Homeopathy (2016) 105, 131–147

http://dx.doi.org/10.1016/j.homp.2016.02.001, available online at http://www.sciencedirect.com

ORIGINAL PAPER

# *Arnica montana* effects on gene expression in a human macrophage cell line. Evaluation by quantitative Real-Time PCR

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***Background:*** ***Arnica montana*** **is a popular traditional remedy widely used in complementary medicine, also for its wound healing properties. Despite its acknowledged action in clinical settings at various doses, the molecular aspects relating to how *A. montana* promotes wound healing remain to be elucidated. To fill this gap, we evaluated the whole plant extract, in a wide range of dilutions, in THP-1 human cells, differentiated into mature macrophages and into an alternative IL-4-activated phenotype involved in tissue remodelling and healing.**

***Methods:*** **Real-time quantitative Reverse Transcription Polymerase Chain Reaction (PCR) analysis was used to study the changes in the expression of a customized panel of key genes, mainly cytokines, receptors and transcription factors.**

***Results:*** **On macrophages differentiated towards the wound healing phenotype, *A. montana* affected the expression of several genes. In particular CXC chemokine ligand 1 (CXCL1), coding for an chief chemokine, exhibited the most consistent increase of expression, while also CXC chemokine ligand 2 (CXCL2), Interleukin8 (IL8) and bone morphogenetic protein (BMP2) were slightly up-regulated, suggesting a positive influence of *A. montana* on neutrophil recruitment and on angiogenesis. MMP1, coding for a metalloproteinase capable of cleaving extracellular matrix substrates, was down-regulated. Most results showed non-linearity of the dose-effect relationship.**

***Conclusions:*** **This exploratory study provides new insights into the cellular and molecular mechanisms of action of *A. montana* as a promoter of healing, since some of the genes it modifies are key regulators of tissue remodelling, inflammation and chemotaxis.** *Homeopathy* (2016) **105**, 131–147.

**Keywords:** *Arnica montana*; Macrophages; Real-Time PCR; Gene expression; Wound healing; Chemokines

## Introduction

*Arnica montana* L. (*A. montana*) is a herbaceous perennial plant belonging to the Asteraceae family, native to the temperate region of Europe and widely distributed in mountainous areas. It is a popular traditional remedy widely used in complementary medicine to treat various pathological conditions such as contusion, swelling associated with trauma, pain, inflammation, wounds and post-operative clinical conditions.[1–3] However, the evidence of its clinical efficacy is subject to debate.[4–6] A recent systematic review of herbal remedies for treating

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Received 8 October 2015; revised 1 January 2016; accepted 1 February 2016

osteoarthritis concluded that topical *A. montana* gel probably improves pain and function in this common condition.[7] There is some experimental evidence, in laboratory animals, of an anti-inflammatory action of *A. montana* administered as crude ethanolic extract[8] or at the 6th centesimal homeopathic dilution (6c).[9,10]

The chemical composition of *A. montana* depends on the part of the plant that is used, principally the flowers and roots, and in general the most pharmacologically active compounds are sesquiterpene lactones, thymol derivatives, flavonoids, acid polysaccharides and their glycoconjugates.[11,12] One of the major components of *A. montana* with acknowledged biological activity is the sesquiterpene lactone helenalin, known for its anti-inflammatory properties. In a lymphoid cellular model, helenalin was found to inhibit the transcription factor NF-kappaB – a central mediator of human immune response – by the alkylation of p65 subunit (RelA), thus preventing its binding to DNA.[13] However the cited studies did not evaluate the contribution of the whole plant extract, nor did they evaluate the pharmaceutical properties of different doses and formulations employed in medicine,[1,3,14] ranging from crude herbal extract or low dilutions, to high dilutions (homeopathic doses).

In fibroblast-like cells, whole ethanolic extract of *A. montana* showed antioxidant activity and a cytoprotective effect against oxidative damage.[15] Defence against oxidative stress was reported in studies where a 30th centesimal dilution (30c) of *A. montana*, administered orally to laboratory rats, decreased oxygen consumption of isolated liver mitochondria and protected from oxidative damage caused by lipid peroxidation.[16] Only one paper reported an inhibitory action of *A. montana* on nitric oxide and TNF-$\alpha$ production by murine macrophages.[17]

Although the action of *A. montana* on wound healing is regarded as a promising therapeutic property of this plant, the current knowledge of the effects of *A. montana* in laboratory models of wound healing is scant. A commercial homeopathic complex containing a low dilution (4th decimal, 4x) of *A. montana*, *Calendula* and *Hypericum* promoted fibroblast growth in a scratch model of cellular wound closure[18]; *A. montana* 3x as a topical gel improved the healing of surgically-induced wounds in Wister rats, but significant differences were noted only when the drug was delivered together with microcurrent application.[19]

Wound healing involves different cell types such as fibroblasts, leukocytes, and monocytes/macrophages, as well as endothelial and epidermal cells which cooperate to restore the damaged tissue through hemostasis, inflammation, angiogenesis and remodelling of the new tissue. The delicate balance between inflammation – with its potentially destructive phases – and tissue repair depends on a number of local and systemic factors and can be pharmacologically influenced. In the early inflammatory phase, a prominent role is played by cytokines derived by epithelial cells and macrophages such as IL8, IL-1$\alpha$, IL-1$\beta$, IL-6, TNF-$\alpha$. A pivotal role in immune defence and repair is played by Interleukin-4 (IL-4), whose pleiotropic effects on leukocytes include TH2 differentiation[20] and class switch of naïve B cells to IgE,[21] immunological events that increase antibody defence. On macrophages, IL-4 is essential for 'alternative' polarization, by which these cells take on characteristic properties functional to immune regulation, wound healing and tissue remodelling.[22–24] In the subsequent phase, additional factors are important to healing, including CXC chemokine ligand 1 (CXCL1) and 2 (CXCL2), CC chemokine ligand 2 (CCL2, also known as monocyte chemotactic protein-1, MCP-1),

**Table 1** List of genes of THP-1 macrophages included in the panel

| *Gene full names* | *Gene code* | *Gene symbol and abbreviation* |
|---|---|---|
| Chemokine (C–C motif) ligand 1 | NM_002981 | CCL1 |
| Chemokine (C–C motif) ligand 2; MCP-1 | NM_002982 | CCL2 |
| Chemokine (C–C motif) ligand 3; MIP-1$\alpha$ | NM_002983 | CCL3 |
| Chemokine (C–C motif) ligand 4; MIP-1B | NM_002984 | CCL4 |
| Chemokine (C–C motif) ligand 5; RANTES | NM_002985 | CCL5 |
| Chemokine (C–C motif) ligand 7; MCP-3 | NM_006273 | CCL7 |
| Chemokine (C–C motif) ligand 17; TARC | NM_002987 | CCL17 |
| Chemokine (C–C motif) ligand 22 | NM_002990 | CCL22 |
| Chemokine (C–C motif) ligand 23 | NM_005064 | CCL23 |
| Chemokine (C–X–C motif) ligand 1; GRO-a | NM_001511 | CXCL1 |
| Chemokine (C–X–C motif) ligand 2; GRO-b | NM_002089 | CXCL2 |
| Chemokine (C–X–C motif) ligand 13; BCA-1 | NM_006419 | CXCL13 |
| Interleukin 1 – beta | NM_000576 | IL1B |
| Interleukin 1, receptor antagonist | NM_000577 | IL1RN |
| Interleukin 8 | NM_000584 | IL8 |
| Interleukin 10 | NM_000572 | IL10 |
| Tumour necrosis factor – alpha | NM_000594 | TNFA |
| Vascular endothelial growth factor A | NM_003376 | VEGFA |
| Bone morphogenetic protein-1 | NM_001200 | BMP2 |
| Colony-stimulating factor 1 | NM_000757 | CSF1 |
| Chemokine (C–C motif) receptor 5 | NM_000579 | CCR5 |
| Coagulation factor XIII, A1 polypeptide | NM_000129 | F13A1 |
| Mannose receptor, C type 1 | NM_002438 | MRC1 |
| Matrix metallopeptidase 1 (collagenase) | NM_002421 | MMP1 |
| V-rel reticuloendotheliosis viral oncogene homolog A (p65) | NM_021975 | RELA |
| Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p50) | NM_003998 | NFKB1 |
| Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | NM_020529 | NFKBIA |
| Inhibitor of kappa light polypeptide gene, kinase beta | NM_001556 | IKBKB |

colony-stimulating factors, CC chemokine ligand 3 (CCL3 also known as macrophage inflammatory protien-1 alpha, MIP-l$\alpha$), vascular endothelial growth factor (VEGF), and others.[25,26] This cascade of events eventually leads to repair and reconstruction of the wounded area, but it needs to be optimally orchestrated, since prolonging the inflammation phase frequently leads to wound impairment.

We formulated the hypothesis that one of the cellular targets of *A. montana* action is the macrophage, a key cell in all phases of inflammation and wound healing, and accordingly decided to evaluate – through an *in vitro* model – the action of several dilutions of this plant extract on gene expression of key regulatory factors. We used the THP-1 human cell line, which is a cell line commonly employed to explore the function and regulation of monocytes and macrophages.[27] These cells resemble primary monocytes, but when treated with low doses of phorbol esters (PMA) differentiate to cells with morphologic and functional features of tissue macrophages, including adhesion, phagocytosis, free-radical production, and release of a number of inflammatory cytokines. Using a cell line has the advantage of assuring a homogeneous genetic background and treatment, greatly reducing the inter-individual variability that is instead present in the cell phenotype of human donors. This uniformity is particularly important when studying the biological function of chemicals at high dilutions.[28–30]

*A. montana* dilutions were tested both on normal PMA-differentiated THP-1 cells (here defined as 'Normal Mac') and on PMA-differentiated THP-1 cells that were further differentiated by IL-4 treatment for 24 h (here defined as 'IL-4 Mac'). As suitable molecular markers of drug activity at the cellular level, we analysed by RT-array PCR the gene expression of a series of cytokines, receptors, and transcription factors that are involved in the wound-healing cellular process.

We evaluated a customized RT-array PCR panel made of 384 microwells that allowed simultaneous detection of 28 different RNA species plus the housekeeping gene Large Ribosomal Protein PO (RPLPO), which in preliminary experiments was found to be the most stable in these cells under different experimental conditions. Besides the responses of many chemokines, the expression of some genes involved in NF-kappaB-related signalling pathways was also included in the panel. It has been shown that gene expression of THP-1 cells mimics that of peripheral blood-derived macrophages and represents a good model system for studying NF-kappaB dependent gene expression.[31] Since *A. montana* is used at several doses in clinical settings,[3] we explored a wide range of dilutions, from the 2nd centesimal dilution of the mother tincture (2c, i.e. diluted $10^4$ times) to the 15c (i.e. diluted $10^{30}$ times).

## Materials and methods

### Materials

The human monocytic leukemia cell line THP-1 was purchased from DSMZ (Germany). Growth media (RPMI 1640) and Ultraglutamine 20 mM solution were purchased from Lonza (Belgium). Foetal bovine serum (FBS), phorbol 12-myristate 13-acetate (PMA), pure ethanol and ultra-pure water (W3500) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Human Interleukin-4 (IL-4) was purchased from Macs-Miltenyi Biotec (Germany). Cell proliferation reagent WST-1 was purchased from Roche Diagnostics GmbH (Germany) and the dye reagent for the Bradford protein assay was purchased from Bio-Rad Laboratories GmbH (Germany). RNeasy Mini kit, RT first strand

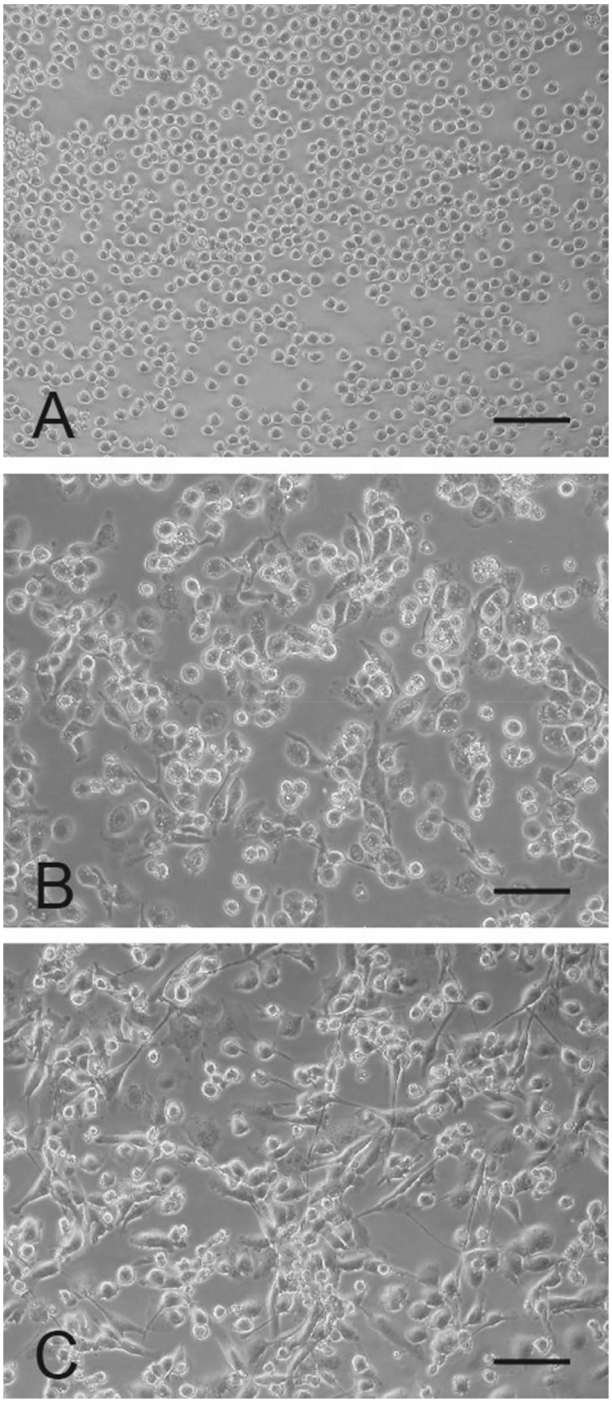


**Figure 1** Morphology of THP-1 cells at different stages of differentiation. The panels show images of THP-1 cells untreated (A), treated with 20 ng/ml PMA (B) and with 20 ng/ml PMA + 50 ng/ml IL-4 (C) as described in Methods. Scale bar = 50 $\mu$m.

kit and RT SYBR Green Rox Master Mix were from Qiagen, (CA, USA). The 384 RT-array custom was made by Sabiosciences (Qiagen Company, CA, USA).

