Fresh unfractionated honey	-	-	-	+	+	+
<3 KDa fraction	-	+	+	+	+	+
3-10 KDa Fraction	+	+	+	+	+	+
10-30 KDa fraction	+	+	+	+	+	+
>30 KDa fraction	+	+	+	+	+	+
Freeze Dried unfractionated honey	-	+	+	+	+	+

Table 2

CLAIMS

1. An isolated immuno stimulatory molecule obtained from honey that has a molecular mass of 5.8 KDa and binds to the TLR4 receptor on monocytes to induce the production of at least one of the following inflammatory cytokines: TNF- α , IL-1 β or IL-6.
2. An isolated immuno stimulatory molecule according to claim 1 wherein said molecule enhances fibroblast proliferation.
3. An isolated immuno stimulatory molecule according to claim 1 or claim 2 wherein said molecule is not anti-bacterial.
4. An isolated immuno stimulatory molecule according to claims 1-3 wherein said honey is manuka honey.
5. An isolated immuno stimulatory molecule according to claim 4 wherein said honey is made from nectar collected from *Leptospermum scoparium*.
6. An isolated immuno stimulatory molecule according to claim 4 wherein the honey is endogenous to New Zealand.
7. An isolated immuno stimulatory molecule according to any preceding claim wherein the molecular weight of the molecule is determined by any one of

the following techniques: microcon centrifugal filtration, gel filtration, dialysis or MALDI-ToF mass spectrometry.

8. An isolated immuno stimulatory molecule according to any preceding claim wherein said molecule is heat sensitive.
9. A dietary supplement comprising the isolated immuno stimulatory molecule according to claims 1-8.
10. A food product augmented with either the isolated immuno stimulatory molecule according to claims 1-8 or the dietary supplement according to claim 9.
11. A wound healing product comprising an isolated immuno stimulatory molecule according to claims 1-8.
12. A wound healing product according to claim 11 wherein said product comprises a dressing which has applied thereon or is impregnated therewith an isolated immuno stimulatory molecule according to claims 1-8.

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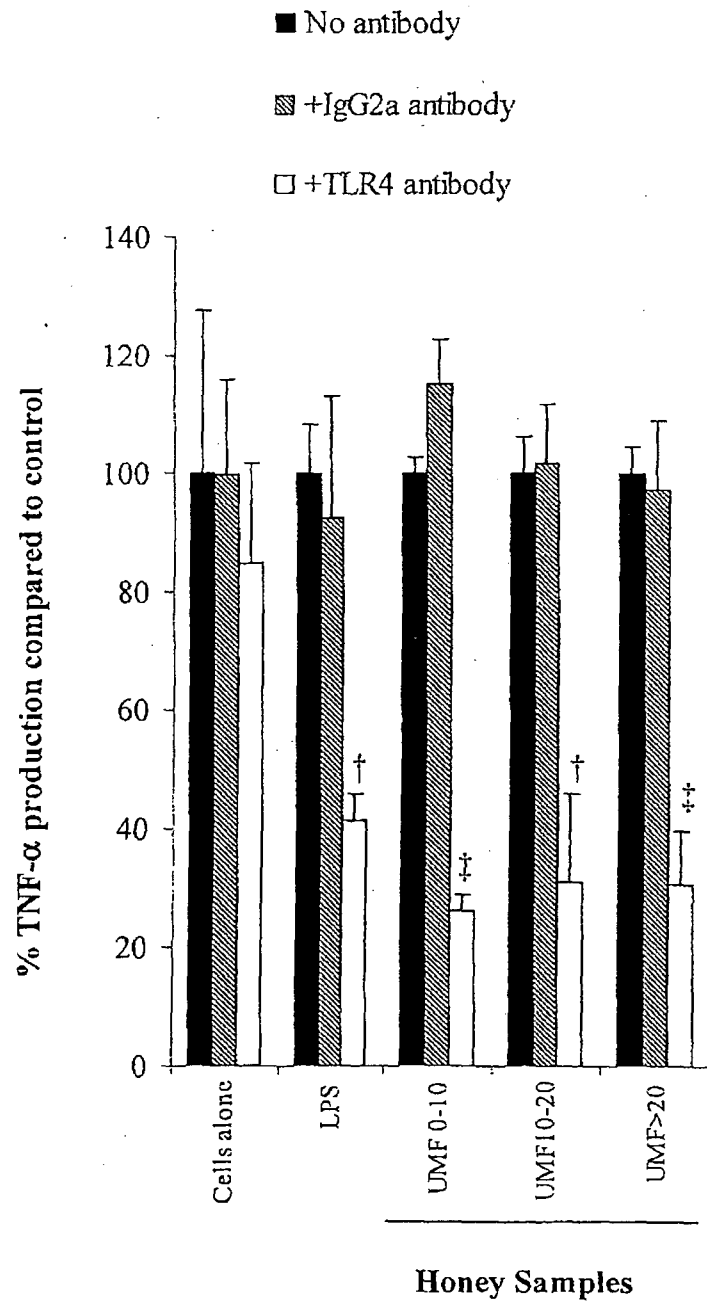


