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(54) **Title**: A SET OF MICRORNAS AS MARKERS AND TARGETS IN CANCER AND IMMUNE SYSTEM RELATED DISEASES, METHODS FOR THEIR APPLICATION AND A KIT

(57) **Abstract**: The present invention involves detection of differential expression of specific microRNAs (miRNAs) in certain biological/pathological conditions, and functional applications of miR-511, miR-193b,miR-99b and other listed miRNAs as markers and targets in cancer and immune system related diseases. The microRNAs are used as an expression modulation targets for influencing proliferative properties of a cell and/or immune functions of an organism.

-5.0 -1.0 3.0

MO I, MO II, MO III, DC I, DC II, DC III, DC + LPS I, DC+LPS II, DC+Curd I, DC+Curd II, MF I, MF II, MF+LPS I, MF+LPS II, MF+Curd I, MF+Curd II

miR-146a
miR-146b-5p
miR-132
let-7e
miR-125b
miR-210
miR-125a-5p
miR-511
miR-34a
miR-212
miR-99b
miR-193b
miR-99a
miR-449a
miR-193a-3p
miR-139-5p
miR-642
miR-570
miR-133a
miR-519e
miR-518e
miR-137
miR-554
miR-135a
miR-182
miR-32
miR-183
miR-147
miR-1
miR-218

miR-548d-3p
miR-196a
miR-454*
miR-150
miR-143
miR-194
miR-582-5p
miR-199b-5p
miR-618
miR-20b

Fig. 2

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TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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A set of microRNAs as markers and targets in cancer and immune system related diseases, methods for their application and a kit

Field of the invention

The present invention relates generally to the field of RNA biology. It involves detection of
5 differential expression of specific microRNAs (miRNAs) in certain biological/pathological conditions, and functional applications of miR-511, miR-193b and miR-99b. This invention provides involvement of specific miRNAs, especially miR-511, in cancers and/or other immune system related diseases. Therefore, the provided miRNAs can be used as diagnostic markers or gene therapy targets or drug targets.

10 **Background of the invention**

MicroRNAs (miRNAs) are 21-23 nt long single-stranded RNAs, which together with partner proteins mainly cause gene silencing by degradation of target mRNAs or inhibition of translation (Amaral, et al. 2008. The eukaryotic genome as an RNA machine. Science N.Y 319:1787-1789). However, upon cell cycle arrest, miRNAs can up-regulate the translation as
15 it was first shown for miR-369-3p, which functions as positive regulator of the TNFalpha protein in response to quiescence (Vasudevan, et al. 2007. Switching from repression to activation: microRNAs can up-regulate translation. Science N.Y 318:1931-1934). Several miRNAs have been shown to be important to the immune system. For instance, miR-146 has been confirmed to regulate inflammatory responses in several different cell types as well as it
20 is implicated in the multiple cancers and inflammatory diseases (Lodish, et al. 2008. Micromanagement of the immune system by microRNAs. Nat Rev Immunol 8:120-130). To date, targeting of miR-122 has potential to reach first into the market as miRNA-based gene therapy method in treatment of hepatitis C (Lanford et al. 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327(5962):198-
25 201). As for most of the other miRNAs, the sequences and genomic localization of mature hsa-miR-511 and the genes, hsa-miR-511-1 and hsa-miR-511-2, have been published previously http://www.mirbase.org/ (Bentwich et al. 2005. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet. 37:766-770). Also, differential expression of miR-511 in different disease conditions have been published before (Kim et al.
30 2010. Deregulation of miR-519a, 153, and 485-5p and its clinicopathological relevance in ovarian epithelial tumours. Histopathology 57(5):734-43; Estep et al. 2010. Differential expression of miRNAs in the visceral adipose tissue of patients with non-alcoholic fatty liver disease. Aliment Pharmacol Ther. 32(3):487-97; Tömböl Z, et al. 2009. Integrative molecular

bioinformatics study of human adrenocortical tumors: microRNA, tissue-specific target prediction, and pathway analysis. Endocr Relat Cancer 16(3):895-906). However, information about specific miRNA functions is very limited and similarly, no functional studies on miR-511 has been carried out previously. miRNAs have been previously shown as potential targets for cancer therapies (Slack, Johnson, Grosshans. Regulation of oncogenesis by microRNAs. WO2006/02967; Suzuki. Method and kit for detection of cancer, and theraopeutic agent for cancer. EP2295598; Lin and WU. Development of universal cancer drugs and vaccines. WO2011/025566; Pagani et al. MicroRNA expression signature in peripheral blood of patients affected by hepatocarcinoma or hepatic cirrhosis and uses thereof. WO2011/027332) or diagnostics (Mambo et al. miRNA biomarkers of lung disease. WO2011/025919), synthetic miRNAs have been introduced into cells for reducing cell viability or proliferation (Brown et al. Methods and compositions involving miRNA and miRNA inhibitor molecules). MicroRNAs have also been involved in diagnostic methods for detecting allergic conditions (Corry et al. miRNA expression in allergic disease. WO2010/129919).

The miRNA studies in relation to this invention are carried out mainly on human CD14+ monocytes (MO) purified from the peripheral blood and differentiated to the Dendritic cells (DCs) and macrophages (MFs) *ex vivo*. In this method, human blood MOs are differentiated in the presence of Granulocyte macrophage colony-stimulating factor (GM-CSF) and IL4 into the cells, which resemble to inflammatory DCs and possess MHCII+DC-SIGN(CD209)+CD14− phenotype. In the presence of GM-CSF only, MOs differentiate into MFs, which hold MHCHDC -SIGN−CD14+ surface markers (Sallusto and Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med, 179(4): 1109-18). After stimulation through pathogen recognition receptors, such as toll-like receptors (TLRs), DCs and MFs achieve activated characteristics and produce higher levels of CD86, CD83 and pro-inflammatory cytokines. In principal, the described differentiation and activation of DCs and MFs mimics *in vivo* situations where pathogens or cancer cells are recognized as danger signals and innate and adaptive immune response are initiated to eliminate the problematic events in the body. Consistently, the *ex vivo* differentiated DCs are efficient in stimulation of CD4 and CD8 positive T cells. These DCs are also the most common type of DCs used in development of immunotherapeutic approaches (Tyagi et al., 2009. RNA-based immunotherapy of cancer: role and therapeutic implications of dendritic cells. Expert Rev

Anticancer Ther. 9(1): 97-114; Melief 2008. Cancer immunotherapy by dendritic cells. Immunity. 29(3): 372-83). Based on early releases of miRBase, the expression profiles of miRNAs in human blood MOs, DCs and MFs have been determined (Ceppi et al. 2009. Several miRNAs have shown to influence the differention and functions of human blood
5 MOs, DCs and MFs. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci U S A,. 106(8): 2735-40; Fulci et al. 2007. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. Blood 109(11): 4944-51; Hashimi et al. 2009. MicroRNA profiling identifies miR-34a and miR-21 and their target genes JAG1 and WNT1 in the coordinate
10 regulation of dendritic cell differentiation. Blood. 114(2): 404-14). However, the expression patterns and roles of more recently discovered miRNAs in DCs and MFs have not been described, and thus their potential applications have to be unveiled.

The development of the malignant tumour is a life-threatening condition. Differential expression of miRNAs in various cancers has been shown earlier (Amaral, et al. 2008. The
15 eukaryotic genome as an RNA machine. Science N.Y 319:1787-1789; Lodish, et al. 2008. Micromanagement of the immune system by microRNAs. Nat Rev Immunol 8:120-130).The only published research regarding miR-511, which we have been studied in relation to the current invention, are related to cancer. miR-511 has been shown to be down-regulated in benign ovarian tumour (Kim et al. 2010. Deregulation of miR-519a, 153, and 485-5p and its
20 clinicopathological relevance in ovarian epithelial tumours. Histopathology 57:734-43), adrenocortical carcinomas and benign adenomas (Tömböl Z, et al. 2009). Integrative molecular bioinformatics study of human adrenocortical tumors: microRNA, tissue-specific target prediction, and pathway analysis. Endocr Relat Cancer 16(3):895-906). According to the recent view, efficient treatment of cancers depends on efficient targeting of cancer-
25 initiating cells or cancer stem cells. One of the main markers of cancer-initiating cell is CD44, an abundantly expressed protein involved in malignancies of haematopoietic and epithelial origin. Previously, targeting of CD44 by CD44 specific antibodies and siRNAs has been used in supression of growth and invasion of several tumours in mouse models (AML, CML, B cell melanoma, mouse melanoma, human ovarian and breast cancer models in mouse etc) and
30 even in patients with chemoresistant breast cancer (Zöller 2011. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? Nat Rev Cancer 11(4):254-67). However, because of unrestricted heterogenity of the cancers, development of more specific targeting methods of CD44 and other tumour growth regulating proteins is further required.

Definitions

In the meaning of the current invention, the following terms are defined below to facilitate understanding of the invention:

microRNA (miRNA) – MicroRNAs (miRNAs) are short RNA molecules, on average only 22 nucleotides long and are found in all eukaryotic cells. miRNAs are post-transcriptional regulators that bind to partially complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression and gene silencing. The human genome may encode up 2000 miRNAs, which may target about 60% of mammalian genes and are abundant in many human cell types. miRNA target sites are usually in the three prime untranslated regions (3'UTR) of the mRNA. In the meaning of the current invention, miRNA may be naturally occurring, synthetic, synthesized from nucleic acid fragments or molecules introduced into a cell, as well as they may be composed of or comprise nucleic acid residues and nucleic acid analogues. miRNAs of the invention may comprise fully or partially identical sequence of the naturally occurring miRNA sequence.

Monocyte (MO) is a type of white blood cell and is part of the human body's immune system. Monocytes play multiple roles in immune function. Such roles include: (1) replenish resident macrophages and dendritic cells under normal states, and (2) in response to inflammation signals, monocytes can move quickly (approx. 8-12 hours) to sites of infection in the tissues and divide/differentiate into macrophages and dendritic cells to elicit an immune response.