*A. montana* was produced by Boiron Laboratoires (Lyon, France) according to the French Homeopathic pharmacopoeia and provided as a first centesimal dilution of the whole hydroalcoholic extract (Mother Tincture, MT) in 30% ethanol/distilled water. The MT was characterised by a content of sesquiterpene lactones of 0.036% (W/V).

### Test solutions

Starting from the 1c dilution of *A. montana*, the 2c dilution was prepared by adding 50 $\mu$l of 1c to 4.95 ml of 30% ethanol/distilled water; this solution was filtered with a 0.22 $\mu$m Millipore filter and subjected to vigorous succussion with a Dyna-A mechanical shaker delivering 20 strokes/s for 7.5 s with an 11-mm travel distance. This process was done in sterile conditions using 10-ml sterile glass test tubes with screw cap. The subsequent serial 100x dilutions were prepared in the same 30% ethanol/distilled water solvent, in a final volume of 5 ml, up to the 14c dilution. After each dilution step, the solutions were subjected to succussion as above described. Before the experiments, the working solutions were prepared in sterile conditions through a further 100x dilution of the 1c, 2c, 4c, 8c and 14c solutions in ultra-pure water, followed by vigorous mixing as above, thus obtaining the 2c, 3c, 5c, 9c and 15c dilutions.

In this way, the ethanol concentration was decreased from 30% to 0.3% in test samples and was 0.03% in the final cell cultures. Preliminary experiments showed that this dose does not affect cell viability and function. The control test solution (pure vehicle) was prepared, before each experiment, using the same 30% ethanol/distilled water batch used for the *A. montana* preparations; it was diluted 100x in ultra-pure water and subjected to shaking with Dyna-A as described above.

### Cell culture and treatments

The THP-1 cell line was cultured in RPMI 1640 medium, supplemented with FBS 10% and 2 mM final concentration of Ultraglutamine, at 37°C in 5% $CO_2$ in a humidified incubator. Cells were sub-cultured twice a week and used between passage 3 and 10. In a typical experiment, on day one the cells were seeded at a density of $2.5 \times 10^5$ cells/mL in 24-well plates in 1 ml medium with 2 mM Ultraglutamine and 2% FBS ('complete culture medium'). On day 2 all the cell cultures were supplemented with 20 ng/mL of PMA in order to develop the macrophage-like phenotype.[32,33]

On day 3 the cells were divided in two groups: a) in the first group the culture was continued for a further 24 h in the complete culture medium containing 20 ng/mL of PMA ('Normal Mac'), b) in the second group, where indicated, IL-4 was added at a concentration of 50 ng/mL to develop the 'wound healing' phenotype (here defined as 'IL-4 Mac').[33] On day 4 the plates were washed twice with complete culture medium and group (b) was again supplemented with 50 ng/mL IL-4 while group (a) continued the culture in complete culture medium. Where indicated, both types of macrophages were exposed for 2 h or 24 h to *A. montana* or control solvent (1 ml cell culture + 110 $\mu$l test solutions). In each experiment, each experimental treatment was performed in triplicate wells.

Pictures of THP-1, Normal Mac and IL-4 Mac in the experimental plates were acquired by means of contrast phase microscopy using an Olympus IX50 microscope with 100x original magnification. Cell viability was checked by the WST-1 assay.[34] Briefly, after 24 h treatment with *A. montana* dilutions or with control, 1:10 (v/v) pre-

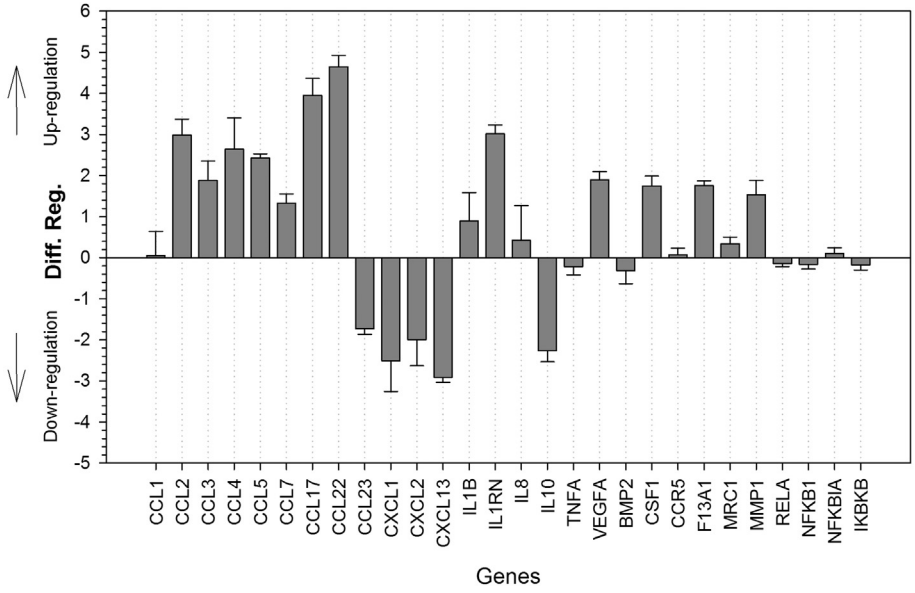


**Figure 2** Changes of gene expression induced by IL-4 treatment of THP-1. Cells were incubated for 26 h in the absence and in the presence of 50 ng/ml of IL-4 and the extent of RNA transcripts of the indicated genes was determined. Differential regulation (Diff. Reg.) of IL-4 Mac versus Normal Mac is expressed as $Log_2$(Fold Change) $\pm$ SE.

**Table 2A** Gene expression by THP-1 macrophages (M0 type) treated for 2 h with *A. montana* dilutions

| *Gene* | *Statistics homog -eneity* | ANOVA | A. montana *2c (n = 6)* | | | | | A. montana *3c (n = 6)* | | | | | A. montana *5c (n = 6)* | | | | | A. montana *9c (n = 6)* | | | | | A. montana *15c (n = 6)* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* |
| CCL1 | 0.224 | 0.086 | −0.13 | 0.16 | 0.027 | – | – | −0.24 | 0.10 | −1.098 | – | – | −0.18 | 0.08 | 0.483 | – | – | 0.09 | 0.14 | 0.403 | – | – | −0.01 | 0.11 | −1.114 | – | – |
| CCL2 | 0.824 | 0.884 | −0.26 | 0.45 | 1.213 | – | – | −0.21 | 0.22 | 1.028 | – | – | −0.32 | 0.28 | 0.416 | – | – | −0.24 | 0.38 | −0.084 | – | – | −0.31 | 0.30 | 0.842 | – | – |
| CCL3 | 0.918 | 0.859 | −0.05 | 0.17 | 1.052 | – | – | 0.08 | 0.11 | 0.128 | – | – | −0.06 | 0.12 | 0.185 | – | – | 0.04 | 0.10 | 0.338 | – | – | −0.06 | 0.18 | 0.311 | – | – |
| CCL4 | 0.984 | 0.617 | −0.13 | 0.13 | 0.399 | – | – | −0.09 | 0.13 | −0.898 | – | – | −0.12 | 0.08 | 0.420 | – | – | −0.04 | 0.10 | 0.540 | – | – | −0.16 | 0.14 | −0.292 | – | – |
| CCL5 | 0.999 | 0.312 | 0.10 | 0.04 | −0.247 | – | – | 0.08 | 0.05 | −1.014 | – | – | 0.04 | 0.02 | 0.360 | – | – | 0.04 | 0.02 | 1.248 | – | – | 0.03 | 0.04 | −0.508 | – | – |
| CCL7 | 0.466 | 0.228 | −0.17 | 0.47 | 1.397 | – | – | −0.54 | 0.21 | 0.091 | – | – | −0.53 | 0.35 | 1.519 | – | – | 0.07 | 0.43 | 0.722 | – | – | −0.35 | 0.46 | 1.036 | – | – |
| CCL17 | 0.805 | 0.083 | −0.51 | 0.39 | −0.255 | – | – | 0.51 | 0.52 | −0.348 | – | – | 0.46 | 0.22 | −1.265 | – | – | 0.08 | 0.23 | −0.313 | – | – | 0.69 | 0.27 | 0.175 | – | – |
| CCL22 | 0.072 | 0.120 | −0.24 | 0.10 | −0.390 | – | – | 0.03 | 0.18 | −0.561 | – | – | 0.08 | 0.09 | −0.035 | – | – | 0.06 | 0.06 | −0.444 | – | – | −0.01 | 0.09 | −0.342 | – | – |
| CCL23 | 0.972 | **0.027** | −0.05 | 0.04 | 0.504 | 1.240 | ns | 0.06 | 0.06 | −0.428 | −1.060 | ns | 0.04 | 0.04 | 0.638 | −1.059 | ns | −0.05 | 0.05 | 0.178 | 1.181 | ns | 0.11 | 0.04 | −0.174 | −3.174 | **<0.05** |
| CXCL1 | 0.944 | 0.720 | −0.14 | 0.19 | −0.538 | – | – | 0.00 | 0.14 | −1.306 | – | – | 0.05 | 0.06 | −0.083 | – | – | 0.09 | 0.12 | −0.050 | – | – | −0.07 | 0.17 | 0.739 | – | – |
| CXCL2 | 0.973 | 0.806 | −0.07 | 0.18 | 0.722 | – | – | −0.07 | 0.11 | −1.074 | – | – | −0.11 | 0.13 | −0.264 | – | – | −0.11 | 0.08 | 0.320 | – | – | 0.00 | 0.13 | 1.338 | – | – |
| CXCL13 | 0.671 | 0.340 | 0.03 | 0.15 | 0.668 | – | – | 0.29 | 0.13 | −0.071 | – | – | 0.16 | 0.12 | 0.074 | – | – | 0.05 | 0.04 | −0.560 | – | – | 0.15 | 0.10 | 1.168 | – | – |
| IL1B | 0.957 | 0.805 | −0.16 | 0.32 | 0.702 | – | – | −0.21 | 0.16 | 0.436 | – | – | −0.26 | 0.25 | 0.395 | – | – | −0.10 | 0.22 | 0.800 | – | – | −0.19 | 0.27 | 0.549 | – | – |
| IL1RN | 0.948 | 0.878 | −0.07 | 0.11 | 0.993 | – | – | −0.10 | 0.10 | −1.309 | – | – | −0.09 | 0.10 | 0.433 | – | – | −0.01 | 0.10 | 0.769 | – | – | −0.04 | 0.13 | 0.259 | – | – |
| IL8 | 0.983 | 0.945 | 0.03 | 0.17 | 0.022 | – | – | 0.03 | 0.14 | 0.761 | – | – | −0.04 | 0.14 | 0.234 | – | – | −0.04 | 0.13 | 0.555 | – | – | −0.10 | 0.18 | 0.100 | – | – |
| IL10 | 0.109 | **0.027** | −0.22 | 0.14 | 0.355 | 1.698 | ns | −0.27 | 0.10 | −0.020 | 3.005 | **<0.05** | −0.21 | 0.24 | 0.128 | 0.956 | ns | 0.07 | 0.11 | 0.477 | −0.706 | ns | 0.07 | 0.11 | −0.330 | −0.749 | ns |
| TNFA | 0.975 | 0.633 | −0.15 | 0.12 | 0.145 | – | – | −0.07 | 0.07 | 0.681 | – | – | −0.06 | 0.10 | 0.048 | – | – | −0.02 | 0.09 | 0.221 | – | – | −0.08 | 0.13 | 0.089 | – | – |
| VEGFA | 1.000 | 0.822 | 0.02 | 0.08 | 1.241 | – | – | 0.12 | 0.06 | 0.138 | – | – | 0.07 | 0.09 | 0.570 | – | – | 0.09 | 0.14 | 1.277 | – | – | 0.00 | 0.15 | 0.517 | – | – |
| BMP2 | 0.683 | 0.607 | 0.02 | 0.17 | 0.476 | – | – | 0.13 | 0.09 | −0.518 | – | – | −0.02 | 0.10 | −0.537 | – | – | −0.04 | 0.11 | −0.453 | – | – | −0.02 | 0.13 | 0.395 | – | – |
| CSF1 | 0.921 | 0.163 | −0.33 | 0.21 | −0.061 | – | – | −0.21 | 0.15 | −0.235 | – | – | −0.08 | 0.14 | 0.749 | – | – | 0.07 | 0.10 | 0.345 | – | – | −0.08 | 0.17 | −0.201 | – | – |
| CCR5 | 0.277 | 0.153 | −0.07 | 0.18 | 0.180 | – | – | −0.03 | 0.13 | −0.672 | – | – | 0.19 | 0.08 | −0.252 | – | – | 0.01 | 0.08 | −0.177 | – | – | 0.14 | 0.14 | −0.164 | – | – |
| F13A1 | 0.233 | 0.337 | −0.04 | 0.13 | 0.676 | – | – | 0.00 | 0.11 | 0.503 | – | – | 0.11 | 0.08 | 0.591 | – | – | 0.17 | 0.09 | 0.243 | – | – | 0.15 | 0.14 | −1.221 | – | – |
| MRC1 | 0.545 | **0.045** | −0.22 | 0.13 | −0.155 | 1.844 | ns | −0.47 | 0.13 | 0.568 | 3.950 | **<0.01** | −0.24 | 0.18 | 1.212 | 1.457 | ns | −0.26 | 0.13 | 0.692 | 2.160 | **<0.05** | −0.08 | 0.09 | −0.072 | 1.004 | ns |
| MMP1 | 0.948 | 0.194 | −0.12 | 0.08 | −0.550 | – | – | −0.05 | 0.12 | −0.892 | – | – | −0.02 | 0.08 | 0.366 | – | – | 0.10 | 0.05 | −1.081 | – | – | 0.09 | 0.09 | 0.554 | – | – |
| RELA | 0.767 | **0.006** | −0.16 | 0.07 | −1.433 | 2.498 | (<0.05) | −0.04 | 0.08 | −0.209 | 0.532 | ns | 0.04 | 0.05 | 0.569 | −0.843 | ns | 0.08 | 0.06 | 0.381 | −1.375 | ns | 0.05 | 0.05 | 0.303 | −1.037 | ns |
| NFKB1 | 0.853 | 0.074 | −0.16 | 0.10 | −0.867 | – | – | −0.17 | 0.10 | 0.039 | – | – | −0.08 | 0.10 | 0.496 | – | – | 0.00 | 0.05 | −0.732 | – | – | 0.00 | 0.09 | 0.404 | – | – |
| NFKBIA | 0.808 | 0.258 | −0.15 | 0.10 | −0.161 | – | – | −0.03 | 0.09 | 0.087 | – | – | −0.03 | 0.09 | 0.944 | – | – | 0.06 | 0.05 | −0.597 | – | – | −0.01 | 0.09 | 0.689 | – | – |
| IKBKB | 0.065 | 0.411 | −0.08 | 0.12 | −0.323 | – | – | −0.01 | 0.14 | 0.274 | – | – | 0.05 | 0.08 | 1.005 | – | – | 0.11 | 0.11 | 0.074 | – | – | 0.05 | 0.07 | 0.408 | – | – |
| Mean differential regulation | | | −0.12 | | | | | −0.05 | | | | | −0.04 | | | | | 0.01 | | | | | 0.00 | | | | |
| Median differential regulation | | | −0.13 | | | | | −0.04 | | | | | −0.04 | | | | | 0.05 | | | | | −0.01 | | | | |
| N. of up-regulated | | | 5 | | | | | 11 | | | | | 11 | | | | | 17 | | | | | 12 | | | | |
| N. of down-regulated | | | 23 | | | | | 17 | | | | | 17 | | | | | 11 | | | | | 16 | | | | |
| Wilcoxon (p-value) | | | **<0.001** | | | | | 0.139 | | | | | 0.187 | | | | | 0.151 | | | | | 0.600 | | | | |