Figure 1a

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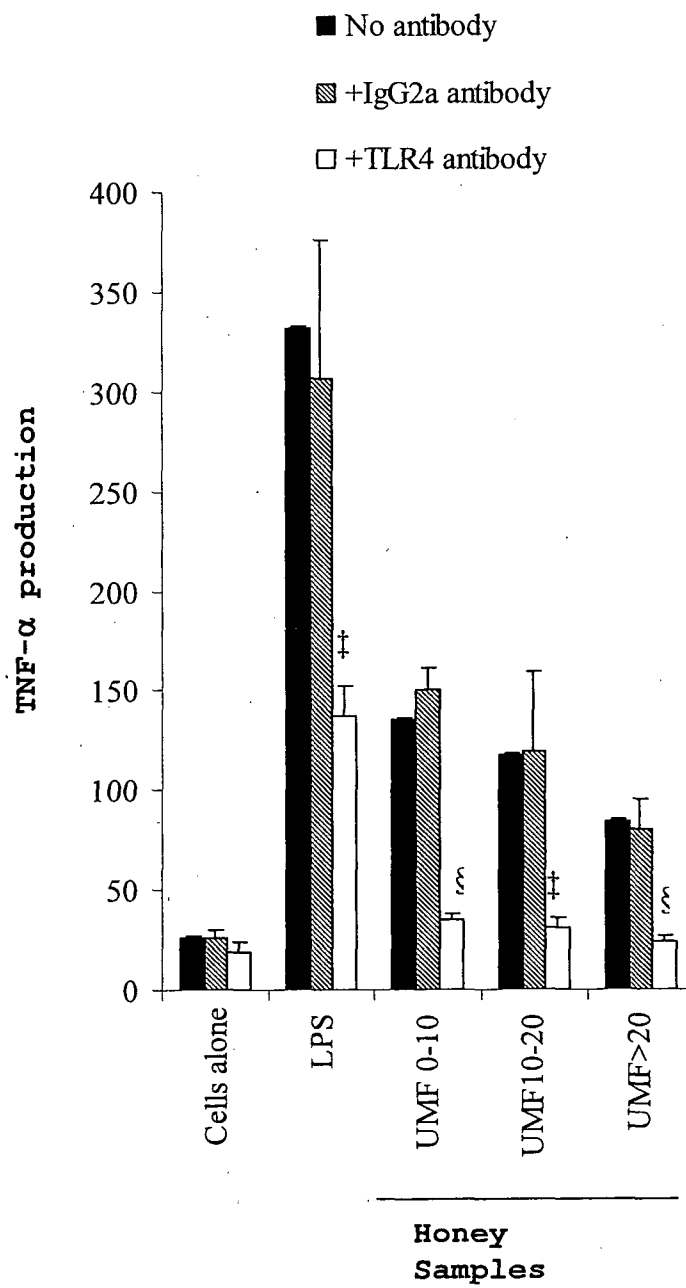


Figure 1b

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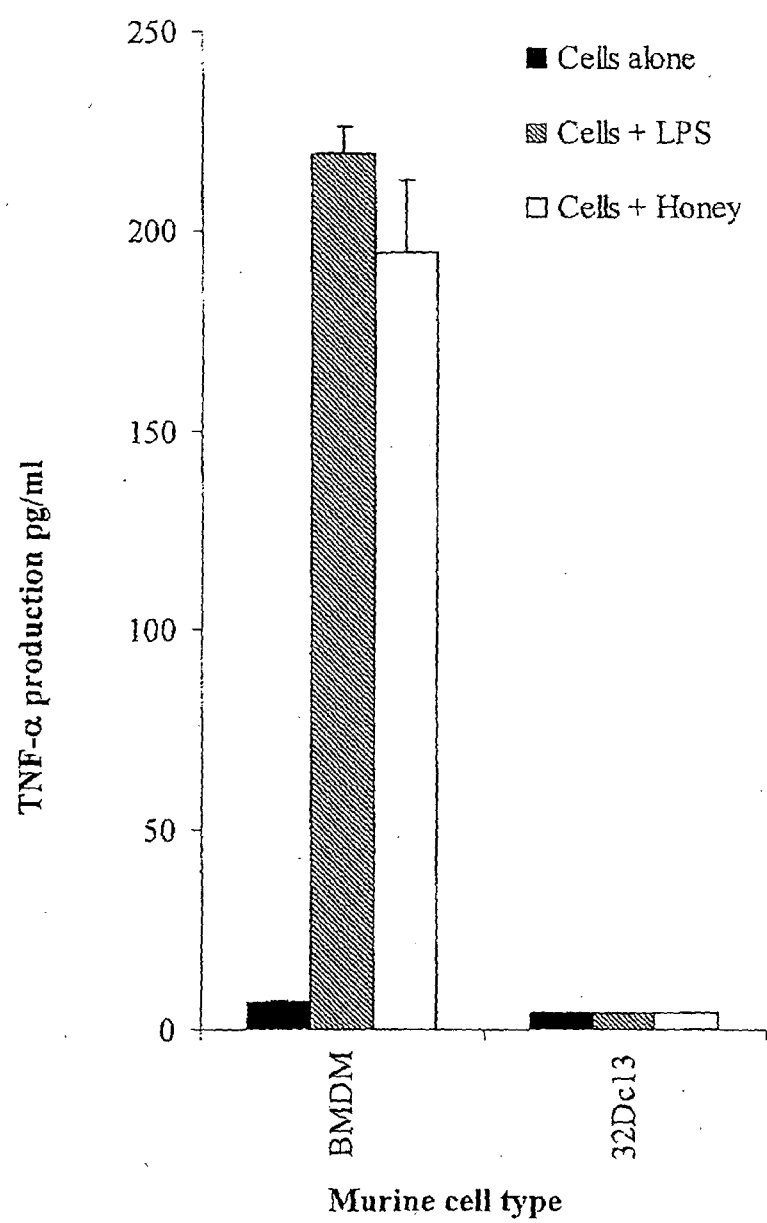