Dendritic cells (DCs) are immune cells forming part of the mammalian immune system. Their main function is to process antigen material and present it on the surface to other cells of the immune system. That is, they function as antigen-presenting cells. They act as messengers between the innate and adaptive immunity. DCs are present in tissues in contact with the external environment, such as the skin (where there is a specialized dendritic cell type called Langerhans cells) and the inner lining of the nose, lungs, stomach and intestines. They can also be found in an immature state in the blood. Once activated, they migrate to the lymph nodes where they interact with T cells and B cells to initiate and shape the adaptive immune response.

Macrophages (MFs) are white blood cells produced by the differentiation of monocytes in tissues. MFs function in both non-specific defense (innate immunity) as well as help initiate specific defense mechanisms (adaptive immunity) of vertebrate animals. MFs are phagocytes, their role is to phagocytose (engulf and then digest) cellular debris and pathogens, either as

stationary or as mobile cells. They also stimulate lymphocytes and other immune cells to respond to pathogens.

Oligonucleotide is a short nucleic acid polymer, typically with fifty or fewer bases. Although they can be formed by bond cleavage of longer segments of DNA or RNA, they are now more
5 commonly synthesized, in a sequence-specific manner from individual nucleoside phosphoramidites. Oligonucleotides are characterized by the sequence of nucleotide residues that comprise the entire molecule. Oligonucleotides readily bind, in a sequence-specific manner, to their respective complementary oligonucleotides, DNA, or RNA to form duplexes or, less often, hybrids of a higher order. In the meaning of the current invention,
10 oligonucleotides may be naturally occurring, synthetic, synthesized from nucleic acid fragments or molecules introduced into a cell, as well as they may be composed of or comprise nucleic acid analogues.

Cancer (malignant neoplasm) is a class of diseases in which a group of cells display uncontrolled growth, invasion that intrudes upon and destroys adjacent tissues, and sometimes
15 metastasis, or spreading to other locations in the body via lymph or blood. These three malignant properties of cancers differentiate them from benign tumors, which do not invade or metastasize.

Modulation means in the current invention a process when by means of direct or indirect intervention in a cell, tissue or an organism, *in vivo*, *in vitro* or *ex vivo*, naturally occurring or
20 synthetic compounds, nucleic acids, nucleic acid analogues are introduced and gene products, nucleic acids, polypeptides and/or downstream components of biological pathways are decreased or increased.

Disclosure of the invention

The present invention discloses a subset of human miRNAs, which are shown to be
25 differentially expressed either in human blood CD14+ monocytes (further MOs), *in vitro* differentiated immature and mature dendritic cells (further DCs) and/or in un-induced and activated macrophages (further MFs). In addition, this invention concerns the proposed and validated targets of specific human miRNAs, miR-511, miR-99b and miR193b. According to the current invention, these miRNAs can be used as expression modulation targets for
30 influencing proliferative properties of a cell and/or immune functions of an organism.

Moreover, the object of the invention is one or more microRNAs, which are selected from the group comprising microRNA miR-511, miR-193b, miR-99b, miR-499, miR-99a, miR-212 , miR-210, miR-139-5p, miR-642, miR-1, miR-218, miR-518e, miR-147, miR-32, miR-570, miR-193a-3p, miR-519e, miR-182, miR-22, miR-137, miR-663, for using as an expression modulation target for influencing proliferative properties of a cell and/or immune functions of an organism.

Another object of the invention is a method, wherein proliferative properties of a cell and/or immune functions of an organism are targeted by modulating the expression of one or more microRNAs selected from the group comprising microRNA miR-511, miR-193b, miR-99b, miR-499, miR-99a, miR-212 , miR-210, miR-139-5p, miR-642, miR-1, miR-218, miR-518e, miR-147, miR-32, miR-570, miR-193a-3p, miR-519e, miR-182, miR-22, miR-137, miR-663.The named one or more microRNAs of the invention can be used as expression modulation targets for influencing proliferative properties of a cell involved in, but not limited to, the following diseases and conditions: Small-cell lung cancer (SCLC) and Non-small-cell lung cancer (NSCLC), Acute lymphoblastic leukemia (ALL) Chronic lymphocytic leukemia (CLL), Acute myelogenous leukemia (AML), Chronic myelogenous leukemia (CML), T-cell prolymphocytic leukemia (T-PLL), Large granular lymphocytic leukemia, Adult T-cell leukemia, and/or modulating immune functions of an organism with allergic asthma and/or chronic obstructive pulmonary disease.

Thus, the present invention comprises a method, wherein proliferative properties of a cell and/or immune functions of an organism are targeted by modulating the expression of one or more microRNAs selected from the group comprising microRNA miR-511, miR-193b, miR-99b, miR-499, miR-99a, miR-212 , miR-210, miR-139-5p, miR-642, miR-1, miR-218, miR-518e, miR-147, miR-32, miR-570, miR-193a-3p, miR-519e, miR-182, miR-22, miR-137, miR-663, for influencing proliferative properties of a cell involved in, but not limited to, the following diseases and conditions: Small-cell lung cancer (SCLC) and Non-small-cell lung cancer (NSCLC), Acute lymphoblastic leukemia (ALL), Chronic lymphocytic leukemia (CLL), Acute myelogenous leukemia (AML), Chronic myelogenous leukemia (CML), T-cell prolymphocytic leukemia (T-PLL), Large granular lymphocytic leukemia, Adult T-cell leukemia, and/or modulating immune functions of an organism with allergic asthma and/or chronic obstructive pulmonary disease.

Yet another object of the invention is a method, wherein proliferative properties of a cell and immune functions of an organism are altered, if the expression of a microRNA selected from

the group comprising microRNA miR-511, microRNA miR-193b and microRNA miR-99b, is modulated. A person skilled in the art will understand, that modulating the expression of a miRNA can be achieved, if synthetic oligonucleotides or gene expression vectors are introduced into the cell or the organism, thus, a detailed object of the invention is a method
5 for targeting proliferative properties of a cell and immune functions of an organism, wherein synthetic oligonucleotides or gene expression vectors are introduced into the cell or the organism, to modulate the functions and expression patterns of a microRNA selected from the group comprising microRNA miR-511, microRNA miR-193b and microRNA miR-99b.

More specifically, this invention first discloses human miR-511 and its direct targets
10 described by differential mRNA expression in DCs in the presence of miR-511 inhibitor. The proposed direct miR-511 targets are TMEM123, RAD21, TRIM33, CD44, NCOA4, SLC12A8, ITPR1, ANKRD28, CRYZL1, LRCH4 and BZRAP1 genes. Thus, a method, wherein the expression of the named genes TMEM123, RAD21, TRIM33, CD44, NCOA4, SLC12A8, ITPR1, ANKRD28, CRYZL1, LRCH4 and BZRAP1, is modulated via
15 modulation of microRNA miR-511, is another object of the invention. This part of the invention also indicates that miR-511 can influence cell proliferation properties and development of neoplasms, including malignant cancers of haematopoietic and epithelial origin in humans via targeting CD44.

Additionally, this invention discloses direct targets of human miR-193b. The proposed direct
20 miR-193b targets are CRKL, CCND1, BCL2L1, RUNX1, CRK, and KRAS genes. Thus, a method, wherein the expression of the named genes CRKL, CCND1, BCL2L1, RUNX1, CRK, and/or KRAS, is modulated via modulation of microRNA miR-193b, is yet another object of the invention.

Moreover, this invention discloses direct targets of human miR-99b. The proposed direct
25 miR-99b targets are AXIN1, BID, CBL, CDK6, DVL3, FZD1, HSP90B1, IGF1R, IKBKG, ITGB1 and STAT5B genes. Thus, a method, wherein the expression of the named genes AXIN1, BID, CBL, CDK6, DVL3, FZD1, HSP90B1, IGF1R, IKBKG, ITGB1 and/or STAT5B, is modulated via modulation of microRNA miR-99b, is yet another object of the invention.

30 A person skilled in the art can understand that using the above named methods will modulate immune response. The modulation can be achieved via activation or inhibition of T helper subsets like Th0, Th1, Th2, Th17 and Th22, Th9 or regulatory T cells, but not limited to these mechanisms.

Moreover, a person skilled in the art will understand that modulation of the immune responses can be related to inhibition of growth and/or invasion of cancers.

The next object of this invention is a method, wherein modulation of miR-511 causes the modulation of protein levels of pathogen recognition receptor TLR4 as well as the DC surface marker and T-cell co-stimulatory molecule CD80, which is located downstream in the toll-like receptor signalling pathway. Thus, this invention provides a method for influencing the strength and direction of adaptive immune responses via modulation of the expression of miR-511.

Further, this invention provides a method, wherein the expression of the following genes and cellular pathways is modulated via microRNA miR-511: Toll-like receptor signalling pathway (TLR4, STAT1, CD80, MAP3K7IP2, CD86, IRAK1, MAP3K7, TIRAP), myeloid cell differentiation (BCL6, IRF4, PPARG, JAK2, SMAD5, TIRAP), JAK-STAT cascade (NLK, STAT1, SOCS2, JAK2, STAT5A, SOCS6, STAT4), regulation of interleukin-2 production (IRF4, CD80, CD86, STAT5A, MAP3K7), cell adhesion (ALCAM, ERBB2IP, ENTPD1, CD36, CDH2, COL8A2, NRP1, OLR1, LPP, ITGA4, CCR1, CD93, ITGB1, RAPH1, ROCK1, CD44, CD9, CD84, VCL), vesicle-mediated transport (ZFYVE16, RAB22A, CD36, FNBP1L, MRC1, MRC1L1, ARFGEF1, RAB6A, PICALM, CD93, RAB2A, PRKCI, AP1S2, RIMS3), pathways in cancer (BID, STAT1, EP300, TGFBR1, HDAC2, PPARG, ITGB1, STAT5A, PTEN, CBL, IGF1R, VHL) and/or cell migration (BTG1, CDH2, TGFBR1, NRP1, ITGA4, ITGB1, IL16, SRF, ROCK1, PTEN, CD44, VHL). In addition, this invention specifically concerns two other miRNAs, miR-99b and miR-193b both highly expressed in DCs, and provides that miR-193b influences genes involved in development of chronic myeloid leukaemia (CRKL CCND1 BCL2L1 RUNX1 CRK KRAS), whereas miR-99b influences the development of various neoplasms or cancers via targeting the expression of AXIN1 BID CBL CDK6 DVL3 FZD1 HSP90B1 IGF1R IKBKG ITGB1 STAT5B genes.