The statistically significant differences between *A. montana* and control are indicated in bold.

**Table 2B** Gene expression by THP-1 macrophages (M0 type) treated for 24 h with *A. montana* dilutions

| Gene | Statistics homogeneity | *ANOVA* | *A. montana* 2c | | | | | *A. montana* 3c | | | | | *A. montana* 5c | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *n* | *Diff. Reg.* | *SE* | *Skewness* | *T-test* *p* | *n* | *Diff. Reg.* | *SE* | *Skewness* | *T-test* *p* | *n* | *Diff. Reg.* | *SE* |
| CCL1 | 0.720 | 0.651 | 6 | 0.34 | 0.37 | −0.915 | – – | 6 | 0.20 | 0.22 | 0.021 | – – | 6 | 0.35 | 0.20 |
| CCL2 | 0.433 | 0.080 | 9 | 0.13 | 0.20 | 0.584 | – – | 10 | −0.27 | 0.13 | 0.005 | – – | 10 | −0.14 | 0.21 |
| CCL3 | 0.867 | **0.005** | 9 | 0.07 | 0.12 | 0.372 | −0.602 ns | 10 | −0.18 | 0.10 | 0.834 | 1.847 ns | 10 | −0.18 | 0.10 |
| CCL4 | 0.988 | 0.298 | 6 | −0.05 | 0.15 | 0.025 | – – | 6 | −0.30 | 0.14 | −0.853 | – – | 6 | −0.25 | 0.16 |
| CCL5 | 0.999 | 0.147 | 6 | 0.10 | 0.05 | 0.450 | – – | 6 | 0.02 | 0.02 | −0.051 | – – | 6 | 0.09 | 0.03 |
| CCL7 | 0.917 | 0.950 | 6 | 0.09 | 0.55 | 0.924 | – – | 6 | 0.14 | 0.51 | 0.026 | – – | 6 | −0.09 | 0.50 |
| CCL17 | 0.470 | 0.651 | 9 | −0.57 | 0.28 | −0.388 | – – | 10 | −0.27 | 0.23 | −0.361 | – – | 10 | −0.50 | 0.27 |
| CCL22 | 0.739 | 0.835 | 6 | 0.10 | 0.13 | 0.193 | – – | 6 | 0.07 | 0.15 | 0.637 | – – | 6 | 0.06 | 0.09 |
| CCL23 | 0.993 | 0.220 | 6 | −0.12 | 0.07 | 0.835 | – – | 6 | −0.07 | 0.07 | 1.445 | – – | 6 | −0.05 | 0.02 |
| CXCL1 | 0.939 | 0.408 | 6 | −0.12 | 0.06 | −0.179 | – – | 6 | −0.12 | 0.11 | −0.735 | – – | 6 | −0.24 | 0.15 |
| CXCL2 | 0.918 | 0.274 | 6 | −0.24 | 0.14 | 0.435 | – – | 6 | −0.12 | 0.14 | −0.105 | – – | 6 | −0.18 | 0.09 |
| CXCL13 | 0.880 | 0.513 | 6 | −0.02 | 0.19 | 0.578 | – – | 6 | 0.10 | 0.23 | 1.173 | – – | 6 | −0.17 | 0.11 |
| IL1B | 0.991 | **0.044** | 9 | −0.21 | 0.13 | −0.166 | 1.683 ns | 10 | −0.16 | 0.13 | 0.395 | 1.338 ns | 10 | −0.16 | 0.11 |
| IL1RN | 0.991 | 0.849 | 6 | 0.01 | 0.04 | −0.322 | – – | 6 | 0.00 | 0.05 | −0.088 | – – | 6 | 0.00 | 0.03 |
| IL8 | 0.963 | 0.201 | 9 | 0.05 | 0.06 | 2.456 | – – | 10 | −0.10 | 0.07 | 0.532 | – – | 10 | −0.11 | 0.07 |
| IL10 | 0.836 | **0.002** | 9 | 0.05 | 0.10 | 2.456 | 0.981 ns | 10 | −0.09 | 0.10 | 0.339 | 0.982 ns | 10 | −0.16 | 0.09 |
| TNFA | 0.943 | **0.017** | 9 | −0.06 | 0.10 | 2.428 | 1.099 ns | 10 | −0.14 | 0.05 | −0.041 | 3.038 **<0.01** | 10 | −0.14 | 0.08 |
| VEGFA | 0.678 | 0.179 | 9 | −0.05 | 0.09 | 2.425 | – – | 10 | −0.08 | 0.08 | 0.073 | – – | 10 | −0.09 | 0.08 |
| BMP2 | 0.689 | 0.546 | 9 | 0.10 | 0.09 | 2.439 | – – | 10 | 0.05 | 0.08 | 0.376 | – – | 10 | 0.03 | 0.07 |
| CSF1 | 0.979 | 0.237 | 6 | −0.05 | 0.09 | 0.610 | – – | 6 | −0.07 | 0.06 | 0.480 | – – | 6 | −0.08 | 0.06 |
| CCR5 | 0.874 | 0.109 | 9 | −0.17 | 0.14 | 2.264 | – – | 10 | −0.08 | 0.16 | 2.230 | – – | 10 | 0.14 | 0.06 |
| F13A1 | 0.992 | 0.481 | 6 | −0.11 | 0.13 | 0.361 | – – | 6 | −0.12 | 0.09 | 0.065 | – – | 6 | 0.00 | 0.04 |
| MRC1 | 0.956 | 0.091 | 6 | 0.07 | 0.09 | −0.634 | – – | 6 | −0.21 | 0.10 | −0.212 | – – | 6 | −0.13 | 0.09 |
| MMP1 | 0.971 | 0.419 | 6 | 0.00 | 0.22 | −0.110 | – – | 6 | −0.07 | 0.18 | 0.326 | – – | 6 | 0.10 | 0.11 |
| RELA | 0.983 | **0.037** | 6 | −0.03 | 0.05 | 0.158 | 0.641 ns | 6 | −0.12 | 0.05 | −0.565 | 2.444 **<0.05** | 6 | −0.06 | 0.04 |
| NFKB1 | 0.875 | 0.182 | 6 | −0.03 | 0.10 | 0.811 | – – | 6 | −0.09 | 0.05 | −0.786 | – – | 6 | −0.11 | 0.06 |
| NFKBIA | 0.943 | 0.122 | 6 | 0.01 | 0.05 | 0.026 | – – | 6 | −0.08 | 0.05 | −0.812 | – – | 6 | −0.16 | 0.06 |
| IKBKB | 0.683 | **0.007** | 6 | 0.00 | 0.08 | 0.439 | −0.020 ns | 6 | −0.14 | 0.07 | −0.218 | 2.120 **<0.05** | 6 | −0.17 | 0.08 |
| Mean differential regulation | | | | −0.02 | | | | | −0.08 | | | | | −0.09 | |
| Median differential regulation | | | | −0.01 | | | | | −0.09 | | | | | −0.11 | |
| N. of up-regulated | | | | 14 | | | | | 7 | | | | | 7 | |
| N. of down-regulated | | | | 14 | | | | | 21 | | | | | 21 | |
| Wilcoxon (p-value) | | | | 0.186 | | | | | **0.002** | | | | | **0.003** | |

The statistically significant differences between *A. montana* and control are indicated in bold.

warmed WST-1 solution was added to the cells cultivated in parallel plates in the same conditions, and the was plate incubated for 20 min. The absorbance (OD) of the samples was measured using a Victor3 multilabel reader (PerkinElmer, Shelton, CT, USA) at 450 nm, and cell metabolic activity was evaluated as the difference between OD of the drugs and control solution. Total proteins of cell extracts were quantified by Bradford assay according to the manufacturer's instructions.