Figure 2a

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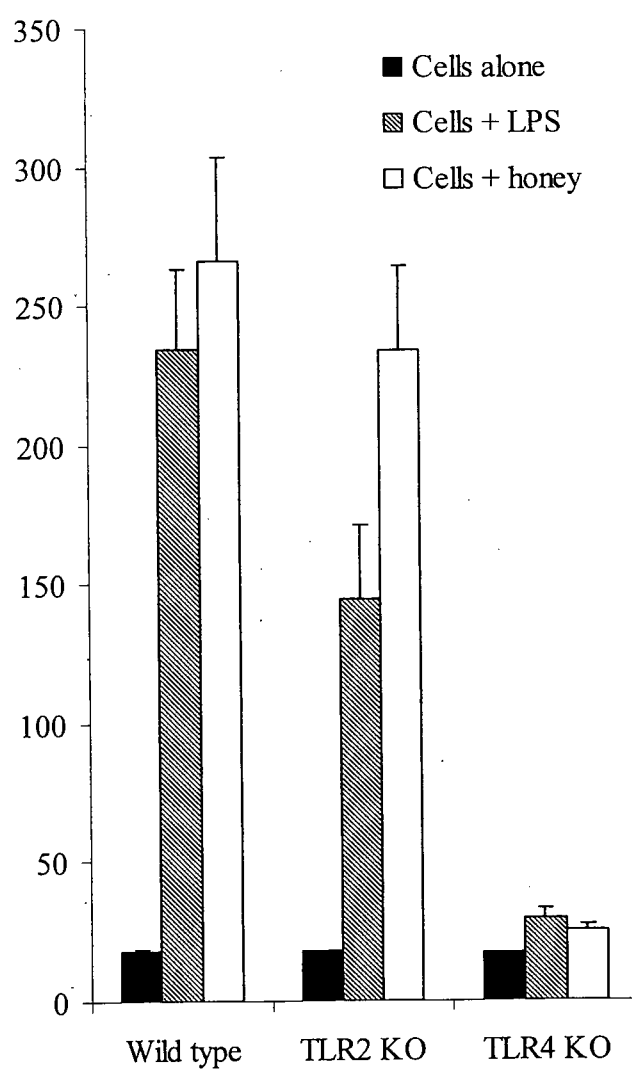