In a preferred embodiment of the invention, the provided method for modulating the levels of a microRNA selected from the group comprising microRNA miR-511, microRNA miR-193b and microRNA miR-99b, is used in human monocyte derived dendritic cells for developing dendritic cell based immunotherapeutic approaches.

Next, this invention has revealed, that certain miRNAs are specifically and strongly up-regulated during the differentiation of DCs (miR-499, miR-99a, miR-193b, miR-212 , miR-210, miR-125b) or MFs (miR-139-5p, miR-642, miR-1, miR-218) or in both of these cell types (miR-511, miR-146a/b, miR-99b, miR-125a-5p) if to compare with MOs. Further the

invention provides that inhibition of miR-511 and miR-99b hinders the protein expression of DC-SIGN, which is DC specific cellular surface marker and is also involved in binding of pathogens and activation of DCs. As a part of this invention, it also provides that miR-518e, miR-147, miR-32, miR-570, miR-193a-3p and miR-519e are upregulated in DCs and MFs
5 upon stimulation of these cells either via TLR4 or DECTIN1 with lipopolysaccharide (LPS) or curdlan, respectively. A certain subset of miRNAs (miR-182, miR-22, miR-137, miR-99b, miR-193b, miR-212, miR-139-5p, miR-99a, miR-642, hmiR-663) is induced by these stimulanting agents only in MFs. Since DC and MF differentiation from the blood MOs occurs in response to cytokines initiated by the presence of pathogens or aberrantly in the case
10 of hematopoietic diseases, this part of invention provides that any of indicated miRNAs can be involved in regulation of inflammatory and tumorigenic processes and therefore any of these differentially regulated miRNAs can be used as a diagnostic and/or prognostic marker or gene therapy target molecule in inflammatory conditions and cancers. Thus, an object of the invention is a method for detection of neoplastic and/or inflammatory condition of an
15 organism, which comprises detection of an expression level in human blood derived CD14+ monocytes and/or in blood serum and/or in blood plasma of a microRNA selected from the group consisting of miR-511, miR-193b, miR-99b, miR-499, miR-99a, miR-212 , miR-210, miR-139-5p, miR-642, miR-1, miR-218, miR-518e, miR-147, miR-32, miR-570, miR-193a-3p, miR-519e, miR-182, miR-22, miR-137, miR-663. In a preferred embodiment of the
20 invention, neoplastic conditions of haematopoietic and epithelial origin are detected by the provided method.

And yet another object of the invention is a kit for detecting neoplastic and/or inflammatory condition of an organism, which comprises at least a means for quantitative detection of the expression level in human blood derived CD14+ monocytes and/or in blood serum and/or in
25 blood plasma of a microRNA selected from the group consisting of miR-511, miR-193b, miR-99b, miR-499, miR-99a, miR-212 , miR-210, miR-139-5p, miR-642, miR-1, miR-218, miR-518e, miR-147, miR-32, miR-570, miR-193a-3p, miR-519e, miR-182, miR-22, miR-137, miR-663.

Brief Description of the Drawings

30 Fig. 1. Characteristics of MO derived DCs and MFs. Flow cytometric analysis of MO derived DCs and MFs. Left panels show DCs and MFs characterized by the expression of CD14 and DC-SIGN on day 6 (6d), histograms represent the geometric mean fluorescent intensities of CD86, HLA-DR and CD83 shown as % of maximum in LPS stimulated DCs and MFs

(patterned) relative to respective values of unstimulated cells (black line). Data from one representative donor are shown.

Fig. 2. MOs, DCs and MFs have distinct miRNA expression profiles. Heatmaps of miRNAs that are up- or down-regulated (differential p-val<0.05) in DCs and/or MFs if to compare with the levels in MOs. The miRNAs, which are shown, have an average expression level of over 100 in at least one condition. Each column represents expression levels of miRNA (shown right) in each sample (shown above). miRNA expression levels are mean-centered across all the analyzed miRNA expression values and are presented as log2 values from -5.0 as the lowest to 3.0 as the highest as indicated.

Fig. 3. Verification of Illumina array results with quantitative RT-PCR. Data from one representative donor are shown and are normalized the to the value in MOs (= 1).

Fig. 4. Reduced expression of selected miRNAs results in downregulation of DC specific surface marker DC-SIGN. (A) Inhibition of individual miRNAs are shown as average miRNA expression level with SEM of three different treatment normalized to the average levels of control transfections (=1, neg). (B) FACS analyses of DCs treated with indicated miRNA inhibitors. Geometric mean fluorescence intensities were normalized to control transfected cells (=1, neg) and are presented as Relative fluorescence intensities (FI), data from 5 different donors are blotted, p-values are calculated using Mann-Whitney test.

Fig. 5. Expression of miR-511 correlates with the enhanced level of the TLR4 protein in differentiating DCs. (A) Schematic of the TLR4 mRNA transcript containing miR-511 target sites, alternative polyadenyation sites and positions of RT-PCR primers (designated with arrows). (B) Inhibition of miR-511 is shown as average miRNA expression level with SEM of two parallel treatment normalized to the levels of control transfection on day 2 (DC2, neg, =1). (C) Western analysis of TLR4 in differentiating DCs in the presence of miR-511 inhibitor (anti-511) or the control inhibitor (neg) are normalized to the GAPDH, the numbers indicate the fold difference compared to control transfected cells on day 2 (DC2, neg). (D) The mRNA expression levels of the TLR4 transcripts in differentiating DCs in the presence of miR-511 inhibitor (anti-511) or the control inhibitor (neg). Data are one representative out of three independent experiments. RT-PCR of specific genes are normalized to HPRT and to the expression levels in MOs (=1).

Fig. 6. Knock-down of miR-511 does not lead to major changes in expression of DC cell activation markers. (A) The levels of CD80, CD86 and CD83 were measured on day 6 (DC6)

of the DC differentiation and after that 24 later hours of LPS treatment (DC6+LPS). Average of geometric mean fluorescence intensities (MFI) with SEM of two parallel treatments is shown. (B) Inhibition of miR-511 is shown as average miRNA expression level with SEM of two parallel treatment normalized to the level of control transfection on differentiation time
5 point day 6 (DC6, neg, =1). (C) Western analyzis of DCs on day 6 (DC6) and after the LPS treatment, miR-511 inhibitor (anti-511) and the control inhibitor (neg) were used as indicated. Western blots are normalized to the GAPDH, the numbers indicate the fold difference compared to control transfected cells on day 6 (DC6, neg). (D) Analysis of TLR4 mRNA levels using indicated primers and conditions. Data are mean with SEM of two parallel
10 transfections.

Fig. 7. The influence of miR-511 to expression of its proposed targets in LUC reporter assays. The luciferase (LUC) activity was measured either 48 (A, normal conditions) or 96 (B, contact inhibited, designated with ci) hours after the transfection of the LUC reporters and pre-miR-511 or the control pre-miRNA. Data are mean with SEM of at least eight different
15 transfections. All data are normalized to Renilla and the LUC values of control transfections (=1). LUC activity measurements of the ARE reporter used as positive control were done 48 hours (norm, =1) and 96 hours (ci) after the transfections and are normalized to Renilla and the levels of pGL3-control. Three different target sequences of TLR4 are cloned in separate LUC constructs and are designated as TLR4 I, II and III. P-values are calculated using Mann-
20 Whitney test.

Description of embodiments

Differential expression of miRNAs in MOs, DCs and MFs

To find novel miRNAs important in DC differentiation and functions, the human peripheral blood CD14+ MOs were purified and differentiated into DCs and MFs and stimulated on day
25 6 with LPS as TLR4 ligand, or curdlan, as Dectin-1 (CLEC7A) ligand. More specifically, PBMCs were prepared by density gradient centrifugation on Ficoll-Paque™ PLUS (GE Healthcare) and freshly collected "buffy coats" obtained from Blood Centre of Tartu University Hospital and MOs were further purified by positive sorting using anti-CD14-conjugated magnetic microbeads using two runs through LS columns (both from Miltenyi
30 Biotech). MOs were differentiated into MFs using 50 ng/ml GM-CSF and into DCs using 50 ng/ml GM-CSF and 25 ng/ml IL-4 (both from PeproTech) by growing 6 days at concentration of 1 million cells/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (all from PAA). For maturation, 1 μg/ml of

Lipopolysaccharide (LPS, InvivoGen) or 0.1 mg/ml of curdlan (Wake Chemicals) was added to the growing media for 24 hours. This protocol is previously essentially described in Sallusto and Lanzavecchia. 1994. (Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med, 179(4): 1109-18). FACS analysis showed well-established phenotype of immature DCs (DC-SIGN^{+}CD14^{-}) and MFs (DC-SIGN^{-}CD14^{+}) on day 6. Following stimulations, DCs had significantly higher expression of CD86, HLA-DR (MHCII) and CD83. For activated MFs, enhancement of CD86 and CD83 was detected. For FACS analysis, fluorescence conjugated antibodies CD83, DC-SIGN, CD86 (BD Biosciences), CD14 and HLA-DR (Miltenyi Biotec) and FACSCalibur (BD Biosciences) were used. Data were analyzed and visualized with FlowJo v. 7.6. (Fig. 1). The study on human blood derived MOs is approved (Approval 166/T-10) by Ethics Review Committee on Human Research of the University of Tartu. All human participants gave written informed consent.