## RT-array PCR

For the RNA extraction procedure, cell cultures from triplicate wells were collected in a single tube. RNA extraction was performed with the RNeasy Mini kit including two DNA elimination steps. The extracted RNA was precipitated for 3 h at −20°C with 0.1 volume of 3M Na-acetate solution and 2.5 volumes of ice-cold 100% ethanol. The pelleted RNA was washed with ice-cold 70% ethanol and dissolved in 15 $\mu$l of ultra-pure water in order to concentrate and clean the nucleic acid. RNA was then immediately stored at −80°C until use. The RNA quality was assessed with Bioanalyzer (Agilent Technologies, USA) and the RNA quantity with Nanodrop (Thermo Fisher Scientific, USA). One $\mu$g of total RNA for each sample was reverse transcribed with random hexamer primers, and the resulting cDNA was amplified in a volume of 10 $\mu$l with RT SYBR Green Rox Master Mix in ViiA7 real time platform (Life Technologies, USA) following the thermal profile suggested by the manufacturer for the 384-well format plate.

The customized RT-array PCR contained the validated intron-spanning primers of the indicated transcripts (see list and abbreviations in Table 1), the internal reference gene RPLPO and as controls the Genomic DNA Contamination (GDC), the Reverse Transcription Control (RTC), and the Positive PCR Control (PPC). Preliminary experiments showed that RPLPO was the most stable reference gene among the 5 candidates (ACTB, B2M, GADPH, HPRT1 and RPLPO). All the genes included in the custom array were sufficiently expressed by THP-1 macrophages and gave only specific amplicons on inspection of melting curve.

The quantification cycle (Cq) for each well was calculated applying the same conditions of baseline and threshold across all the PCR-Array runs, since all genes had same reaction efficiency; runs were analysed in the PCR-Array data analysis web portal (http://www.SABiosciences.com/pcrarraydataanalysis.php) for the validation of the control wells.

| *A. montana* 5c | | | *A. montana* 9c | | | | | | *A. montana* 15c | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Skewness* | *T-test* | *p* | *n* | *Diff. Reg.* | *SE* | *Skewness* | *T-test* | *p* | *n* | *Diff. Reg.* | *SE* | *Skewness* | *T-test* | *p* |
| 0.036 | – | – | 6 | 0.24 | 0.44 | −0.408 | – | – | 6 | 0.59 | 0.23 | 2.363 | – | – |
| 0.357 | – | – | 11 | −0.16 | 0.15 | 0.898 | – | – | 11 | 0.16 | 0.14 | 1.305 | – | – |
| −0.145 | 1.878 | **<0.05** | 11 | −0.26 | 0.18 | 0.994 | 1.475 | ns | 11 | 0.18 | 0.12 | −0.049 | −1.515 | ns |
| 0.285 | – | – | 6 | −0.45 | 0.35 | 1.549 | – | – | 6 | 0.13 | 0.23 | −0.493 | – | – |
| 1.716 | – | – | 6 | 0.06 | 0.05 | −1.160 | – | – | 6 | 0.05 | 0.03 | −0.190 | – | – |
| 1.316 | – | – | 6 | 0.31 | 0.62 | 0.368 | – | – | 6 | 0.32 | 0.49 | 0.871 | – | – |
| −0.951 | – | – | 11 | −0.41 | 0.47 | −0.263 | – | – | 11 | 0.19 | 0.41 | 0.452 | – | – |
| −0.131 | – | – | 6 | 0.09 | 0.09 | −0.820 | – | – | 6 | 0.16 | 0.13 | −0.071 | – | – |
| −1.215 | – | – | 6 | −0.04 | 0.02 | −0.204 | – | – | 6 | −0.06 | 0.03 | −1.346 | – | – |
| 0.118 | – | – | 6 | −0.30 | 0.35 | 1.527 | – | – | 6 | 0.23 | 0.22 | −1.319 | – | – |
| −0.654 | – | – | 6 | −0.34 | 0.32 | 1.604 | – | – | 6 | 0.25 | 0.22 | −1.349 | – | – |
| −0.741 | – | – | 6 | −0.19 | 0.11 | 0.200 | – | – | 6 | 0.01 | 0.11 | 0.748 | – | – |
| 0.531 | 1.498 | ns | 11 | −0.36 | 0.17 | 0.536 | 2.275 | **<0.05** | 11 | 0.06 | 0.15 | −1.058 | −0.436 | ns |
| −0.052 | – | – | 6 | −0.04 | 0.03 | 0.287 | – | – | 6 | −0.04 | 0.03 | −0.277 | – | – |
| 0.641 | – | – | 11 | −0.22 | 0.19 | 1.715 | – | – | 11 | 0.10 | 0.15 | −0.637 | – | – |
| −0.763 | 1.767 | ns | 11 | 0.07 | 0.08 | 0.195 | −0.970 | ns | 11 | 0.17 | 0.05 | −0.342 | −3.698 | **<0.01** |
| 0.128 | 1.920 | **<0.05** | 11 | −0.18 | 0.11 | 0.274 | 1.787 | ns | 11 | 0.10 | 0.08 | −1.660 | −1.244 | ns |
| 0.253 | – | – | 11 | −0.01 | 0.07 | 1.081 | – | – | 11 | 0.03 | 0.08 | −0.368 | – | – |
| −0.639 | - | – | 11 | 0.07 | 0.05 | −0.141 | – | – | 11 | 0.08 | 0.06 | 0.280 | – | – |
| −0.111 | – | – | 6 | −0.01 | 0.04 | −0.323 | – | – | 6 | 0.09 | 0.06 | −0.080 | – | – |
| −0.711 | – | – | 11 | 0.13 | 0.07 | −0.125 | – | – | 11 | −0.06 | 0.09 | 0.685 | – | – |
| −0.385 | – | – | 6 | 0.00 | 0.05 | −0.818 | – | – | 6 | −0.11 | 0.08 | −1.077 | – | – |
| 0.323 | – | – | 6 | −0.04 | 0.07 | 0.199 | – | – | 6 | −0.02 | 0.11 | 0.250 | – | – |
| 0.256 | – | – | 6 | −0.08 | 0.13 | 0.009 | – | – | 6 | 0.23 | 0.15 | −0.054 | – | – |
| −0.006 | 1.808 | ns | 6 | 0.01 | 0.03 | −1.205 | −0.281 | ns | 6 | 0.00 | 0.03 | −0.689 | −0.122 | ns |
| 0.684 | – | – | 6 | −0.09 | 0.08 | 0.976 | – | – | 6 | 0.10 | 0.08 | −0.437 | – | – |
| −0.633 | – | – | 6 | −0.11 | 0.16 | 1.503 | – | – | 6 | 0.15 | 0.09 | −0.941 | – | – |
| −1.246 | 2.423 | (<0.05) | 6 | −0.04 | 0.06 | −0.900 | 0.788 | ns | 6 | −0.11 | 0.05 | −0.062 | 2.267 | **<0.05** |
| | | | | −0.08 | | | | | | 0.11 | | | | |
| | | | | −0.04 | | | | | | 0.10 | | | | |
| | | | | 8 | | | | | | 22 | | | | |
| | | | | 20 | | | | | | 6 | | | | |
| | | | | **0.031** | | | | | | **0.005** | | | | |

**Statistical analysis**

The experimental model had a dose-response setup, comprising 5 dilutions of *A. montana* and the control solvent. Data from 11 independent experiments were considered for Normal Mac at 24 h of treatment; data from 6 independent experiments were considered for Normal Mac at 2 h of treatment and for IL-4 Mac at 2 h and 24 h of treatment. We considered 28 different genes, and for each gene we had four different categories of analysis: Normal Mac (2 h), Normal Mac (24 h), IL-4 Mac (2 h), IL-4 Mac (24 h).

The main statistical working variable was the normalized Cq (ΔCq), calculated using the formula $\Delta Cq = Cq_{\text{Gene of Interest}} - Cq_{\text{RPLPO}}$. The effect of treatment (here defined as Differential Regulation, Diff. Reg.) was calculated by the $\text{Log}_2$(Fold Change) where Fold Change is $2^{(-\Delta\Delta Cq)}$ and $\Delta\Delta Cq = \Delta Cq_{\text{treated sample}} - \Delta Cq_{\text{control}}$. By this calculation a Diff. Reg. = 0 means no treatment effect, while a Diff. Reg. = 1 or = −1 means that the RNA content in the treated sample is respectively twice or half that of the control sample.

For each gene and each category, we considered 6 conditions: 5 different treatments (*A. montana* 2c, 3c, 5c, 9c and 15c) and one control consisting of the solvent. We thus obtained a total of 772 (28 × 4 × 6) samples of data, each corresponding to a single gene/treatment combination. The sample size was not constant, ranging from a minimum of n = 5 to a maximum of n = 11. We compared the ΔCq of each treatment (T) with that of the control (C).

In this way, each treatment value was paired with the control value recorded at the same time, and we then checked whether the differences between them were significantly different from zero. We started by performing an evaluation of equality of variance by Levene's test; since data for each gene and category proved to have similar variance, a parametric ANOVA was done, simultaneously comparing the six conditions of treatment. We then singled out the gene/category combinations for which ANOVA proved significant. Next, we carried out all the T-test pairwise comparisons between treatment and control, calculating the Pearson skewness index to check for possible large deviations from normality. Comparisons with skewness index <−1.0 or >1.0 were not considered to be reliable even if statistically significant. This occurred very rarely (see Tables 2 and 3B). The null hypothesis was then $\delta = 0$, where $\delta$ is the difference between treatment and control in the whole population.

For each treatment, we thus checked four features: the statistical homogeneity, the global comparison by means