Figure 2b

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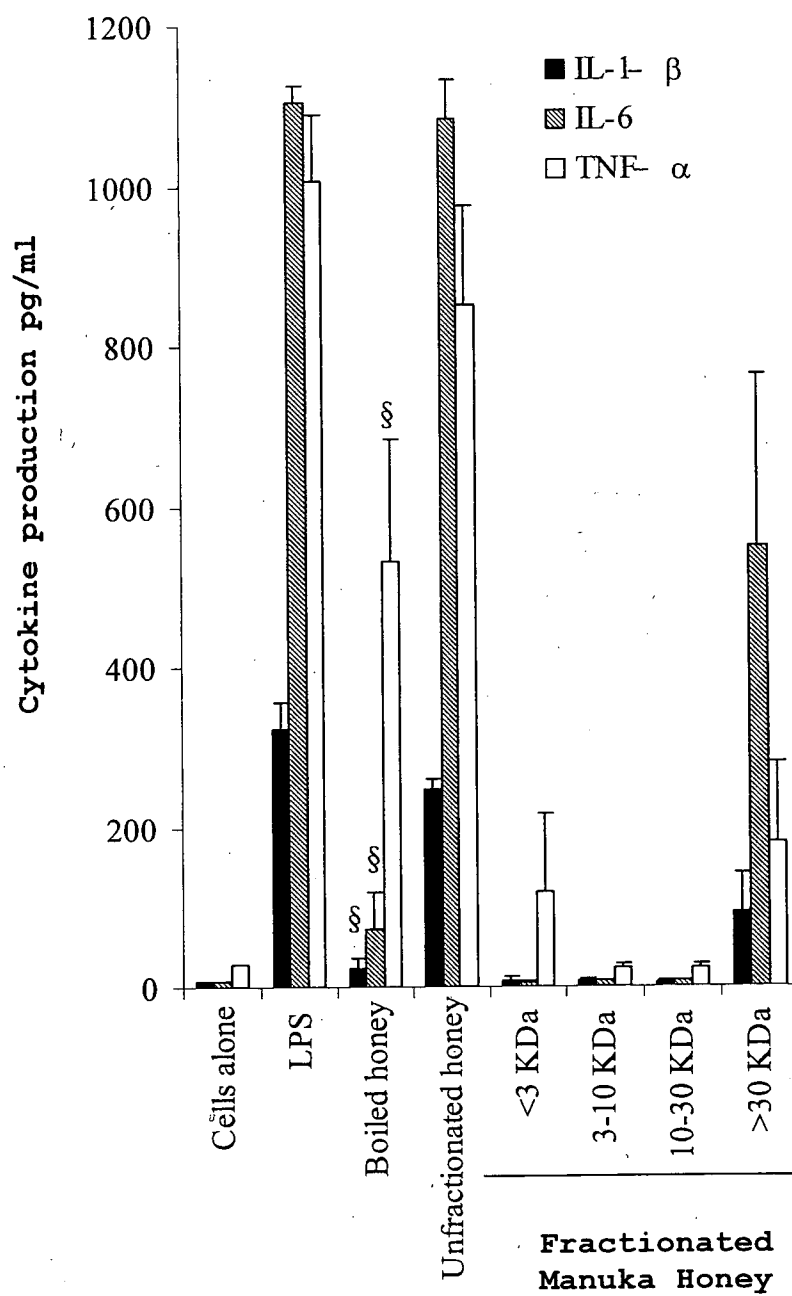


Figure 3

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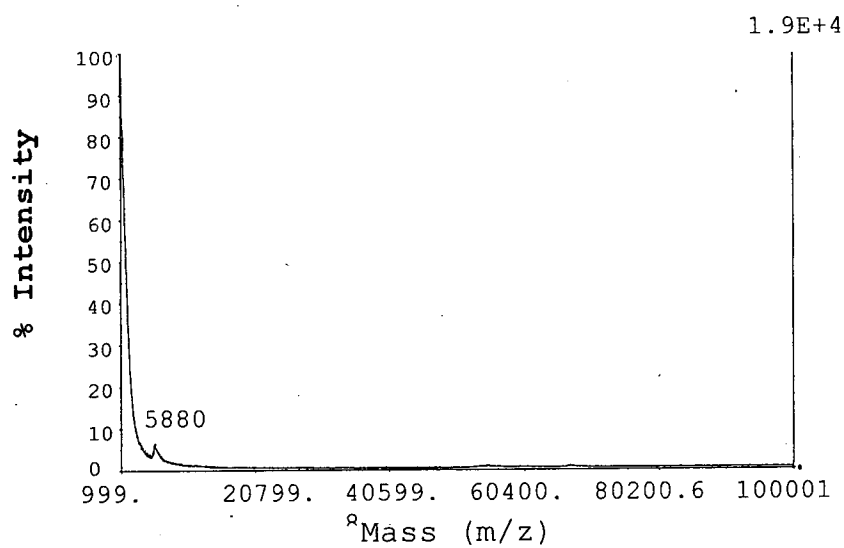
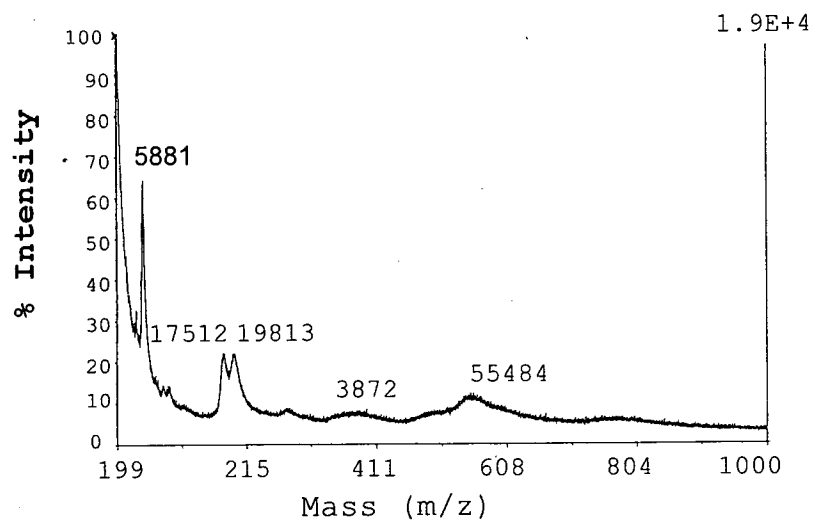