From the described cell subsets, total RNA was purified and updated miRNA expression profiles were determined (Fig. 2, Table 1). RNA was purified using Trizol (Invitrogen) and when needed, further purified with RNAeasy Mini Kit (Qiagen). To maintain small RNA fraction, 3.5 volume of 100% ethanol was added to the samples before loading on Qiagen mini columns. Alternatively, miRNAeasy Mini Kit (Qiagen) was used. The concentration and quality of RNA was assessed with NanoDrop ND-1000 spectrophotometer and Agilent RNA 6000 Nano Kit on Agilent 2100 Bioanalyzer.miRNA profiling was carried out on Illumina miRNA Universal-16 BeadChips (miRBase version 12.0). The data were analyzed with BeadStudio Gene Expression Module v3.3.7 (Illumina) using Average Normalization for miRNA data. and Illumina's custom rank invariant method for mRNA arrays. Genes with differential expression p-value <0.05 were considered differentially expressed. Further analyses and visualizations were carried out using Microsoft Excel and Multi Experiment Viewer 4.0. Unsupervised hierarchical clustering was done using Euclidean distance and average linkage analysis. The miRNA microarray data are available at ArrayExpress as E-TABM-968.

Table 1: Differential expression of miRNAs in MOs, DCs and MFs[a]

ILMN_GENE	MO	DC	DC LPS	DC curd	MF	MF LPS	MF curd
hsa-miR-193b	40,70	5 367,60	5 263,40	3 937,00	54,50	126,30	380,20
hsa-miR-551b	1,00	84,90	119,40	55,00	1,00	1,60	1,00
hsa-miR-548d-3p	192,50	64,00	70,00	31,30	1,00	55,50	52,50
hsa-miR-32	31,00	37,30	211,20	63,30	1,00	24,00	56,20
hsa-miR-616*	223,80	590,20	513,00	380,20	29,30	366,50	538,40
hsa-miR-194	558,70	268,20	236,00	268,70	15,80	392,00	676,10
hsa-miR-99a	152,90	2 723,20	2 486,70	2 257,10	184,70	466,80	680,20
hsa-miR-196a	231,30	62,40	40,10	22,40	7,20	31,30	10,40
hsa-miR-147	1,00	79,40	589,30	352,30	11,80	305,10	321,00
hsa-miR-582-5p	3 075,60	892,00	936,90	590,20	138,30	608,20	783,70
hsa-miR-125b	1 071,30	6 830,60	6 223,20	5 121,30	1 067,80	1 487,20	2 222,80
hsa-miR-182	8,00	97,90	140,10	94,30	18,70	54,60	79,60
hsa-miR-212	52,70	4 895,30	4 767,00	5 205,10	1 065,80	3 158,90	4 864,70
hsa-miR-143	822,00	200,30	303,80	193,20	46,10	172,80	233,50
hsa-miR-150	8 618,10	3 508,80	3 082,90	2 272,30	837,60	1 521,10	2 342,90
hsa-miR-570	83,00	135,60	718,40	470,00	34,00	346,20	354,70
hsa-miR-454*	3 370,60	1 635,40	1 436,80	1 332,40	502,40	608,90	562,50
hsa-miR-449a	49,40	565,80	773,90	710,60	196,80	270,40	290,60
hsa-miR-199b-5p	2 136,40	554,80	647,50	367,40	193,30	332,30	534,90
hsa-miR-20b	2 117,10	300,60	457,40	370,10	107,40	183,50	153,30
hsa-miR-210	269,30	2 815,90	3 501,00	2 426,20	1 066,40	1 070,50	2 310,40
hsa-miR-618	1 512,30	384,30	408,50	297,10	160,70	290,90	500,50
hsa-miR-554	17,50	164,70	152,10	120,40	69,90	127,10	167,70
hsa-miR-663	105,90	229,20	116,30	95,50	108,60	455,40	430,30
hsa-miR-378*	1 808,70	4 861,20	3 922,30	3 110,20	2 357,70	3 518,00	3 982,00
hsa-miR-22	3 251,30	6 211,00	6 746,10	6 116,60	3 120,10	6 353,80	6 538,90
hsa-miR-642	55,60	743,10	638,30	461,50	382,50	767,10	1 155,70
hsa-miR-137	34,40	133,50	135,10	152,20	85,90	202,80	409,10
hsa-miR-222	3 998,10	6 821,20	9 217,40	7 845,10	4 493,40	6 929,80	7 511,90
hsa-miR-99b	15,30	4 023,10	4 042,00	3 680,10	2 809,90	6 120,90	7 056,00
hsa-miR-365	2 365,90	4 285,00	5 055,30	4 700,00	3 032,90	5 733,30	5 552,10
hsa-miR-132	4 071,00	15 177,70	15 138,60	16 988,90	11 307,00	14 735,20	14 608,00
hsa-miR-511	222,10	13 372,70	12 476,50	12 493,10	10 133,10	11 919,30	12 679,20
hsa-miR-342-3p	6 306,50	14 896,10	15 397,30	14 405,20	12 793,00	11 654,50	12 276,30
hsa-miR-146b-5p	3 703,00	15 830,20	16 210,30	15 930,60	13 775,10	16 574,70	15 348,10
hsa-miR-135a	20,40	112,40	168,50	112,50	99,20	21,70	23,70
hsa-miR-29a	3 299,00	6 692,90	7 404,90	7 209,30	6 153,50	7 881,40	8 886,40
hsa-miR-193a-3p	313,90	220,60	451,50	219,20	218,30	802,10	696,70
hsa-miR-34a	65,20	3 424,50	3 154,20	2 487,20	3 551,40	3 650,70	3 452,80
hsa-let-7e	3 822,80	11 357,00	12 381,90	13 072,40	12 464,70	15 568,90	14 345,10
hsa-miR-125a-5p	578,70	9 156,00	9 304,40	9 994,50	10 676,00	12 310,60	11 906,20
hsa-miR-146a	7 858,00	15 571,40	18 463,20	19 151,40	20 947,30	21 589,30	20 816,40
hsa-miR-518e	19,40	40,10	359,20	254,70	56,80	192,90	230,50
hsa-miR-519e	30,60	96,80	189,10	155,30	144,70	474,90	559,60
hsa-miR-139-5p	123,50	462,80	440,40	339,70	870,90	2 054,40	2 713,70
hsa-miR-133a	68,50	111,70	98,90	103,00	373,50	631,60	655,60
hsa-miR-455-3p	31,10	36,40	38,70	51,10	135,60	105,40	127,50
hsa-miR-218	1,00	159,90	143,30	119,60	656,50	1 088,10	1 263,90
hsa-miR-1	1,00	84,20	135,40	80,40	617,40	1 127,80	682,70

ILMN_GENE	MO	DC	DC LPS	DC curd	MF	MF LPS	MF curd
hsa-miR-193b	40,70	5 367,60	5 263,40	3 937,00	54,50	126,30	380,20
hsa-miR-551b	1,00	84,90	119,40	55,00	1,00	1,60	1,00
hsa-miR-548d-3p	192,50	64,00	70,00	31,30	1,00	55,50	52,50
hsa-miR-32	31,00	37,30	211,20	63,30	1,00	24,00	56,20
hsa-miR-616*	223,80	590,20	513,00	380,20	29,30	366,50	538,40
hsa-miR-194	558,70	268,20	236,00	268,70	15,80	392,00	676,10
hsa-miR-99a	152,90	2 723,20	2 486,70	2 257,10	184,70	466,80	680,20
hsa-miR-196a	231,30	62,40	40,10	22,40	7,20	31,30	10,40
hsa-miR-147	1,00	79,40	589,30	352,30	11,80	305,10	321,00
hsa-miR-582-5p	3 075,60	892,00	936,90	590,20	138,30	608,20	783,70
hsa-miR-125b	1 071,30	6 830,60	6 223,20	5 121,30	1 067,80	1 487,20	2 222,80
hsa-miR-182	8,00	97,90	140,10	94,30	18,70	54,60	79,60
hsa-miR-212	52,70	4 895,30	4 767,00	5 205,10	1 065,80	3 158,90	4 864,70
hsa-miR-143	822,00	200,30	303,80	193,20	46,10	172,80	233,50
hsa-miR-150	8 618,10	3 508,80	3 082,90	2 272,30	837,60	1 521,10	2 342,90
hsa-miR-570	83,00	135,60	718,40	470,00	34,00	346,20	354,70
hsa-miR-454*	3 370,60	1 635,40	1 436,80	1 332,40	502,40	608,90	562,50
hsa-miR-449a	49,40	565,80	773,90	710,60	196,80	270,40	290,60
hsa-miR-199b-5p	2 136,40	554,80	647,50	367,40	193,30	332,30	534,90
hsa-miR-20b	2 117,10	300,60	457,40	370,10	107,40	183,50	153,30
hsa-miR-210	269,30	2 815,90	3 501,00	2 426,20	1 066,40	1 070,50	2 310,40
hsa-miR-618	1 512,30	384,30	408,50	297,10	160,70	290,90	500,50
hsa-miR-554	17,50	164,70	152,10	120,40	69,90	127,10	167,70
hsa-miR-663	105,90	229,20	116,30	95,50	108,60	455,40	430,30
hsa-miR-378*	1 808,70	4 861,20	3 922,30	3 110,20	2 357,70	3 518,00	3 982,00
hsa-miR-22	3 251,30	6 211,00	6 746,10	6 116,60	3 120,10	6 353,80	6 538,90
hsa-miR-642	55,60	743,10	638,30	461,50	382,50	767,10	1 155,70
hsa-miR-137	34,40	133,50	135,10	152,20	85,90	202,80	409,10
hsa-miR-222	3 998,10	6 821,20	9 217,40	7 845,10	4 493,40	6 929,80	7 511,90
hsa-miR-99b	15,30	4 023,10	4 042,00	3 680,10	2 809,90	6 120,90	7 056,00
hsa-miR-365	2 365,90	4 285,00	5 055,30	4 700,00	3 032,90	5 733,30	5 552,10
hsa-miR-132	4 071,00	15 177,70	15 138,60	16 988,90	11 307,00	14 735,20	14 608,00
hsa-miR-511	222,10	13 372,70	12 476,50	12 493,10	10 133,10	11 919,30	12 679,20
hsa-miR-342-3p	6 306,50	14 896,10	15 397,30	14 405,20	12 793,00	11 654,50	12 276,30
hsa-miR-146b-5p	3 703,00	15 830,20	16 210,30	15 930,60	13 775,10	16 574,70	15 348,10
hsa-miR-135a	20,40	112,40	168,50	112,50	99,20	21,70	23,70
hsa-miR-29a	3 299,00	6 692,90	7 404,90	7 209,30	6 153,50	7 881,40	8 886,40
hsa-miR-193a-3p	313,90	220,60	451,50	219,20	218,30	802,10	696,70
hsa-miR-34a	65,20	3 424,50	3 154,20	2 487,20	3 551,40	3 650,70	3 452,80
hsa-let-7e	3 822,80	11 357,00	12 381,90	13 072,40	12 464,70	15 568,90	14 345,10
hsa-miR-125a-5p	578,70	9 156,00	9 304,40	9 994,50	10 676,00	12 310,60	11 906,20
hsa-miR-146a	7 858,00	15 571,40	18 463,20	19 151,40	20 947,30	21 589,30	20 816,40
hsa-miR-518e	19,40	40,10	359,20	254,70	56,80	192,90	230,50
hsa-miR-519e	30,60	96,80	189,10	155,30	144,70	474,90	559,60
hsa-miR-139-5p	123,50	462,80	440,40	339,70	870,90	2 054,40	2 713,70
hsa-miR-133a	68,50	111,70	98,90	103,00	373,50	631,60	655,60
hsa-miR-455-3p	31,10	36,40	38,70	51,10	135,60	105,40	127,50
hsa-miR-218	1,00	159,90	143,30	119,60	656,50	1 088,10	1 263,90
hsa-miR-1	1,00	84,20	135,40	80,40	617,40	1 127,80	682,70