**Table 3A** Gene expression by THP-1 macrophages (M2 type) treated for 2 h with *A. montana* dilutions

| *Gene* | *Statistics homog eneity* | ANOVA | A. montana *2c (n = 6)* | | | | | A. montana *3c (n = 6)* | | | | | A. montana *5c (n = 6)* | | | | | A. montana *9c (n = 6)* | | | | | A. montana *15c (n = 6)* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* |
| CCL1 | 0.742 | **0.001** | −0.06 | 0.20 | 1.145 | 0.940 | ns | **−0.28** | 0.17 | 0.505 | 2.760 | **<0.05** | −0.23 | 0.09 | 0.322 | 2.049 | ns | 0.16 | 0.12 | −0.447 | −0.842 | ns | 0.18 | 0.13 | 0.808 | −0.942 | ns |
| CCL2 | 0.916 | 0.659 | 0.09 | 0.23 | 0.022 | −0.171 | − | −0.19 | 0.21 | 0.378 | 0.717 | − | −0.31 | 0.06 | 0.812 | 4.665 | − | −0.04 | 0.32 | −0.672 | −0.410 | − | −0.17 | 0.10 | −0.396 | 0.549 | − |
| CCL3 | 0.963 | 0.099 | −0.01 | 0.11 | 0.502 | 0.486 | − | −0.05 | 0.10 | 0.333 | 2.026 | − | 0.01 | 0.07 | 0.528 | −0.501 | − | −0.03 | 0.10 | −0.009 | 0.726 | − | 0.02 | 0.09 | 0.659 | 0.292 | − |
| CCL4 | 0.899 | **0.033** | −0.10 | 0.09 | 0.755 | 1.304 | ns | −0.16 | 0.13 | −0.085 | 3.794 | **<0.01** | −0.10 | 0.07 | 0.333 | 1.834 | ns | −0.10 | 0.07 | −0.677 | 1.729 | ns | −0.05 | 0.09 | 0.647 | 1.033 | ns |
| CCL5 | 0.978 | **0.049** | −0.04 | 0.03 | −0.715 | 1.275 | ns | −0.06 | 0.04 | −0.114 | 3.697 | **<0.01** | −0.04 | 0.05 | 0.397 | 1.283 | ns | −0.07 | 0.04 | 0.466 | 2.968 | **<0.05** | 0.02 | 0.04 | 0.537 | 0.067 | ns |
| CCL7 | 0.952 | 0.283 | 0.06 | 0.24 | 0.057 | −0.200 | − | −0.42 | 0.10 | 0.454 | 2.746 | − | −0.36 | 0.08 | −0.857 | 2.426 | − | 0.24 | 0.32 | −1.212 | −0.648 | − | 0.09 | 0.22 | 0.296 | −0.509 | − |
| CCL17 | 0.919 | **0.036** | −0.01 | 0.16 | 0.045 | −0.018 | ns | 0.07 | 0.10 | 0.436 | −1.284 | ns | 0.36 | 0.23 | 0.389 | −1.693 | ns | 0.02 | 0.17 | 0.877 | −0.188 | ns | 0.40 | 0.09 | −0.588 | −4.200 | **<0.01** |
| CCL22 | 0.989 | 0.312 | 0.11 | 0.07 | 0.000 | −1.361 | − | 0.18 | 0.10 | −0.448 | −0.928 | − | 0.07 | 0.08 | −0.391 | −0.310 | − | 0.01 | 0.06 | −0.302 | 2.929 | − | 0.08 | 0.07 | −0.451 | −0.837 | − |
| CCL23 | 0.731 | 0.326 | 0.06 | 0.05 | 0.129 | −2.328 | − | −0.01 | 0.04 | 1.049 | 0.116 | − | 0.08 | 0.09 | 0.316 | −1.084 | − | −0.05 | 0.09 | 0.475 | 0.440 | − | 0.06 | 0.11 | 0.326 | −0.556 | − |
| CXCL1 | 0.957 | **0.038** | 0.22 | 0.09 | 0.129 | −3.242 | **<0.05** | 0.27 | 0.11 | −0.710 | −4.165 | **<0.01** | 0.29 | 0.08 | 0.482 | −5.867 | **<0.01** | 0.15 | 0.08 | −0.036 | −2.077 | **<0.05** | 0.19 | 0.09 | 0.474 | −2.110 | **<0.05** |
| CXCL2 | 0.739 | **0.038** | −0.05 | 0.12 | 0.418 | 0.210 | ns | 0.01 | 0.13 | −0.466 | −0.642 | ns | 0.13 | 0.11 | −0.200 | −2.298 | **<0.05** | −0.01 | 0.16 | −0.412 | −0.315 | ns | −0.01 | 0.11 | 0.209 | 0.267 | ns |
| CXCL13 | 0.992 | 0.080 | 0.09 | 0.11 | 0.729 | −0.750 | − | 0.15 | 0.07 | −1.012 | −3.006 | − | 0.23 | 0.05 | 0.937 | −0.131 | − | −0.23 | 0.16 | 0.306 | 1.771 | − | 0.12 | 0.10 | −0.060 | −1.306 | − |
| IL1B | 0.939 | 0.413 | 0.19 | 0.11 | −0.151 | −1.606 | − | 0.10 | 0.05 | −0.401 | −1.225 | − | 0.07 | 0.03 | 0.034 | −3.338 | − | 0.05 | 0.12 | −0.110 | 0.163 | − | 0.13 | 0.10 | 0.508 | −0.819 | − |
| IL1RN | 0.948 | 0.517 | 0.05 | 0.06 | −0.253 | −0.705 | − | 0.04 | 0.07 | −0.486 | 1.181 | − | 0.02 | 0.04 | 0.084 | −0.123 | − | 0.00 | 0.06 | −0.318 | 0.426 | − | 0.07 | 0.07 | 0.196 | −0.767 | − |
| IL8 | 0.912 | 0.159 | −0.02 | 0.15 | 0.553 | 1.173 | − | 0.10 | 0.14 | 0.226 | −0.181 | − | 0.06 | 0.08 | 0.953 | −0.717 | − | −0.09 | 0.12 | −0.206 | 1.165 | − | 0.02 | 0.12 | 1.248 | 0.068 | − |
| IL10 | 0.616 | 0.481 | −0.13 | 0.19 | 0.948 | 0.400 | − | −0.14 | 0.11 | 1.063 | 0.951 | − | −0.17 | 0.10 | −0.570 | 1.741 | − | 0.16 | 0.15 | −0.301 | −1.122 | − | 0.02 | 0.16 | −0.311 | −0.124 | − |
| TNFA | 0.644 | **0.048** | −0.11 | 0.09 | −0.338 | 1.815 | ns | −0.10 | 0.07 | −0.258 | 2.047 | **<0.05** | −0.07 | 0.07 | 0.303 | 0.708 | ns | 0.03 | 0.10 | 0.085 | −0.112 | ns | 0.07 | 0.06 | 0.017 | −0.715 | ns |
| VEGFA | 0.896 | 0.200 | −0.09 | 0.10 | 0.300 | 0.962 | − | −0.06 | 0.09 | 0.046 | 2.333 | − | −0.06 | 0.07 | −0.032 | 0.150 | − | 0.00 | 0.07 | 0.147 | −0.075 | − | 0.03 | 0.05 | −0.909 | −0.661 | − |
| BMP2 | 0.480 | 0.353 | 0.05 | 0.14 | 0.695 | 0.379 | − | −0.01 | 0.14 | −0.019 | 1.270 | − | −0.07 | 0.13 | 0.131 | 1.558 | − | 0.19 | 0.09 | 0.206 | −0.508 | − | 0.15 | 0.09 | 0.132 | −0.391 | − |
| CSF1 | 0.877 | 0.228 | 0.00 | 0.11 | 0.288 | 0.227 | − | −0.10 | 0.09 | 0.217 | 3.379 | − | −0.08 | 0.10 | −0.124 | 2.886 | − | −0.02 | 0.12 | −0.638 | 0.727 | − | 0.06 | 0.12 | 0.805 | −0.125 | − |
| CCR5 | 0.820 | **0.028** | 0.05 | 0.12 | −0.027 | −0.671 | ns | −0.08 | 0.09 | 0.372 | 0.713 | ns | −0.01 | 0.08 | 0.565 | −0.096 | ns | 0.00 | 0.05 | 0.254 | 0.607 | ns | 0.16 | 0.06 | −0.394 | −4.916 | **<0.01** |
| F13A1 | 0.961 | 0.114 | −0.11 | 0.07 | −0.068 | 3.065 | − | −0.08 | 0.10 | 0.846 | 2.226 | − | −0.07 | 0.09 | 0.358 | 3.215 | − | −0.10 | 0.11 | 0.392 | 2.208 | − | 0.01 | 0.06 | 0.179 | 0.475 | − |
| MRC1 | 0.977 | 0.089 | 0.07 | 0.11 | 0.106 | 0.080 | − | −0.15 | 0.19 | −0.260 | 0.396 | − | 0.12 | 0.10 | −0.246 | −1.057 | − | −0.24 | 0.13 | −0.059 | 1.466 | − | 0.17 | 0.06 | −0.043 | −2.448 | − |
| MMP1 | 0.986 | **0.000** | −0.07 | 0.06 | −0.008 | 1.295 | ns | −0.28 | 0.18 | −0.119 | 4.416 | **<0.01** | −0.10 | 0.10 | 0.195 | 1.827 | ns | −0.04 | 0.09 | 0.133 | 1.967 | ns | 0.04 | 0.05 | 0.024 | 0.265 | ns |
| RELA | 0.699 | 0.675 | 0.03 | 0.05 | −0.078 | −0.376 | − | 0.00 | 0.05 | 0.238 | 0.859 | − | −0.02 | 0.07 | 0.106 | 0.342 | − | −0.01 | 0.06 | 0.100 | 1.116 | − | 0.01 | 0.05 | −0.271 | 0.707 | − |
| NFKB1 | 0.102 | **0.005** | 0.14 | 0.04 | 0.142 | −3.992 | **<0.01** | 0.00 | 0.05 | −0.156 | 1.199 | ns | 0.12 | 0.06 | 0.017 | −1.711 | ns | 0.07 | 0.04 | −0.764 | −1.395 | ns | 0.11 | 0.05 | 0.183 | −1.533 | ns |
| NFKBIA | 0.447 | 0.584 | −0.01 | 0.05 | −0.579 | 0.049 | − | −0.04 | 0.05 | 0.012 | 0.835 | − | −0.02 | 0.06 | 0.218 | 0.136 | − | 0.01 | 0.06 | −0.234 | −0.117 | − | 0.06 | 0.03 | 0.717 | −2.045 | − |
| IKBKB | 0.361 | 0.311 | 0.02 | 0.09 | −0.443 | −0.217 | − | 0.00 | 0.09 | −0.044 | 1.047 | − | 0.02 | 0.10 | −0.035 | −0.355 | − | 0.08 | 0.07 | −0.122 | −1.107 | − | 0.12 | 0.07 | −0.912 | −1.457 | − |
| Mean differential regulation | | | 0.02 | | | | | −0.05 | | | | | 0.00 | | | | | 0.01 | | | | | 0.08 | | | | |
| Median differential regulation | | | 0.01 | | | | | −0.05 | | | | | −0.02 | | | | | 0.00 | | | | | 0.06 | | | | |
| N. of up-regulated | | | 15 | | | | | 10 | | | | | 13 | | | | | 13 | | | | | 25 | | | | |
| N. of down-regulated | | | 13 | | | | | 18 | | | | | 15 | | | | | 15 | | | | | 3 | | | | |
| Wilcoxon (p-value) | | | 0.716 | | | | | **0.016** | | | | | 0.219 | | | | | 0.495 | | | | | **0.004** | | | | |

The statistically significant differences between *A. montana* and control are indicated in bold.