Figure 4

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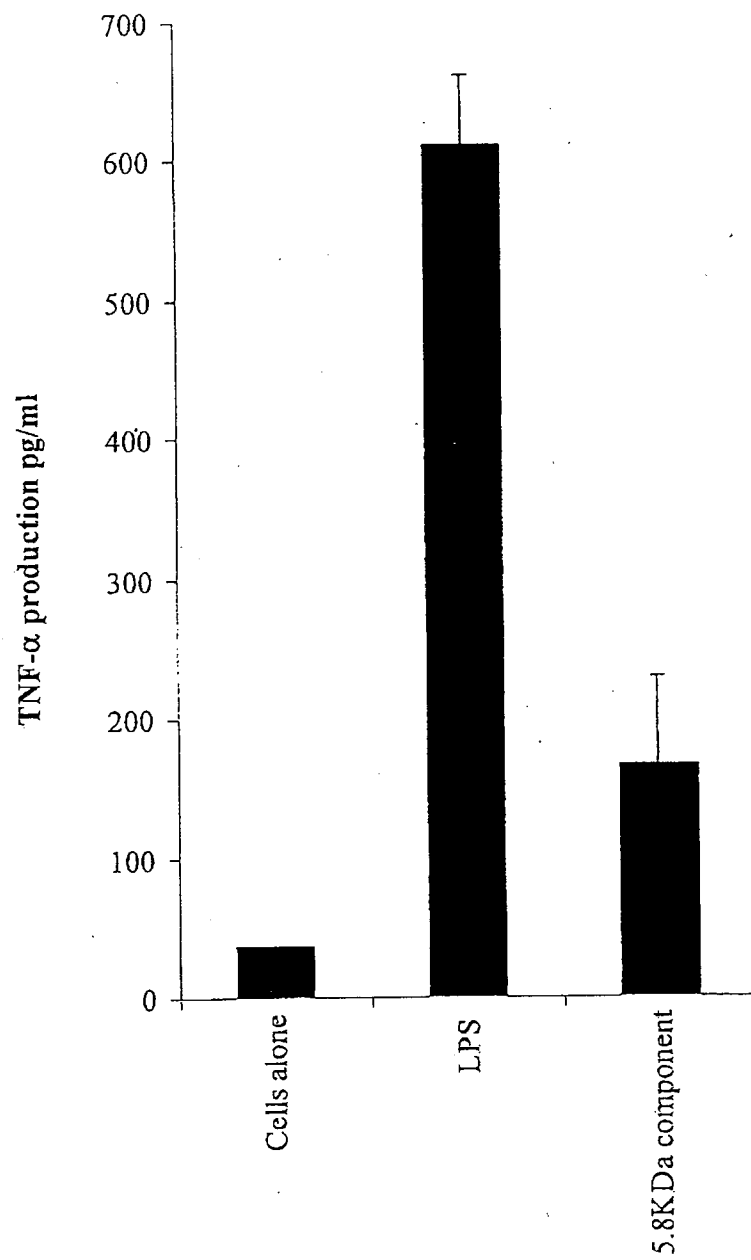