[a]Average expression signals of miRNAs with differential p-value<0.05 and which average expression level reached over 100 at least in one condition are shown.

Confirmation of differential expression of selected miRNAs in MOs, DCs and MFs

For 12 miRNAs, the expression differences were confirmed by qPCR (Fig. 3). miRNA expression was analyzed using Taqman MicroRNA Assays, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and 5X HOT FIREPol Probe RT-PCR Mix Plus (Solis Biodyne). All RT-PCRs were carried out on ABI Prism 7900, the relative gene expression levels were calculated using the comparative $\Delta\Delta$Ct) (method (Applied Biosystems).

Together the expression studies revealed that certain miRNAs are specifically and strongly up-regulated during the differentiation of DCs (miR-499, miR-99a, miR-193b, miR-212 , miR-210, miR-125b) or MFs (miR-139-5p, miR-642, miR-1, miR-218) or in both of these cell types (miR-511, miR-146a/b, miR-99b, miR-125a-5p). Upon stimulation of DCs and MFs either via TLR4 ligand lipopolysaccharide (LPS) or DECTIN1 ligand curdlan, miR-518e, miR-147, miR-32, miR-570, miR-193a-3p and miR-519e are upregulated. A certain subset of miRNAs (miR-182, miR-22, miR-137, miR-99b, miR-193b, miR-212, miR-139-5p, miR-99a, miR-642, miR-663) is induced by these stimulants only in MFs (Table 2).

Table 2. miRNAs up-regulated after stimulation with endotoxins[a]

Up-regulated after induction both in MFs and DCs				
	DC LPS/DC	DC curd/DC	MF LPS/MF	MF curd/MF
miR-518e	9.0	6.4	3.4	4.1
miR-147	7.4	4.4	25.9	27.2
miR-32	5.7	1.7	24.0	56.2
miR-570	5.3	3.5	10.2	10.4
miR-193a-3p	2.0	1.0	3.7	3.2
miR-519e	2.0	1.6	3.3	3.9
Up-regulated after induction only in MFs				
	DC LPS/DC	DC curd/DC	MF LPS/MF	MF curd/MF
miR-182	1.4	1.0	2.9	4.3
miR-22	1.1	1.0	2.0	2.1
miR-137	1.0	1.1	2.4	4.8
miR-99b	**1.0**	**0.9**	**2.2**	**2.5**
miR-193b	**1.0**	**0.7**	**2.3**	**7.0**
miR-212	**1.0**	**1.1**	**3.0**	**4.6**
miR-139-5p	**1.0**	**0.7**	**2.4**	**3.1**
miR-99a	**0.9**	**0.8**	**2.5**	**3.7**

miR-642	0.9	0.6	2.0	3.0
miR-663	0.5	0.4	4.2	4.0

[a]miRNA expression levels are shown as fold differences compared to the respective average values in DCs or MFs. For miRNAs designated with bold, induction with LPS and curdlan was also determined by RT-PCR

***ex vivo* differentiation assay of miRNAs in MOs, DCs and MFs**

5 To further study the functions of specific miRNAs, *ex vivo* DC differentiation was performed in the presence of sequence specific miRNA inhibitors for miR-511 and miR-99b, as the two most highly up-regulated miRNAs in both DCs and MFs, and for DC specific miR-193b. Pre-designed anti-miRNA inhibitors and respective negative controls (Applied Biosystems) and LNA based miR-511 inhibitor and unlabeled control A (Exiqon) were used. All transfections 10 were carried out at the concentration of 120 nM miRNA inhibitors using 3 µl of siPORT NeoFX Transfection agent for 106 cells/1 ml medium (Applied Biosystems). After the transfection procedure, MOs were differentiated as usual. Transfection efficiency was controlled by fluorescence microscopy of separate transfections by Cy3 labeled negative control miRNA inhibitor or negative control siRNAs and was estimated to be between 90-15 100%. The transfection of miRNA inhibitors resulted in 50-90% reduced expression of specific miRNAs if measured by RT-PCR (Fig. 4A), which led to statistically significantly reduced DC-SIGN protein levels when the inhibitors for miRNA-511 and miR-99b were used (Fig. 4B).

***in silico* selection of** miRNA-511, miR-193b and miR-99a **targets**

20 Next, prediction and computational analysis of possible targets in DCs was carried out. We first selected all TargetScan (http://www.targetscan.org/) predicted targets with total context score <-0.2 corresponding to the top one third of the full lists. To search for immunologically important targets, we only included 4274 human genes related to immune function according to Immport Database (https://www.immport.org). As another approach, we compiled lists of 25 potential targets based on 5 different algorithms. The compiled miRNA target lists were generated based on Diana microT (v3.0), miRanda (downloaded in September 2008), PicTar (downloaded in August 2009), rna22 (downloaded in August 2009), Targetscan conserved targets (5.1). The putative targets were ranked by their position in any of the input lists, the top 500 genes from this ranking were used in subsequent analysis.

Genes with very low expression, average signal intensity < 50 based on our Illumina mRNA expression data available at (E-TABM-976), were excluded. The remaining working lists, containing 135 - 247 targets per each studied miRNA, were analyzed using the g:GOSt tool at g:Profiler website (http://biit.cs.ut.ee/gprofiler/) that retrieves most significant Gene Ontology (GO) terms, KEGG and REACTOME pathways. This analysis revealed number of genes with known immune functions or potentially involved in regulation of pathways related to different cancers and are listed in Table 3.

Table 3. DC and MF specific miRNAs potentially target genes from functional pathways or groups important in the differentiation or immune functions

miRNA	Nr of targets	Functional group and nr of genes		ID	Putative targets in functional group	Significance[b]	Method[a]
miR-511	135	Toll-like receptor signaling pathway	107	KEGG:04620	TLR4 STAT1 CD80 MAP3K7IP2 CD86 IRAK1 MAP3K7 TIRAP	2.56e-05	T
		myeloid cell differentiation	93	GO:0030099	BCL6 IRF4 PPARG JAK2 SMAD5 TIRAP	4.11e-05	T
		JAK-STAT cascade	47	GO:0007259	NLK STAT1 SOCS2 JAK2 STAT5A SOCS6 STAT4	2.84e-08	T
		regulation of interleukin-2 production	28	GO:0032663	IRF4 CD80 CD86 STAT5A MAP3K7	1.16e-06	T
		cell adhesion	860	GO:0007155	ALCAM ERBB2IP ENTPD1 CD36 CDH2 COL8A2 NRP1 OLR1 LPP ITGA4 CCR1 CD93 ITGB1 RAPH1 ROCK1 CD44 CD9 CD84 VCL	5.61e-06	T
		vesicle-mediated transport	570	GO:0016192	ZFYVE16 RAB22A CD36 FNBP1L MRC1 MRC1L1 ARFGEF1 RAB6A PICALM CD93 RAB2A PRKCI AP1S2 RIMS3	3.39e-05	T

		Pathways in cancer	334	KEGG:05200	BID STAT1 EP300 TGFBR1 HDAC2 PPARG ITGB1 STAT5A PTEN CBL IGF1R VHL	3.36e-04	T
		cell migration	282	GO:0016477	BTG1 CDH2 TGFBR1 NRP1 ITGA4 ITGB1 IL16 SRF ROCK1 PTEN CD44 VHL	5.10e-07	T
		regulation of transcription from RNA polymerase II promoter	732	GO:0006357	HIPK2 TIAL1 EP300 RYBP BCL6 AHR YY1 HDAC2 IRF4 PPARG STAT5A SRF SMAD5 IRF2 VHL LITAF	3.73e-05	T
	193	negative regulation of gene expression	497	GO:0010629	ATBF1 CBX1 DEDD DEDD2 GABPA NAB2 ORC2L POU2F1 RUNX2 RYBP SATB2 SIN3A ZNF281 TARBP2 TGIF1 TNRC6B	2.29e-05	C
miR-193b	234/ 247	Chemokine signaling pathway	189	KEGG:04062	CRK CRKL KRAS SOS2	1.56e-06	T
		Chronic myeloid leukemia	76	KEGG:05220	CRKL CCND1 BCL2L1 RUNX1 CRK KRAS	5.23e-04	C
miR-99b	225	beta-catenin binding	27	GO:0008013	NUMB DVL3 SMAD7 C5ORF22 AXIN1	8.49e-06	C
		Pathways in cancer	334	KEGG:05200	AXIN1 BID CBL CDK6 DVL3 FZD1 HSP90B1 IGF1R IKBKG ITGB1 STAT5B	7.03e-04	C

[a] The target lists used for analysis were generated either based on top third of Targetscan (T) or by compiling five different algorithms, Targetscan 5.1 , Miranda, DIANA-microT, Pictar and rna22, predictions (C).