**Table 3B** Gene expression by THP-1 macrophages (M2 type) treated for 24 h with *A. montana* dilutions

| *Gene* | *Statistics* ANOVA *homog -eneity* | | A. montana *2c (n = 5)* | | | | | A. montana *3c (n = 6)* | | | | | A. montana *5c (n = 6)* | | | | | A. montana *9c (n = 6)* | | | | | A. montana *15c (n = 6)* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* | *Diff. Reg.* | *SE* | *Skew -ness* | *T-test* | *p* |
| CCL1 | 0.934 | 0.060 | −0.24 | 0.12 | −0.197 | 2.099 | – | −0.27 | 0.18 | 0.043 | 1.544 | – | −0.05 | 0.17 | 0.475 | 0.292 | – | 0.05 | 0.14 | 0.216 | −0.351 | – | 0.14 | 0.15 | −0.085 | −0.910 | – |
| CCL2 | 0.802 | **0.028** | 0.21 | 0.12 | 0.206 | −1.975 | ns | −0.11 | 0.15 | −0.376 | 0.704 | ns | −0.19 | 0.16 | 0.513 | 1.158 | ns | 0.20 | 0.21 | −0.640 | −0.915 | ns | 0.33 | 0.12 | −0.152 | −2.709 | **<0.05** |
| CCL3 | 0.816 | 0.053 | −0.08 | 0.18 | −0.167 | 0.517 | – | −0.13 | 0.12 | −0.116 | 1.063 | – | 0.09 | 0.13 | −0.160 | −0.710 | – | −0.02 | 0.13 | −0.254 | 0.186 | – | 0.30 | 0.20 | −0.352 | −1.484 | – |
| CCL4 | 0.967 | 0.121 | −0.22 | 0.24 | −0.242 | 1.013 | – | −0.16 | 0.14 | 0.098 | 1.132 | – | 0.00 | 0.17 | −0.134 | −0.002 | – | 0.04 | 0.15 | −0.206 | −0.277 | – | 0.27 | 0.32 | −0.499 | −0.844 | – |
| CCL5 | 0.997 | **0.001** | −0.22 | 0.05 | −0.174 | 4.983 | **<0.01** | −0.12 | 0.04 | −0.467 | 3.102 | **<0.05** | −0.05 | 0.05 | 0.544 | 1.033 | ns | −0.02 | 0.03 | 1.042 | 0.593 | ns | −0.09 | 0.05 | −0.280 | 1.705 | ns |
| CCL7 | 0.805 | 0.069 | 0.21 | 0.17 | −0.076 | −1.383 | – | −0.09 | 0.15 | 0.095 | 0.643 | – | −0.08 | 0.23 | 0.135 | 0.347 | – | 0.29 | 0.20 | −0.761 | −1.424 | – | 0.27 | 0.15 | −0.279 | −1.780 | – |
| CCL17 | 0.983 | **0.009** | −0.26 | 0.21 | −0.009 | 1.330 | ns | 0.04 | 0.08 | 0.357 | −0.459 | ns | 0.28 | 0.15 | −0.066 | −1.838 | ns | 0.09 | 0.11 | 0.055 | −0.747 | ns | −0.10 | 0.07 | −0.429 | 1.480 | ns |
| CCL22 | 0.524 | 0.153 | −0.07 | 0.14 | −0.120 | 0.570 | – | 0.10 | 0.10 | −0.017 | −1.044 | – | 0.22 | 0.08 | −0.267 | −2.660 | – | 0.10 | 0.10 | 0.407 | −0.957 | – | 0.03 | 0.13 | −0.880 | −0.256 | – |
| CCL23 | 0.940 | 0.358 | 0.16 | 0.20 | 0.102 | −0.896 | – | 0.14 | 0.14 | 0.411 | −1.005 | – | 0.14 | 0.10 | 0.738 | −1.438 | – | 0.04 | 0.15 | 1.244 | −0.248 | – | 0.22 | 0.08 | −0.360 | −2.595 | – |
| CXCL1 | 0.980 | 0.222 | 0.30 | 0.33 | −0.071 | −1.009 | – | 0.58 | 0.18 | 0.342 | −3.197 | – | 0.55 | 0.17 | 0.357 | −3.151 | – | 0.41 | 0.14 | 0.642 | −2.840 | – | 0.59 | 0.34 | −0.999 | −1.726 | – |
| CXCL2 | 0.960 | 0.450 | 0.45 | 0.25 | −0.105 | −1.946 | – | 0.13 | 0.20 | −0.558 | −0.678 | – | 0.22 | 0.15 | 0.519 | −1.420 | – | 0.08 | 0.14 | 0.207 | −0.572 | – | 0.41 | 0.41 | −1.138 | −1.000 | – |
| CXCL13 | 0.468 | 0.375 | 0.40 | 0.19 | −0.045 | −2.324 | – | 0.43 | 0.18 | 0.124 | −2.439 | – | 0.17 | 0.21 | 0.896 | −0.817 | – | 0.15 | 0.22 | 0.854 | −0.694 | – | 0.07 | 0.19 | 0.618 | −0.379 | – |
| IL1B | 0.964 | 0.499 | −0.09 | 0.24 | −0.160 | 0.395 | – | 0.11 | 0.15 | −0.041 | −0.712 | – | 0.14 | 0.16 | 0.120 | −0.871 | – | 0.04 | 0.13 | 0.483 | −0.281 | – | 0.18 | 0.26 | −0.526 | −0.662 | – |
| IL1RN | 0.704 | 0.179 | −0.27 | 0.16 | −0.210 | 1.836 | – | −0.13 | 0.12 | −0.043 | 1.049 | – | −0.03 | 0.13 | −0.270 | 0.246 | – | 0.02 | 0.12 | −0.291 | −0.157 | – | 0.00 | 0.16 | −0.706 | 0.026 | – |
| IL8 | 0.991 | **0.033** | 0.14 | 0.38 | −0.152 | −0.421 | ns | 0.01 | 0.19 | −0.578 | −0.074 | ns | 0.09 | 0.19 | −0.754 | −0.506 | ns | 0.31 | 0.13 | −0.743 | −2.378 | **<0.05** | 0.72 | 0.40 | −0.815 | −1.820 | ns |
| IL10 | 0.693 | **0.649** | 0.11 | 0.14 | 0.051 | −0.878 | – | −0.03 | 0.17 | −0.254 | 0.157 | – | 0.03 | 0.14 | −0.583 | −0.256 | – | 0.04 | 0.15 | −0.476 | −0.261 | – | 0.10 | 0.14 | −0.473 | −0.709 | – |
| TNFA | 0.890 | **0.022** | −0.18 | 0.07 | −0.037 | 2.749 | **<0.05** | −0.21 | 0.09 | 0.189 | 2.384 | **<0.05** | −0.11 | 0.10 | 1.149 | 1.091 | ns | 0.03 | 0.10 | −0.103 | −0.328 | ns | 0.05 | 0.10 | −0.557 | −0.446 | ns |
| VEGFA | 0.160 | 0.226 | −0.17 | 0.12 | −0.060 | 1.617 | – | −0.09 | 0.11 | 0.250 | 0.844 | – | −0.04 | 0.09 | −0.403 | 0.414 | – | 0.06 | 0.08 | 0.502 | −0.654 | – | −0.08 | 0.11 | 0.473 | 0.664 | – |
| BMP2 | 0.803 | **0.003** | 0.28 | 0.12 | 0.239 | −2.475 | **<0.05** | 0.05 | 0.12 | 0.549 | −0.431 | ns | 0.11 | 0.14 | 0.400 | −0.754 | ns | 0.11 | 0.12 | 0.118 | −0.977 | ns | 0.33 | 0.14 | 0.317 | −2.336 | **<0.05** |
| CSF1 | 0.701 | 0.461 | −0.17 | 0.23 | −0.183 | 0.824 | – | 0.01 | 0.19 | −0.302 | −0.081 | – | 0.14 | 0.18 | −0.371 | −0.750 | – | 0.16 | 0.18 | −1.034 | −0.914 | – | 0.25 | 0.31 | −0.957 | −0.798 | – |
| CCR5 | 0.834 | 0.178 | 0.21 | 0.16 | 0.176 | −1.429 | – | −0.13 | 0.20 | 0.770 | 0.676 | – | 0.08 | 0.06 | 0.183 | −1.378 | – | 0.00 | 0.08 | 0.369 | 0.059 | – | 0.13 | 0.03 | 0.112 | −3.872 | – |
| F13A1 | 0.839 | 0.329 | 0.19 | 0.19 | 0.161 | −1.120 | – | 0.07 | 0.12 | 0.115 | −0.571 | – | 0.08 | 0.15 | 0.831 | −0.536 | – | −0.03 | 0.11 | 1.245 | 0.256 | – | 0.12 | 0.05 | −0.163 | −2.597 | – |
| MRC1 | 0.737 | **0.003** | 0.28 | 0.11 | 0.118 | −2.853 | **<0.05** | 0.14 | 0.07 | 0.241 | −1.998 | ns | 0.18 | 0.09 | −0.509 | −1.993 | ns | 0.05 | 0.08 | −0.111 | −0.637 | ns | 0.32 | 0.11 | 0.161 | −2.890 | **<0.05** |
| MMP1 | 0.997 | **0.008** | −0.30 | 0.13 | −0.032 | 2.545 | **<0.05** | −0.23 | 0.15 | 0.364 | 1.529 | ns | 0.13 | 0.09 | 0.346 | −1.430 | ns | −0.14 | 0.18 | 1.177 | 0.772 | ns | −0.19 | 0.10 | 0.846 | 1.867 | ns |
| RELA | 0.968 | **0.004** | 0.12 | 0.01 | 0.551 | −8.737 | **<0.001** | 0.08 | 0.04 | 0.519 | −2.265 | **<0.05** | 0.15 | 0.02 | −0.328 | −6.529 | **<0.001** | 0.11 | 0.04 | 0.215 | −2.572 | **<0.05** | 0.05 | 0.01 | −0.454 | −3.890 | **<0.01** |
| NFKB1 | 0.873 | **0.001** | 0.30 | 0.07 | 0.084 | −4.644 | **<0.01** | 0.24 | 0.06 | −0.647 | −4.041 | **<0.01** | 0.31 | 0.06 | 0.380 | −5.638 | **<0.01** | 0.18 | 0.05 | 0.530 | −3.378 | **<0.01** | 0.18 | 0.10 | −0.523 | −1.735 | ns |
| NFKBIA | 0.892 | 0.140 | 0.00 | 0.08 | −0.163 | 0.025 | – | −0.04 | 0.08 | −0.118 | 0.438 | – | 0.09 | 0.08 | 0.218 | −1.074 | – | 0.08 | 0.06 | 0.393 | −1.468 | – | 0.13 | 0.10 | −0.252 | −1.351 | – |
| IKBKB | 0.909 | 0.085 | 0.13 | 0.04 | 0.086 | −3.303 | – | 0.12 | 0.05 | 0.726 | −2.331 | – | 0.19 | 0.08 | −0.196 | −2.364 | – | 0.16 | 0.07 | 0.417 | −2.352 | – | 0.03 | 0.07 | 0.567 | −0.479 | – |
| Mean differential regulation | | | 0.04 | | | | | 0.02 | | | | | 0.10 | | | | | 0.09 | | | | | 0.17 | | | | |
| Median differential regulation | | | 0.12 | | | | | 0.01 | | | | | 0.10 | | | | | 0.07 | | | | | 0.13 | | | | |
| N. of up-regulated | | | 15 | | | | | 15 | | | | | 21 | | | | | 23 | | | | | 23 | | | | |
| N. of down-regulated | | | 13 | | | | | 13 | | | | | 7 | | | | | 5 | | | | | 5 | | | | |
| Wilcoxon (p-value) | | | 0.054 | | | | | 0.946 | | | | | **0.001** | | | | | **<0.0001** | | | | | **<0.0001** | | | | |

The statistically significant differences between *A. montana* and control are indicated in bold.

of ANOVA, the level of skewness, and the result of the T-test comparisons between control sample and treatment.

The differences between expression profiles of the whole panel of genes in various treatment conditions was calculated by the Wilcoxon signed-rank test for paired data. For each *A. montana* dilution, the differences versus control were ranked and statistically compared using the specific Wilcoxon tables. The null hypothesis was that the median of differences is equal to zero. If the resulting sums were significantly different, the null hypothesis of the absence of treatment effect was rejected.

## Results

### Characterization of THP-1 macrophages and IL-4 differentiation

The macrophage populations used in the experiments are described in Figure 1. Original myelomonocytic cells of THP-1 line (panel A) have a rounded shape with homogeneous morphology. After differentiation with PMA (Normal Mac, panel B) the cells are characterized by slightly larger dimensions and many cells exhibit an evident adhesion attitude and flat shape. IL-4 Mac (panel C) show an elongated profile and some long cytoplasmic extensions.

The polarization induced by IL-4 was analysed by monitoring the gene expression panel of several well-known marker genes, such as interleukins, chemokines, transcription factors involved in inflammation, coagulation and angiogenic factors (Table 1). Comparative analysis of the transcripts profile of Normal Mac and IL-4 Mac (Figure 2) demonstrated that IL-4 treatment caused a marked increase (Diff. Reg > 2.0) of the chemokines transcripts for CCL2, CCL4, CCL5, CCL17, CCL22 and a marked decrease (Diff. Reg < −2.0) of transcripts for CXCL1, CXCL2, CXCL13 and IL10. Moreover, other genes were found to be enhanced by IL-4, such as the cytokines CCL3, CCL7, IL1B, the growth factors VEGFA and CSF1, the metalloproteinase MMP1, the coagulation factor F13A1, and the mannose receptor MRC1.

### *A. montana* effects on normal macrophages

In parallel experiments not designed for RNA extraction, the cell viability and growth of Normal Mac and IL-4 Mac treated for 24 h with *A. montana* were assessed by WST method. Both cell types were unaffected by the doses of *A. montana* used in this study (data not shown). Table 2A and 2B report the complete set of results obtained with *A. montana* treatment of Normal Mac for 2 h and 24 h respectively. After 2 h of treatment (Table 2A), most genes, namely 23 out of a total of 28 analysed, were slightly down-regulated by *A. montana* 2c, indicating a rapid inhibitory effect of this plant on the panel of selected genes. Considering the whole panel as a gene-set, the prevalent down-regulation by *A. montana* 2c was statistically significant at Wilcoxon test ($p < 0.001$). Changes in differential regulation were quantitatively small, ranging from a down-regulation of −0.51 (CCL17) to an up-regulation of 0.10 (CCL5). The expression of 4 genes, namely CCL23, IL10, MRC1 and RELA, was significantly changed according to ANOVA analysis, but only 5 samples proved to be statistically significant at some dilutions according to T-test comparing *A. montana* with the respective control solvent (CCL23 at 15c, IL10 at 3c, MRC1 at 3c and 9c, RELA at 2c). The significance of RELA down-regulation by *A. montana* 2c was affected by high skewness of data and therefore is not reliable.

After 24 h of treatment (Table 2B), no significant effects were done by *A. montana* 2c, while a trend to down-regulation — significant by the Wilcoxon test — was observed in the dilutions 3c ($p < 0.01$), 5c ($p < 0.01$), and 9c ($p < 0.05$). With the dilution 15c a trend to up-regulation was found ($p < 0.01$). According to ANOVA, the expression of 6 genes — CCL3, IL1B, IL10, TNFA, RELA and IKBKB — was significantly changed, but only in 8 samples the differences were statistically significant with T-test (CCL3 at 5c, IL1B at 9c, IL10 at 15c, TNFA at 3c and 5c, RELA at 3c and IKBKB at 3c and 15c). All these changes but IL10 at 15c were in the direction of gene down-regulation.

Figure 3 reports the most interesting findings extracted from the general dataset of Normal Mac, considering only those genes where ANOVA revealed some statistically significant difference and where the direction of effect was consistent at different times (2 h and 24 h) and for different dilutions. A slight down-regulation of IL1B, TNFA and MRC1 was caused by 2c, 3c, 5c and 9c test solutions in almost all conditions. However, only 9c on IL1B at 24 h, 3c and 5c on TNFA at 24 h and 3c and 9c on MRC1 at 2 h reached the statistically significant threshold.

### *A. montana* effects on IL-4 Macrophages

The effects of *A. montana* on IL-4 Mac at 2 h and 24 h are reported in Table 3A and 3B respectively. In general, ANOVA analysis showed that a higher number of genes was modified in macrophages differentiated towards the wound healing phenotype: after 2 h of treatment (Table 2A), 10 genes were significantly affected — CCL1, CCL4, CCL5, CCL17, CXCL1, CXCL2, TNFA, CCR5, MMP1, NFKB1 — with a total of 15 samples statistically significant at the T-test. Considering the whole panel as a gene-set, statistically significant at Wilcoxon test indicated a prevalent down-regulation of genes in cells treated with 3c ($p = 0.016$) and prevalent up-regulation of genes in cells treated with 15c ($p = 0.004$). After 24 h of treatment (Table 3B), 10 genes were significantly affected — CCL2, CCL5, CCL17, IL8, TNFA, BMP2, MRC1, MMP1, RELA and NFKB1 — with a total of 20 samples statistically significant according to the T-test. In IL-4 Mac, after 24 h of treatment, most changes were in the direction of up-regulation and at the dilutions 5c, 9c and 15c the Wilcoxon test was highly significant. The genes TNFA, CCL5 and MMP1 were down-regulated by low dilutions of *A. montana* (2c and 3c).