Figure 5a

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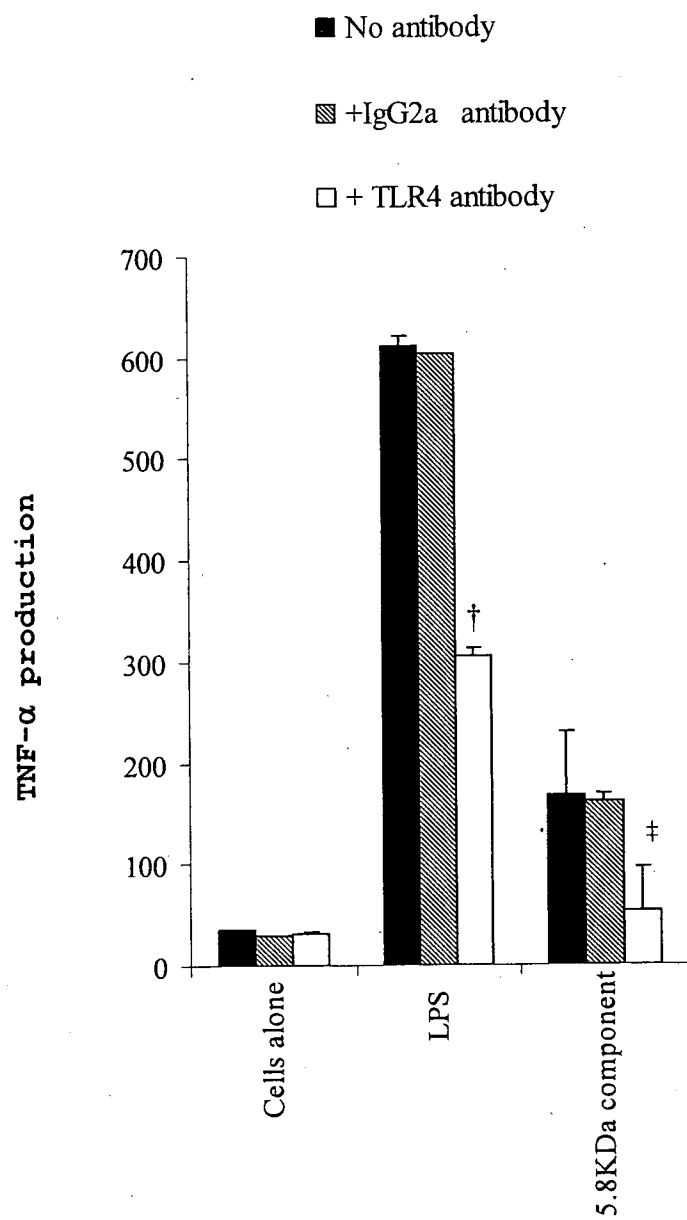


Figure 5b

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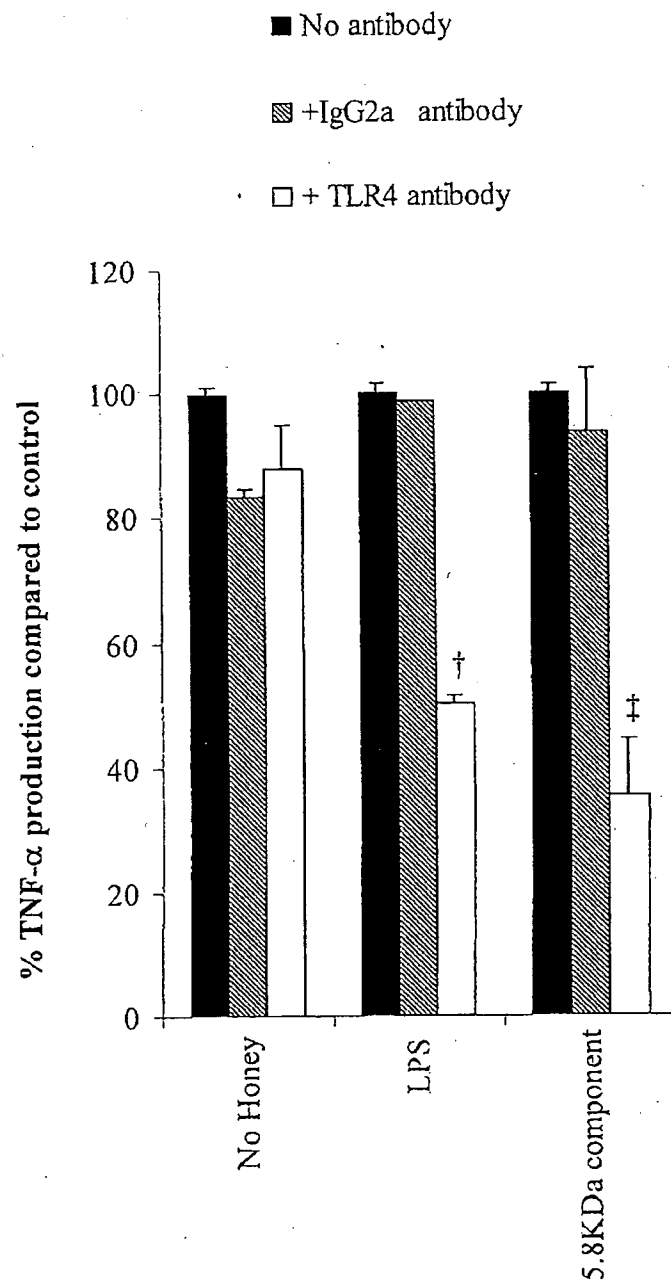