[b]The p-value form Fisher exact test showing the significance of the overlap between the target list and indicated functional category.

Validation of *miRNA-511* ***targets***

Further studies were concentrated on miR-511 targets, which were validated by three different methods. First, to analyze miR-511 influence on mRNA levels in DCs, we have carried out Illumina expression analyzes on mRNA from DCs grown either in the presence or absence of miR-511 inhibitors. Total RNA was purified and mRNA profiling by Illumina Human-6 v2 BeadChips in Core Facility at Department of Biotechnology, University of Tartu was carried out. The data were analyzed with BeadStudio Gene Expression Module v3.3.7 (Illumina) Illumina's custom rank invariant method. From this analysis, we have detected 48 genes with p-value <0.05 either up-regulated (Differential Score>13.0, equal with p-value<0.05) or down-regulated (Differential Score<-13.0, equal with p-value<0.05) in the presence of miR-511 inhibitors (Table 4). Of these genes, 8 down-regulated and 3 up-regulated genes contain miR-511 target sites (Table 5), which indicates that at least these 8 differentially regulated genes are direct targets of miR-511. Moreover, since more genes are downregulated (8) than upregulated (3) in mRNA level after knock-down of miR-511, this result suggests that in the differentiating DCs, miR-511 is rather positive than negative regulator of the expression of its direct targets.

Table 4. miR-511 targets genes detected from mRNA array analysis of DCs transfected with miR-511 inhibitors

ILMN_GENE	contr. AVG Signal	contr. ARRAY STDEV*	anti-511 AVG Signal	anti-511 ARRAY STDEV	anti-511 Diff Score
SLC25A34	67.3	4.641	173.9	8.094	63.643
BZRAP1	45.8	8.36	157.3	27.368	45.727
MYBPC3	510.2	79.68	1039.3	130.466	35.998
CETP	41.7	3.922	105.2	7.644	29.084
TPCN2	565.5	4.034	885.4	27.75	27.461
LOC440900	66.9	4.401	154.8	27.898	21.212
LRCH4	168.7	12.345	278.7	14.624	21.212
TAGLN	795.7	47.074	1200	49.638	20.184
ULK3	35.6	3.527	88.4	10.541	19.891
GPBAR1	92.3	1.108	164.8	12.576	19.808
NPM3	359.5	15.284	538.4	29.758	17.798
BCL7C	420.5	0.592	626.1	21.127	17.798
ARPC4	1895.8	79.784	2740.3	296.715	17.798
STUB1	749.2	1.817	1086.4	73.7	16.722
CCNB2	101.4	4.913	171.3	12.401	15.924
MICAL1	1021.9	11.758	1466.5	42.015	15.924
STAG3L2	426.3	22.76	621.2	25.155	15.284
SNRP70	128.2	4.641	205.4	22.929	15.07
FLJ43093	43.1	5.217	92.1	5.027	14.78
ABHD12	1189.3	23.628	1691.5	192.063	14.78
MYO5C	13.6	3.653	59.5	13.229	13.827
IL11RA	221	15.83	375.5	60.142	13.827
HS.542993	464.3	13.065	661.9	69.541	13.231
CCDC17	50.9	1.534	103.1	16.707	13.006
CRYZL1	151.6	11.84	248.5	36.397	13.006
HS.580797	253	26.639	390.1	36.257	13.006
SGSH	1387.9	26.534	2026.9	258.67	13.006
MRPL12	581.7	14.171	821.4	75.736	13.006
ANKRD28	69.2	4.595	29.4	3.093	-13.487
ZNF143	271.2	2.827	190.1	11.942	-13.621
AP3B1	2179.3	83.084	1638.1	38.824	-13.827
ITPR1	369.2	29.002	251.2	19.987	-13.827
SLC12A8	263.7	44.74	116.1	23.35	-14.305
HIST1H2BK	156.8	9.949	99.2	12.227	-15.07
NCOA4	7132.8	297.604	5080.1	721.428	-15.182
CD44	1418.8	0.517	1053.2	54.129	-15.284
PDCD10	881.4	10.588	648	66.116	-15.484
PDGFC	493.2	4.654	353.1	32.281	-16.796
SEMA3C	337.3	21.9	230.8	21.961	-16.897
CA2	1225.1	36.01	847.5	125.016	-17.798
TRIM33	610	20.087	436.6	21.659	-18.159
RAD21	569.7	6.826	406.8	40.505	-18.159
FLJ20160	1090.6	15.79	786.4	22.964	-19.891
EVI2A	568.3	32.464	396.7	46.427	-20.057
TMEM123	2446.7	148.145	1674.7	98.833	-27.461
LOC390466	230.3	11.062	140.1	6.649	-33.391
SPINK1	801.4	2.06	468.4	90.583	-35.131
SLC39A12	137	0.756	20	1.273	-224.919

*DCs were transfected either with LNA based control inhibitors or miR-511 inhibitors (Exiqon). The efficiency of miR-511 knock-down was measured as on Fig. 2B. Average signals and SDEVs of 2 control samples (contr AVG Signal, contr ARRAY SDEV) and 3 knock-down samples (anti-511 AVG Signal, anti-511 ARRAY SDEV) are shown.

Differential Score is shown to control transfection (anti-511 Diff.Score). Data were analyzed with BeadStudio Gene Expression Module v3.3.7 (Illumina) using Illumina's custom rank invariant normalization method.

Table 5. Proposed miR-511 direct target genes detected from mRNA array analysis of DCs transfected with miR-511 inhibitors

ILMN_GENE	contr AVG Signal	contr ARRAY STDEV*	anti-511 AVG Signal	anti-511 ARRAY STDEV	anti-511 Diff. Score	Nr of miR-511 Target sites**
TMEM123	2446.7	148.145	1674.7	98.833	-27.461	2
RAD21	569.7	6.826	406.8	40.505	-18.159	1
TRIM33	610	20.087	436.6	21.659	-18.159	1
CD44	1418.8	0.517	1053.2	54.129	-15.284	1
NCOA4	7132.8	297.604	5080.1	721.428	-15.182	1
SLC12A8	263.7	44.74	116.1	23.35	-14.305	1
ITPR1	369.2	29.002	251.2	19.987	-13.827	1
ANKRD28	69.2	4.595	29.4	3.093	-13.487	1
CRYZL1	151.6	11.84	248.5	36.397	13.006	1
LRCH4	168.7	12.345	278.7	14.624	21.212	1
BZRAP1	45.8	8.36	157.3	27.368	45.727	1

*DCs were transfected either with LNA based control inhibitors or miR-511 inhibitors (Exiqon). The efficiency of miR-511 knock-down was measured as on Fig. 5B. Average signals and SDEVs of 2 control samples (contr AVG Signal, contr ARRAY SDEV) and 3 knock-down samples (anti-511 AVG Signal, anti-511 ARRAY SDEV) are shown. Differential Score is shown to control transfection (anti-511 Diff.Score). Data were analyzed with BeadStudio Gene Expression Module v3.3.7 (Illumina) using Illumina's custom rank invariant normalization method.

**Number of miR-target sites predicted by TargetScanHuman 5.1 (http://www.targetscan.org)

<u>Analysis of TLR4 and CD80 as miR-511 targets in DCs</u>

Next, two putative target genes TLR4 and CD80 were validated on protein level using inhibition of miR-511 in differentiating DCs. From the experiment, where DCs were differentiated in the presence of miR-511 inhibitor and the control inhibitor revealed that levels of the TLR4 protein were about 2 fold higher in control-transfected cells in comparison with miR-511 inhibited samples on day 4 and day 6 of the differentiation when more miR-511 had accumulated (Fig. 5B and 5C). No significant difference was detected in TLR4 different transcript levels in miR-511 knock-down samples in comparison to the control transfections

(Fig. 5D). The schematic of TLR4 different transcripts and miR-511 sites are presented in Fig. 5A. Although knock-down of miR-511 resulted in lower TLR4 protein level also in activated DCs, it did not lead to major changes in main DC activation markers CD86 and CD80 (Fig. 6). For Western blots, rabbit polyclonal anti human TLR4, (sc-10741, Santa Cruz Biotec) in 2% milk, mouse monoclonal anti human GAPDH (ab8245, Abcam) in 5% milk were used. Signals were detected with the ECL Advance Western Blotting Detection Kit (GE Healthcare) and captured and quantified by ImageQuant TM-RT ECL image analysis system. PCR was carried out with Maxima SYBR green/Rox Master Mix (Fermentas). miRNA expression was analyzed using Taqman MicroRNA Assays, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and 5X HOT FIREPol Probe RT-PCR Mix Plus (Solis Biodyne). All RT-PCRs were carried out on ABI Prism 7900, the relative gene expression levels were calculated using the comparative ΔΔCt) (method (Applied Biosystems). The RT-PCR primers were the following:

TLR4-1 Forward: ATCCCCTGAGGCATTTAGGC,
Reverse: TCAATTGTCTGG ATTTCACACCTG;
TLR4-2 Forward: TCCCTCCCCTGTACCCTTCT,
Reverse: AGCATTGCCCA ACAGGAAAC;
TLR4-3 Forward: ATCCCTGGGTGTGTTTCCAT,
Reverse: TGCGGACACACACACTTTCA.

Data were normalized to house-keeping gene HPRT expression. Primers for HPRT were
Forward: GACTTTGCTTTCCTTGG TCAGG,
Reverse: AGTCTGGCTTATATCCAACACTTCG

These data together show that there is correlation between high expression of miR-511 and enhanced level of the TLR4 protein, which suggests that miR-511 positively influences the TLR4 protein level in MO derived DCs.