The histograms in Figures 4–6 show the results for some genes where ANOVA was significant and where the direction of effect was consistent for different dilutions. This category includes three important chemokines of

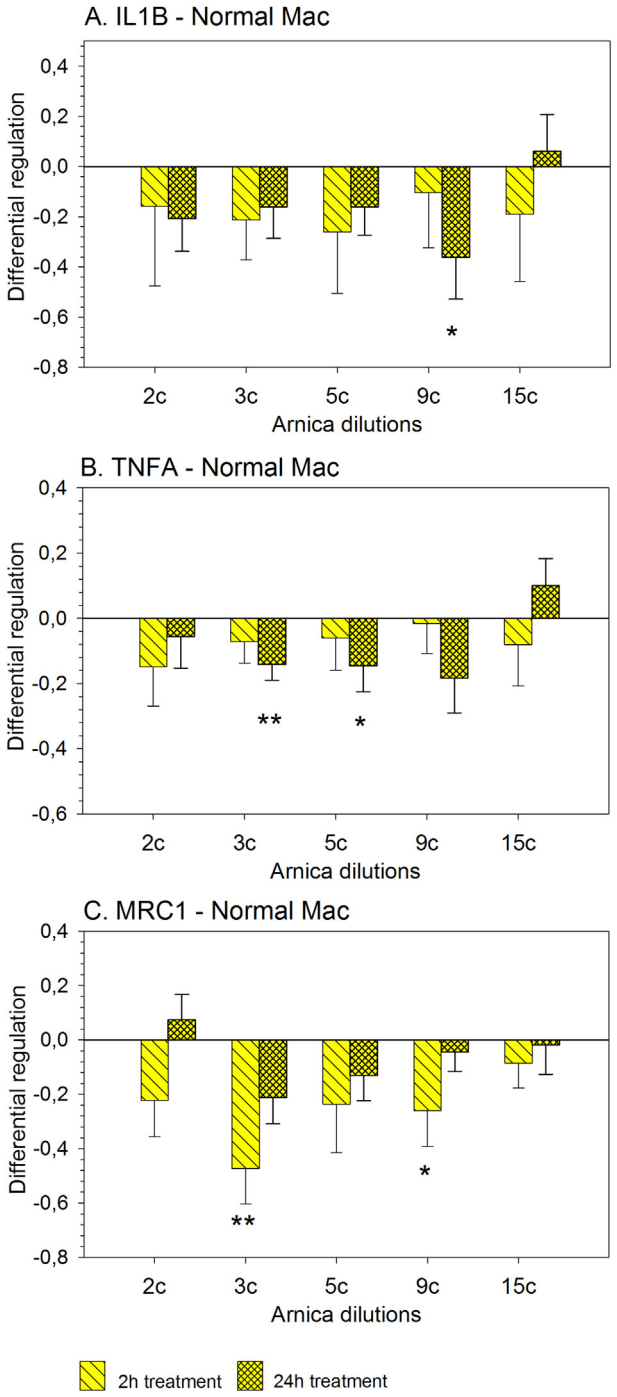


**Figure 3** Effects of *A. montana* on Normal Mac IL1B (A), TNFA (B) and MRC1 (C) gene expression after 2 h and 24 h of treatment. * = $p < 0.05$, **$p < 0.01$. Differential regulation is expressed as $Log_2$(Fold Change) ± SE.

CXC type (CXCL1, CXCL2 and IL8) (Figure 4). The most consistent findings concerned the CXCL1 gene whose expression was increased in all conditions, with statistically significant values at the 2 h time point and with higher values also at the 24 h time point, where ANOVA ($p = 0.222$) did not reach the statistical threshold probably due to high variance of data. The CXCL2 and IL8 genes also showed quantitatively higher stimulating effects of *A. montana*, but only after 24 h of treatment. Broadly, the effects were scattered for the different dilutions, without a clear dose-dependence. Furthermore, *A. montana* exhibited a remarkable tendency to increase bone morphogenetic protein (BMP2) and to decrease MMP1 gene expression in IL-4 Mac (Figure 5), with different effect sizes in different dilutions without dose-dependence for BMP2 and with higher effects at lower dilutions for MMP1. Figure 6 reports the results of the NF-kappaB complex genes modified by *A.*

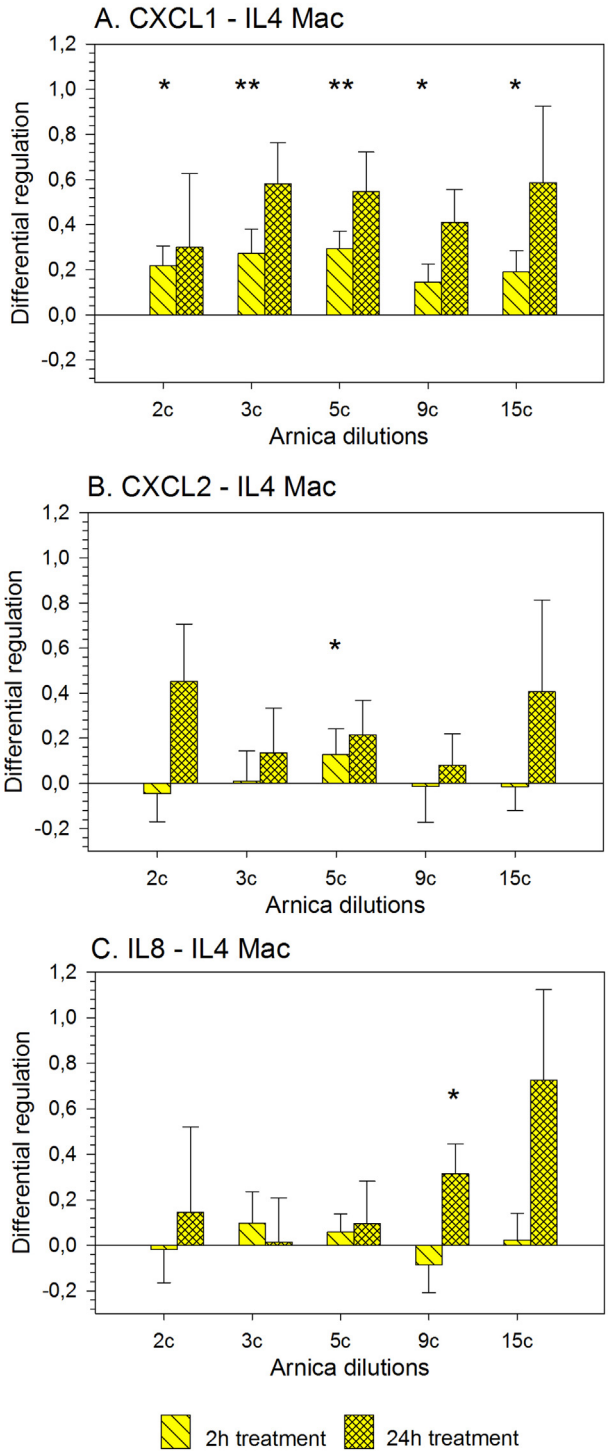


**Figure 4** Effects of *A. montana* on IL-4 Mac CXCL1 (A), CXCL2 (B), IL8 (C) gene expression after 2 h and 24 h of treatment. * = $p < 0.05$, **$p < 0.01$. Differential regulation is expressed as $Log_2$(Fold Change) ± SE.

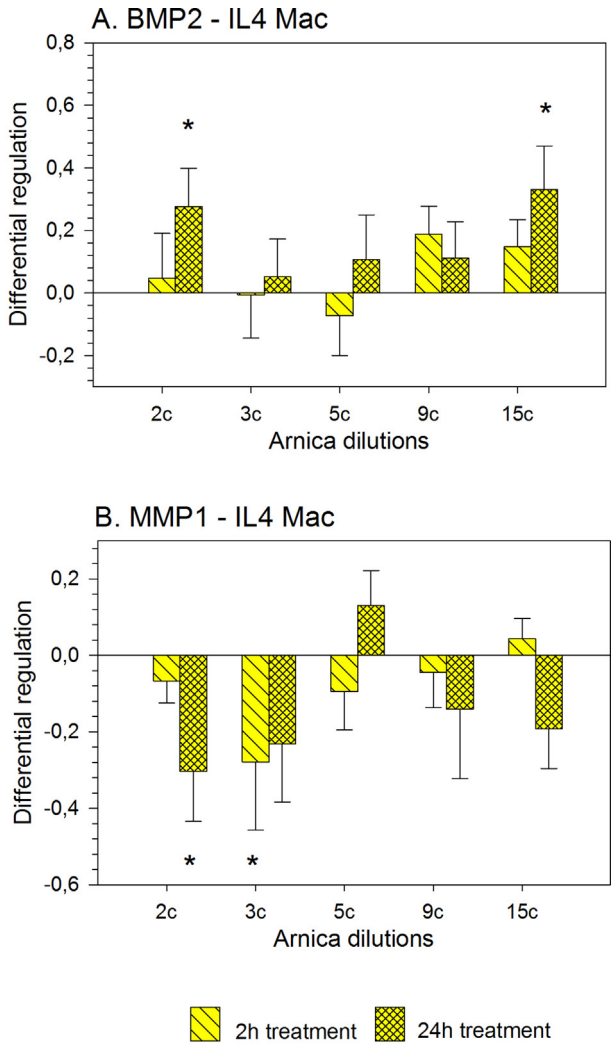


**Figure 5** Effects of *A. montana* on IL-4 Mac BMP2 (A) and MMP1 (B) gene expression after 2 h and 24 h of treatment. * = $p < 0.05$. Differential regulation is expressed as $Log_2$(Fold Change) ± SE.

*montana* in IL-4 Mac. These genes were consistently up-regulated, especially after 24 h of treatment.

A summary of the major findings concerning the pharmacological effects of *A. montana* on both types of macrophages and their possible dealings with the functional changes of connective tissue during the processes of inflammation and tissue repair is shown in Figure 7.

## Discussion

The use of complementary medicines is currently widespread in the population for treating various kinds of ailments, in the hope of recovering whilst reducing the use of conventional drugs which may cause adverse effects. Among herbal medicines, those containing extracts of Asteraceae (Compositae) are especially popular in the primary-care setting. However, there remains a gap between the growing acceptance of these remedies and the lack of data concerning their action mechanism.

Several efforts have been made to understand the molecular mechanism(s) of biological responses of homeopathic drugs,[35–37] but many aspects remain to be

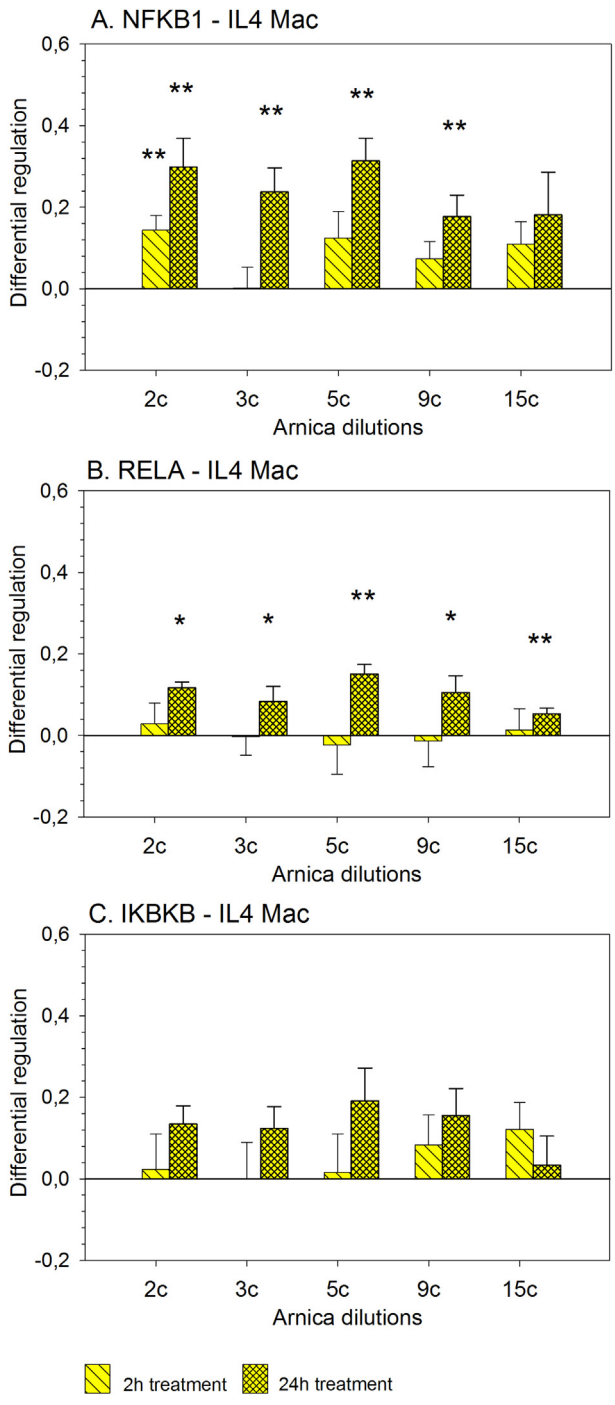


**Figure 6** Effects of *A. montana* on IL-4 Mac NFKB1 (A), RELA (B) and IKBKB (C) gene expression after 2 h and 24 h of treatment. * = $p < 0.05$, **$p < 0.01$. Differential regulation is expressed as $Log_2$(Fold Change) ± SE.

clarified. Recent research has demonstrated the anti-inflammatory effects of *A. montana* plant extract and one of its active principles, helenalin. Furthermore, this plant is widely employed for another therapeutic property that plays a central role for human health, i.e. the promotion of wound healing.