Figure 6a

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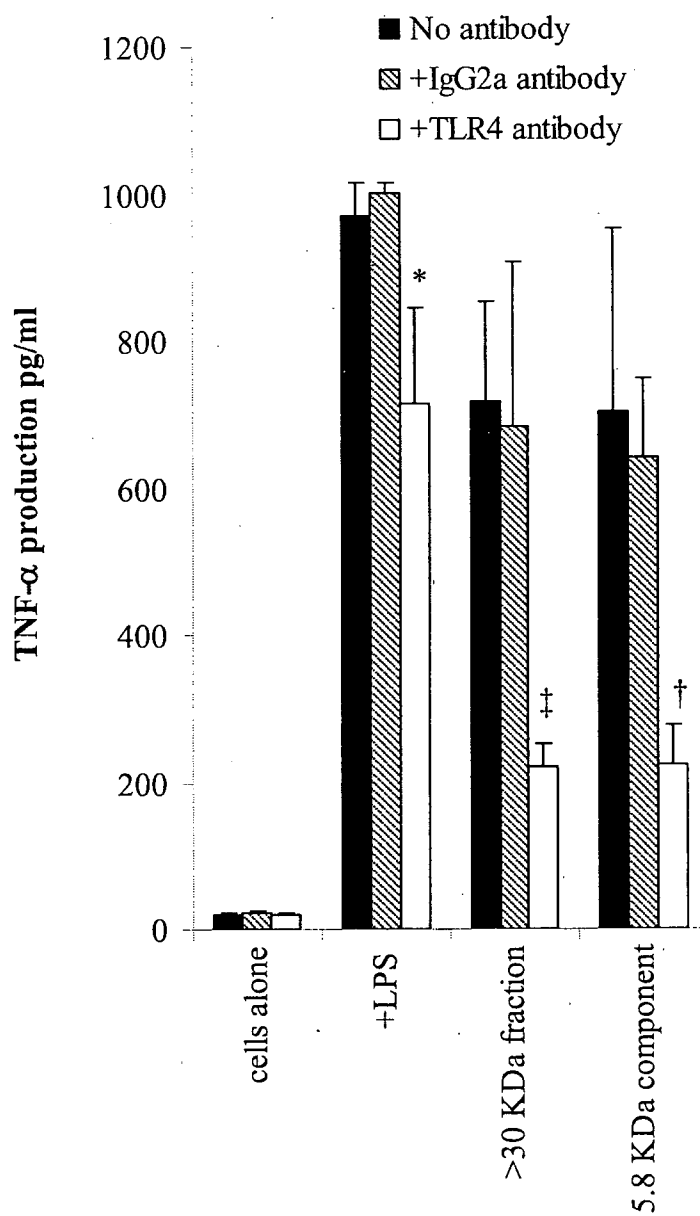


Figure 6b

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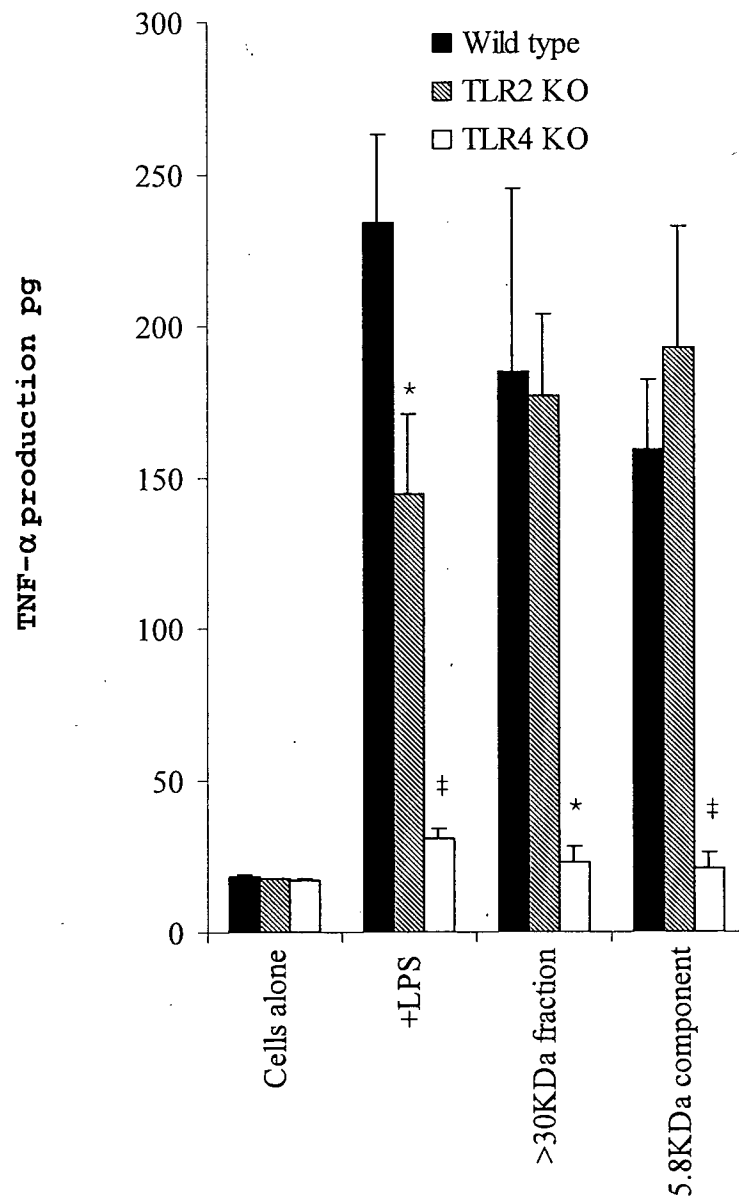