Luciferase reporter gene assays.

We also analyzed whether TLR4 and CD80 are targeted by miR-511 in luciferase (LUC) reporter assays. Since TLR4 contains polyadenylation signals between each potential miR-511 target sequence (Fig. 5A), three different fragments of TLR4 3'UTR were inserted downstream the LUC coding region into the pGL3-contr vector. In addition, we cloned the 3'UTR fragment of CD80 containing two putative miR-511 target sites. For pGL3-3'UTR reporters, the following 3'UTR fragments of TLR4 (NM_138554.3): TLR4-I (3096-3736),

TLR4-II (4588-5000), TLR4-III (5028-5384) and CD80 (NM_005191.3.) (2184-2525) were PCR amplified, digested with *Fse*I (New England Biolabs) and *Xba*I (Fermentas) and inserted downstream the LUC coding region into the same restriction sites of pGL3-Control (Promega). The cloned plasmids were verified by sequencing, ARE plasmid was a kind gift of S. Vasudevan and is published before (Vasudevan, et al. 2007. Switching from repression to activation: microRNAs can up-regulate translation. Science N.Y 318:1931-1934)._ The cloning primers:

TLR4-I: Forward: ATATCTAGAAAAGACAGAGAAAACAGAAAGAGACA,
Reverse: ATAGGCCGGCCTTCCTTCCTGCCTCTAG CCC;
TLR4-II: Forward: ATATCTAGACCCGGAGGCCATTATGCTAT,
Reverse: ATAGGCCGGCC CAATTTGATGAGTTTAGACATAGTCAC;
TLR4-III: CAAACAGCC; CD80
Forward: ATATCTAG ACCATAGGGCCTCCTTAGATCCC,
Reverse: ATAGGCCGGCCGCAAGGTTTGTGAAGCAGCA.

Next, the LUC-3'UTR reporters were transfected either alongside with pre-miR-511 or the control pre-miRNA into the HEK293 cells. The transfection protocol was the following: 8×10^4 HEK293 cells were plated into 24-well plates and transfected after 24 h with 20 ng of renilla encoding pRL-TK (Promega), 100 ng pGL3-3'UTR reporters and either with 50 nM pre-miR-511 precursor or the FAM labeled pre-miR-control (Applied Biosystems) using siPORT NeoFX Transfection Agent (Applied Biosystems).

It has been shown that in cell cycle arrested conditions miRNAs can up-regulate the translation (Vasudevan, et al. 2007. Switching from repression to activation: microRNAs can up-regulate translation. Science N.Y 318:1931-1934). Since human blood MOs do not proliferate during the differentiation, and since we observed positive correlation of miR-511 expression and the TLR4 protein level in differentiating DCs, we carried out LUC assays both 48 and 96 hours after the transfection, thus in normal condition and in contact inhibited cells when cells are arrested in G0 phase. Fig. 7A shows that in normal growth conditions, the expression of the LUC constructs with 3'UTR fragments containing first (TLR4 I) and second predicted TLR4 target site of miR-511 (TLR4 II) and 3'UTR fragment of CD80 containing two miR-511 target sites were down-regulated in the presence of miR-511 if to compare with the levels of control transfected cells. No significant influence on the control vector or constructs containing TLR4 III site was detected (Fig. 7A). Interestingly, in contact inhibited cells, in the presence of transfected miR-511, the LUC expression levels were not reduced any

more, whereas for the construct containing TLR4 III site, the LUC expression was even significantly enhanced in comparison with the control transfected cells. The LUC activity of the construct containing 3'UTR of CD80 was not reduced in the presence of miR-511 in contact inhibited cells. The expression of the ARE plasmid used as a positive control for cell 5 cycle arrest was enhanced in contact inhibited cells if to compare with the control plasmid and the normal conditions (Fig. 7B). The LUC activity measurements together suggest that CD80 and TLR4 are indeed direct targets of miR-511. In addition, these data indicate that miR-511, depending on the target mRNA 3'UTR and the conditions, can either up- or down-regulate the target gene expression.

10 Most interestingly, it seems that under the cell cycle arrest conditions in non-proliferating DCs and contact inhibited HEK293 cells, miR-511 functions as positive regulator of the TLR4 protein and various other mRNAs, including CD44.

Claims

1. A method for targeting proliferative properties of a cell and/or immune functions of an organism, wherein the expression of one or more microRNAs selected from the group comprising microRNA miR-511, miR-193b, miR-99b, miR-499, miR-99a, mir-139b, miR-212, miR-210, miR-139-5p, miR-642, miR-1, miR-218, miR-518e, miR-147, miR-32, miR-570, miR-193a-3p, miR-519e, miR-182, miR-22, miR-137, miR-663, is modulated for influencing proliferative properties of a cell and/or immune functions of an organism.
2. A method of claim 1 for for influencing proliferative properties of a cell involved in Small-cell lung cancer (SCLC) and Non-small-cell lung cancer (NSCLC), Acute lymphoblastic leukemia (ALL), Chronic lymphocytic leukemia (CLL), Acute myelogenous leukemia (AML), Chronic myelogenous leukemia (CML), T-cell prolymphocytic leukemia (T-PLL), Large granular lymphocytic leukemia, Adult T-cell leukemia, and/or modulating immune functions of an organism with allergic asthma and/or chronic obstructive pulmonary disease.
3. A method for targeting proliferative properties of a cell and/or immune functions of an organism, wherein synthetic oligonucleotides or gene expression vectors are introduced into the cell or the organism, to modulate the functions and expression patterns of one or more microRNAs of claim 1.
4. A method of claims 1 to 3, wherein the microRNAs are selected from the group comprising microRNA miR-511, microRNA miR-193b and microRNA miR-99b.
5. A method of claims 1 to 3, wherein the microRNA is miR-511, and subsequently the expression of TMEM123, RAD21, TRIM33, CD44, NCOA4, SLC12A8, ITPR1, ANKRD28, CRYZL1, LRCH4 and/or BZRAP1 is modulated.
6. A method of claims 1 to 3, wherein the microRNA is miR-193b, and subsequently the expression of CRKL, CCND1, BCL2L1, RUNX1, CRK, and/or KRAS, is modulated.
7. A method of claims 1 to 3, wherein the microRNA is miR-99b, and subsequently the expression of AXIN1, BID, CBL, CDK6, DVL3, FZD1, HSP90B1, IGF1R, IKBKG, ITGB1 and/or STAT5B, is modulated.
8. A method of claims 1 to 3, wherein the expression of CD44 in a neoplastic cell is modulated.
9. A method of claims 1 to 5, wherein the expression of CD44 is targeted via microRNA miR-511 in malignant cancers of haematopoietic and epithelial origin in humans.
10. A method of claims 1 to 6, wherein immune response is modulated.

11. A method of claim 10, wherein the immune responses can be selected, but not limited to, from the following mechanisms: activation or inhibition of T helper subsets like Th0, Th1, Th2, Th17 and Th22, Th9 or regulatory T cells.
12. A method of claim 10 to 11, wherein the immune responses are related to inhibition of growth and/or invasion of cancers.
13. A method of claim 6, wherein targeting of miR-193b is performed in the condition of chronic myeloid leukaemia.
14. A method of claims 1 to 3, wherein the expression of the following genes and cellular pathways is modulated via microRNA miR-511: Toll-like receptor signalling pathway (TLR4, STAT1, CD80, MAP3K7IP2, CD86, IRAK1, MAP3K7, TIRAP), myeloid cell differentiation (BCL6, IRF4, PPARG, JAK2, SMAD5, TIRAP), JAK-STAT cascade (NLK, STAT1, SOCS2, JAK2, STAT5A, SOCS6, STAT4), regulation of interleukin-2 production (IRF4, CD80, CD86, STAT5A, MAP3K7), cell adhesion (ALCAM, ERBB2IP, ENTPD1, CD36, CDH2, COL8A2, NRP1, OLR1, LPP, ITGA4, CCR1, CD93, ITGB1, RAPH1, ROCK1, CD44, CD9, CD84, VCL), vesicle-mediated transport (ZFYVE16, RAB22A, CD36, FNBP1L, MRC1, MRC1L1, ARFGEF1, RAB6A, PICALM, CD93, RAB2A, PRKCI, AP1S2, RIMS3), pathways in cancer (BID, STAT1, EP300, TGFBR1, HDAC2, PPARG, ITGB1, STAT5A, PTEN, CBL, IGF1R, VHL) and cell migration (BTG1, CDH2, TGFBR1, NRP1, ITGA4, ITGB1, IL16, SRF, ROCK1, PTEN, CD44, VHL).
15. A method of claim 1 to 9 for modulating the levels of a microRNA selected from the group comprising microRNA miR-511, microRNA miR-193b and microRNA miR-99b in human monocyte derived dendritic cells for developing dendritic cell based immunotherapeutic approaches.
16. A method for detection of neoplastic and/or inflammatory condition of an organism, which comprises detection of an expression level in human blood derived CD14+ monocytes and/or in blood serum and/or in blood plasma of a microRNA of claim 1.
17. A method of claim 16, wherein neoplastic conditions of haematopoietic and epithelial origin are detected.
18. A kit for detecting neoplastic and/or inflammatory condition of an organism, which comprises at least a means for quantitative detection of the expression level in human blood derived CD14+ monocytes and/or in blood serum and/or in blood plasma of a microRNA of claim 1.