In a randomized double-blind study conducted on humans, *A. montana* at low homeopathic dilution (4x) reduced wound irritation (redness, swelling and heat) after hallux valgus surgery and ameliorated post-operative

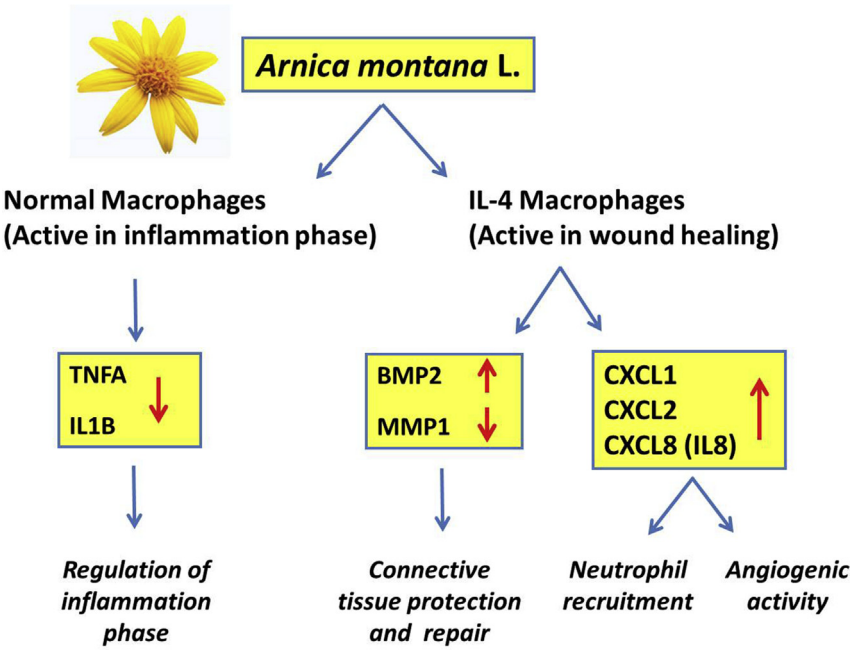


**Figure 7** Most important functions of genes whose expression is adjusted by *A. montana* on macrophages at two stages of differentiation/polarization.

conditions in fewer days than did diclofenac.[1] An anti-inflammatory effect of *A. montana* (6c dilution) on carrageenan-induced foot oedema in rats has been reported by some authors[10] and a complex homeopathic product based on *A. montana* plus other compounds at low dilutions (4x−6x) was effective for blood-induced experimental oedema.[38]

To better investigate the targets and mechanisms of *A. montana* action at cell level, we used a molecular biology approach, testing several dilutions of the plant extract on a panel of genes possibly involved in the function of human macrophages during inflammation and tissue repair. Based on the literature, human and mouse macrophages can be polarized by IL-4 into a wound-healing type,[22,33] and we confirmed this effect both morphologically and transcriptionally. Our cells were profoundly changed by IL-4 treatment, as shown the by differential expression of many genes (CCL4, CCL17, CCL22, F13A1, and MMP1) that others have previously described as markers of a polarization typical of 'wound healing' macrophages.[23] The two functional and genetic states of the cell were well differentiated as described in literature and responded in different ways to *A. montana* treatment.

The evaluation of gene expression by inflammatory cells provides a means of probing the effects of a drug using up-to date and reliable assay tools. In fact, analysis of gene expression by microarrays just 5 h after an arterial injury showed that the most differentially expressed genes were primarily associated with inflammation, cell proliferation, migration, and adhesion.[39] Similarly, internal damage such as traumatic brain injury can exhibit a significant overrepresentation of genes belonging to the inflammation, stress, and signalling categories, such as CCL3, CXCL1, IL1B.[40] Being key-cells of inflammatory processes, macrophages are involved in both tissue damage and repair, and their pharmacological regulation may have a profound impact on the progression of wound healing.

The results of the present study suggest a possible regulating role of *A. montana*, involving a direct effect on both types of macrophages, with a slight but significant influence on the expression of some relevant genes belonging to inflammatory pathways and connective tissue defence and repair (see a summary in Figure 7). One important and novel finding was the marked difference between the effects of *A. montana* in Normal Mac and IL-4 Mac. In the basal state (Normal Mac), the main significant change was a slight down-regulation of the genes of two pro-inflammatory cytokines, namely IL1B and TNFA. This effect can be linked to a decrease in expression of genes of the NF-kappaB complex, in agreement with the claimed anti-inflammatory effect of this plant. IL1B plays a major role in regulating inflammatory mediator production in wounds by stimulating the production of multiple cytokines and chemokines, with important aspects of wound biology.[41]

One of the most striking and unexpected effects of *A. montana* on IL-4 Mac was the increase of chemokines of the CXC family (CXCL1, CXCL2, IL8) (Figure 4 A,B,C respectively). The effects were higher after 24 h of treatment, but generally were visible already after 2 h, indicating a rapid response of the cellular genome. If confirmed, this finding suggests a new property of this plant in wound-healing processes. Cytokines and chemokines promote inflammation and angiogenesis, facilitate the passage of leukocytes from circulation into the tissue, and contribute to the regulation of epithelialization.[42] Thus, their pharmacological fine-tuning can be regarded as a possible way to regulate this complex series of events. CXCL1 and CXCL2 stimulate the expression of specific integrin

proteins on the surface of human neutrophils, which can be rapidly induced to adhere to the extracellular matrix and migrate into injured tissue.[43,44] Besides stimulating neutrophil adhesion and chemotaxis, a positive effect of CXC chemokine up-regulation could also concern angiogenesis. The chemokine-mediated regulation of angiogenesis is highly sophisticated, finely tuned, and involves pro-angiogenic chemokines, including CXCL1,-3,-5,-8 and their receptors, CXCR1 and CXCR2. CXCL1 and CXCR2 are expressed in normal human epidermis and are further induced during the wound healing process of human burn wounds, especially during the inflammatory, epithelialization and angiogenic processes.[42,45,46] The topical application of pantothenic acid (vitamin B5) is widely used in skin care and in treating various dermatological diseases because it stimulates skin regeneration and promotes wound healing. Up-regulation of gene expression of CXCL1, CCL18, IL-6, IL1B and other factors are among the molecular mechanisms responsible for the effect of pantothenic acid in wound healing.[47]

A positive influence on connective tissue reinforcement can be also exercised by the decrease of metalloproteinase (MMP1), coding for an enzyme capable of cleaving extracellular matrix substrates and involved in many pathologic processes.[48] Our finding is in keeping with a previous study showing that *A. montana* preparations suppress MMP1 and MMP13 mRNA levels in bovine and human articular chondrocytes.[49] Also the slight increase inBMP2 gene expression after treatment with *A. montana* is important because BMP2 is required for the initiation of bone repair[25,50] or other regenerative processes *in vitro*,[51] and BMP2 gene expression is activated in the wounds caused by tooth extraction in mice treated with alendronate, an antiresorptive agent widely used for the cure of osteoporosis.[52]

The extent of differential regulation by *A. montana* on both types of macrophages was generally low and reached statistical significance only in a minority of the considered genes and dilutions. The slight effect in this *in vitro* model does not mean that the modulating effect will also be small *in vivo*, in whole organisms. Whereas conventional anti-inflammatory drugs are designed to suppress the underlying enzymatic mechanism of inflammation (e.g. prostaglandins, cytokines) and act at considerably high doses, homeopathic treatment is designed to regulate only the pathological excess of inflammation, because the phenomenon in itself is seen as an expression of natural healing dynamics. Given the variety of *A. montana* effects and the multiplicity of its alkaloids, flavonoids and sesquiterpene lactones,[53] it is conceivable that the picture of its action is much more complex and could involve modulation of further pathways. These aspects can be addressed using 'omic' approaches such as total RNA sequencing and proteomic analysis, which are under investigation in our laboratory.

The ability of highly diluted compounds to modulate gene expression in human/animal cells and unicellular organisms has previously been reported by some authors. A complex homeopathic medication (Canova) used as an immunostimulant produced a differential expression in 147 genes of macrophages involved in transcription/translation, cell structure and dynamics, immune response, cytoprotection, enzymatic process, and receptors/ligands[54]; one of the down-regulated transcripts was CCL5, as also observed in this study. In experiments using microbiological models, ultradiluted *A. montana* 30c modified the expression of specific genes that are the targets UV irradiation injury.[55]

Preethi and co-workers investigated the effects of antitumour homeopathic medicines through the analysis of expression changes in the whole genome using DNA microarray.[56] In human epithelial cell line, highly diluted preparations obtained from *Apis mellifica* (3c, 5c and 7c) modulated hundreds of genes including those involved in cytokine expression, inflammatory processes, and antioxidative responses.[57] These findings support the hypothesis that homeopathic remedies could turn some important genes on or off, initiating a cascade of gene actions to correct the gene expression that has gone wrong and so produced the disorder or disease.

A final aspect to be discussed is the unusual dose-effect relations of the effect of *A. montana* on the expression of many genes. The statistical evaluation of the set of genes observed in this RT-array panel (Wilcoxon test in Tables 2 and 3) showed that the lower dilutions (2c, 3c, 5c) have a tendency to inhibition of gene expression, while the highest (particularly the 15c) always lead to an up-regulation (statistically significant at 24 h in the Normal Mac and at 2 h and 24 h in IL-4 Mac). This observation could be in line with the views that gene expression is modulated by a hormetic effect as recently suggested by others.[58] However, this type of inversion of effects applies on the median behaviour of the gene set and on some genes in particular (CCL3, L1B and TNFA in Table 2B; TNFA in Table 3B), while other important genes (see Figures 3–6), including CXC chemokines have no hormetic trends, showing remarkable consistency between the responses to higher and lower dilutions. The role of hormesis in homeopathic effects is still a matter of investigation and discussion,[59–62] particularly because in this pharmacologic approach also high dilutions are used, where the conventional dose-response paradigm should be revised in view of physicochemical factors such as water clusters and nanoparticles.[63–65]

Genes of CXC chemokines and NF-kappaB complex were modulated in a non-linear way, and often showed some effects that were higher in higher dilutions (Figures 4A, C and 5A). Among the genes that were affected by *A. montana* treatment, in 5 cases the 15c was the only significantly active dilution (see CCL23 in Table 2A, IL10 in 2B, CCL17 and CCR5 in 3A, CCL2 in 3B). This kind of non-linearity of homeopathic drugs has previously been observed by us[66,67] and others.[57,68–74] High sensitivity to external regulations and non-linear responses are frequently reported also with non-homeopathic dilutions. For example, mouse fibroblasts responded to TNF-$\alpha$ at concentrations as low as few pg/ml ($10^{-12}$ g, corresponding approximately to a 6c dilution) and in a non-linear manner, since early NF-$\kappa$appaB gene expression was not dependent on the concentration of the

inducing signal.[75] Moreover, the cell response was expressed as a dynamical oscillations of NF-kappaB translocations, whose frequency can produce distinct gene expression profiles.[76]

On the basis of DNA microarray data, it has been suggested that gene regulatory networks may be regarded as dynamically 'critical' systems poised near the transition phase between order and chaos[77,78] where extreme sensitivity to initial conditions and small perturbations are known to occur. Genetic regulatory networks may be the target of subtle messages by virtue of their flexibility of response to environmental stimuli.[78] In such a situation, a variability of responses to low doses of drugs is also to be expected: if a treatment acts by influencing the cell genetic networks by means of small doses, this action could be highly sensitive to even small changes in experimental conditions.[79,80]

## Conclusion

This study examined, for the first time, several *A. montana* dilutions on human macrophages, using real-time RT-array PCR of a panel of cytokines and chemokines. A small but significant down-regulation of inflammatory genes was detected in normal macrophages, confirming previous findings, while in IL-4 treated macrophages *A. montana* promoted the down-regulation of genes for metalloproteinase-1 and the up-regulation of those for chemokines that are involved in connective tissue healing after injury. These findings lead to new working hypotheses concerning the action of this plant on macrophages, and thus on the complex mechanisms of tissue repair.

## Conflict of interest statement

The authors do not have conflict of interest.

## Acknowledgements

This work was supported by grants from Boiron Laboratories (Lyon, F) (grant 2013-4) to the University of Verona and from the Italian Research Ministry (grant FUR 2012-13).

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