Figure 7

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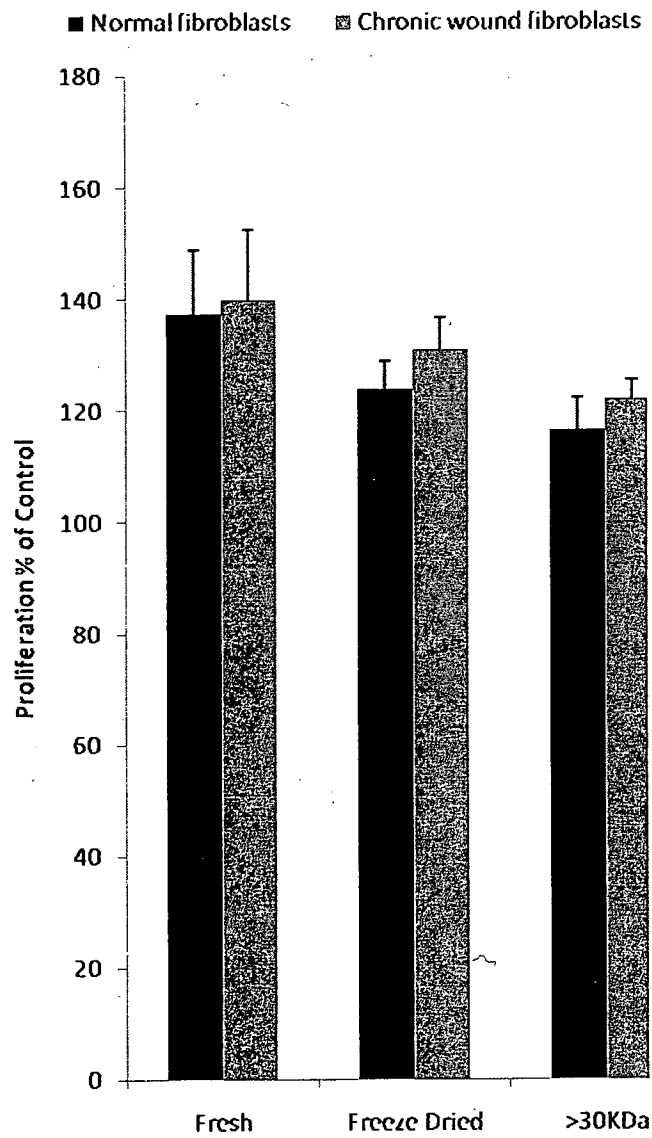


Figure 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/000934

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/64 A61P37/04

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

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

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

EPO-Internal, WPI Data, BIOSIS, COMPENDEX, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	TONKS A J ET AL: "A 5.8-kDa component of manuka honey stimulates immune cells via TLR4" JOURNAL OF LEUKOCYTE BIOLOGY, vol. 82, no. 5, November 2007 (2007-11), pages 1147-1155, XP009101128 ISSN: 0741-5400 page 1149 - page 1150	1-12
X	WO 2005/120250 A (UNIV WAIKATO [NZ]; SNOW MELANIE JANE [NZ]; MANLEY-HARRIS MERILYN [NZ];) 22 December 2005 (2005-12-22)	9-12
Y	page 11; claims 26-30; example 4	1-8
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

9 June 2008

Date of mailing of the international search report

11/07/2008

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INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2008/000934

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/067419 A (MNL PHARMA LTD [GB]; NASH ROBERT JAMES [GB]) 29 June 2006 (2006-06-29)	9-12
Y	page 7, line 32 - page 8, line 25; claims 7-10	1-8
X	WO 01/41776 A (WAIKATOLINK LTD [NZ]; MOLAN PETER [NZ]) 14 June 2001 (2001-06-14) page 17, line 14	9-12
Y	TONKS A J ET AL: "Honey stimulates inflammatory cytokine production from monocytes." CYTOKINE, vol. 21, no. 5, 7 March 2003 (2003-03-07), pages 242-247, XP002483390 ISSN: 1043-4666 page 244 - page 245	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2008/000934

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			US 2004054313 A1	18-03-2004