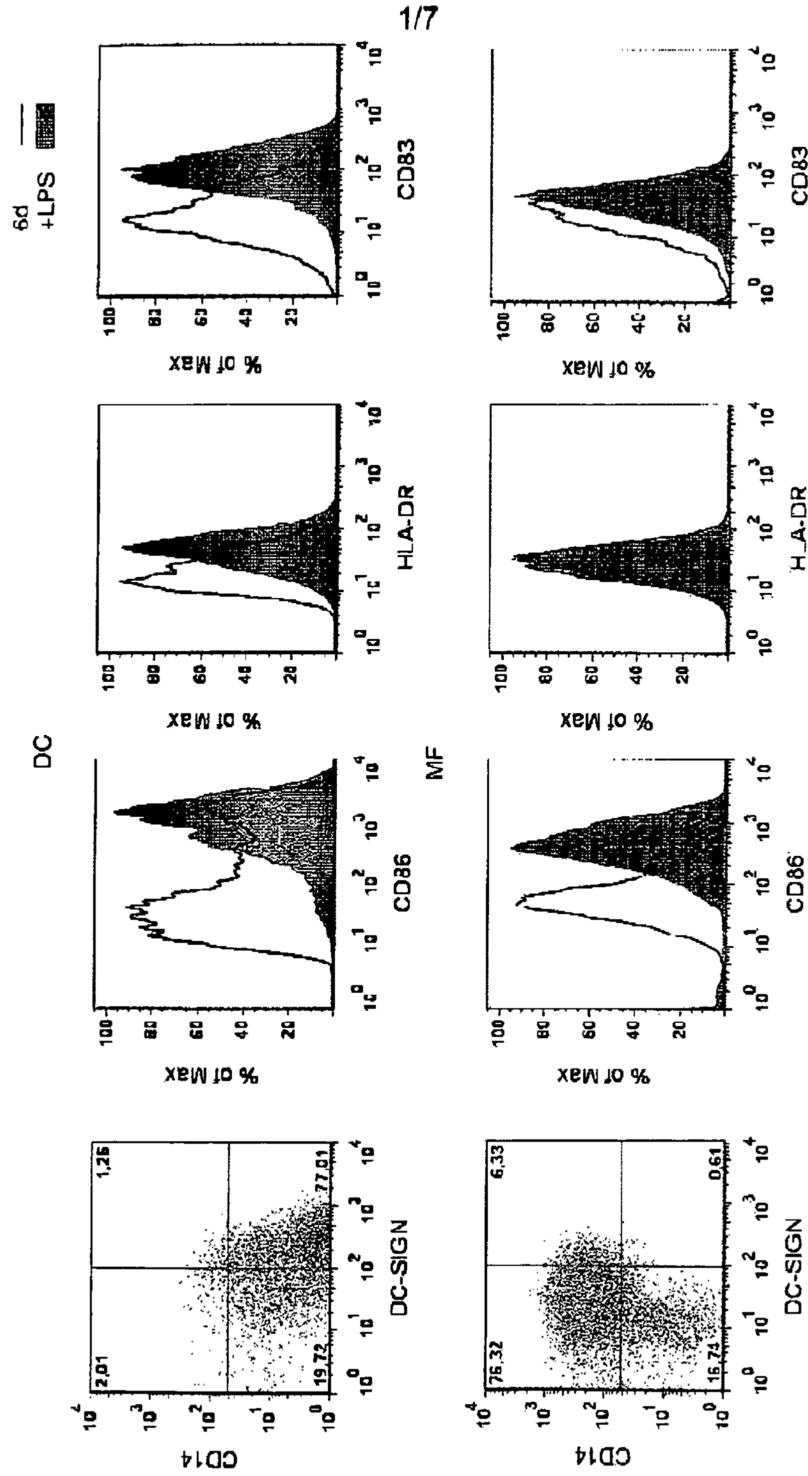

Fig. 1

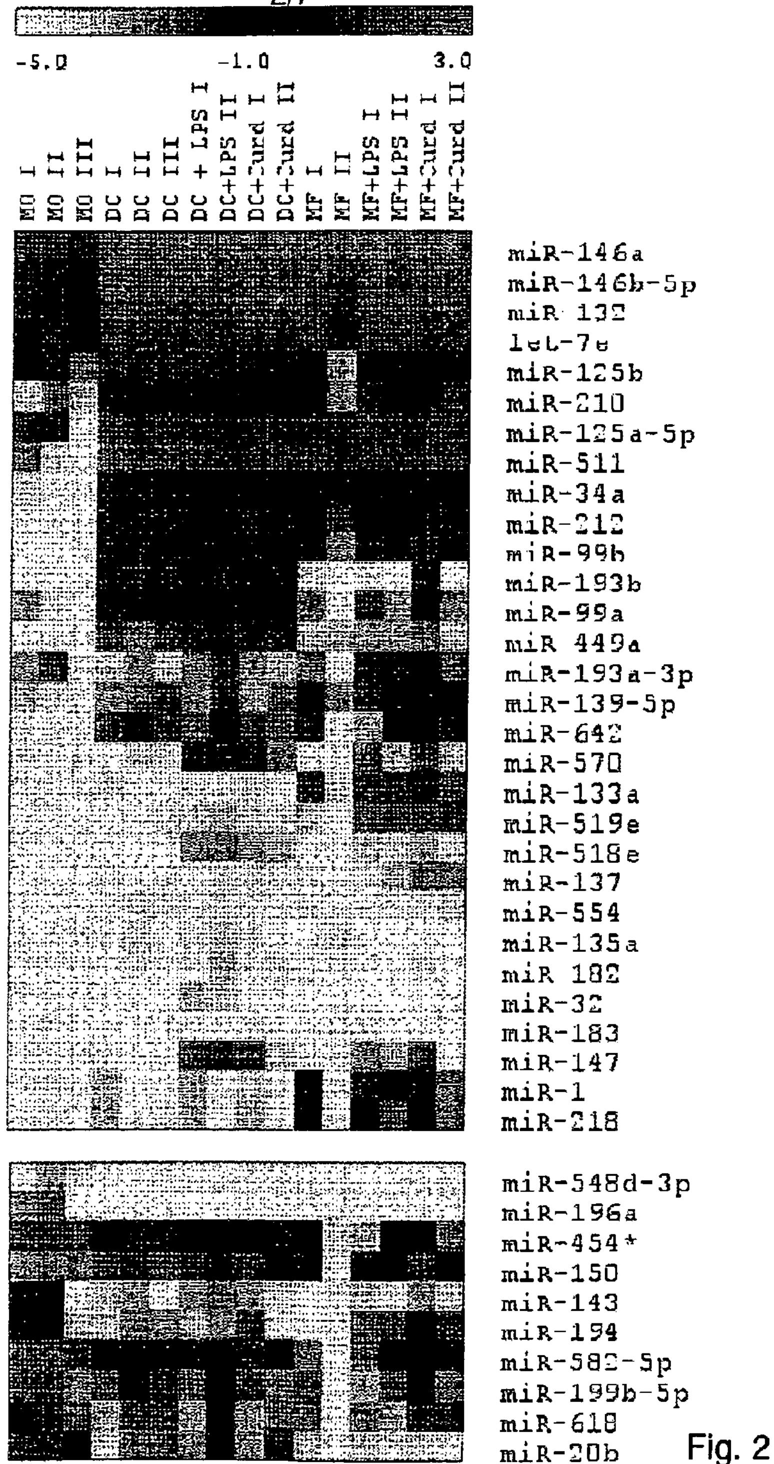

Fig. 2

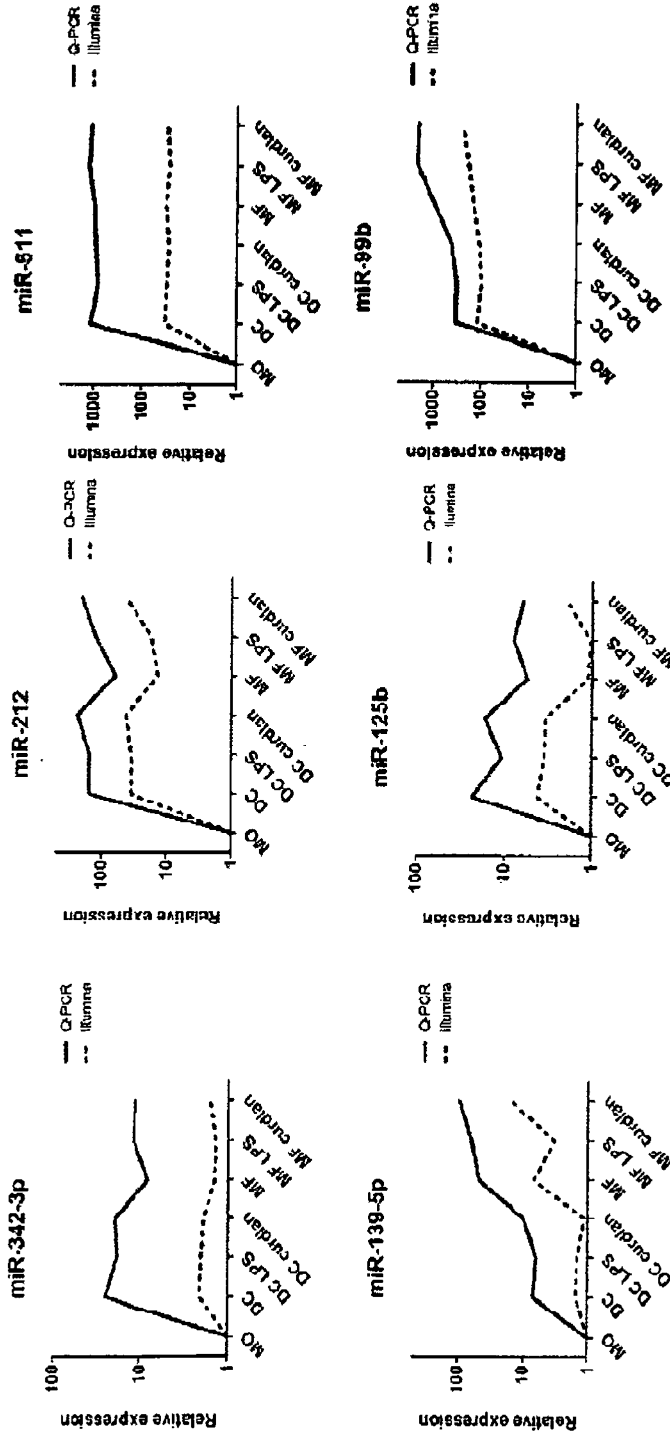

Fig. 3-1

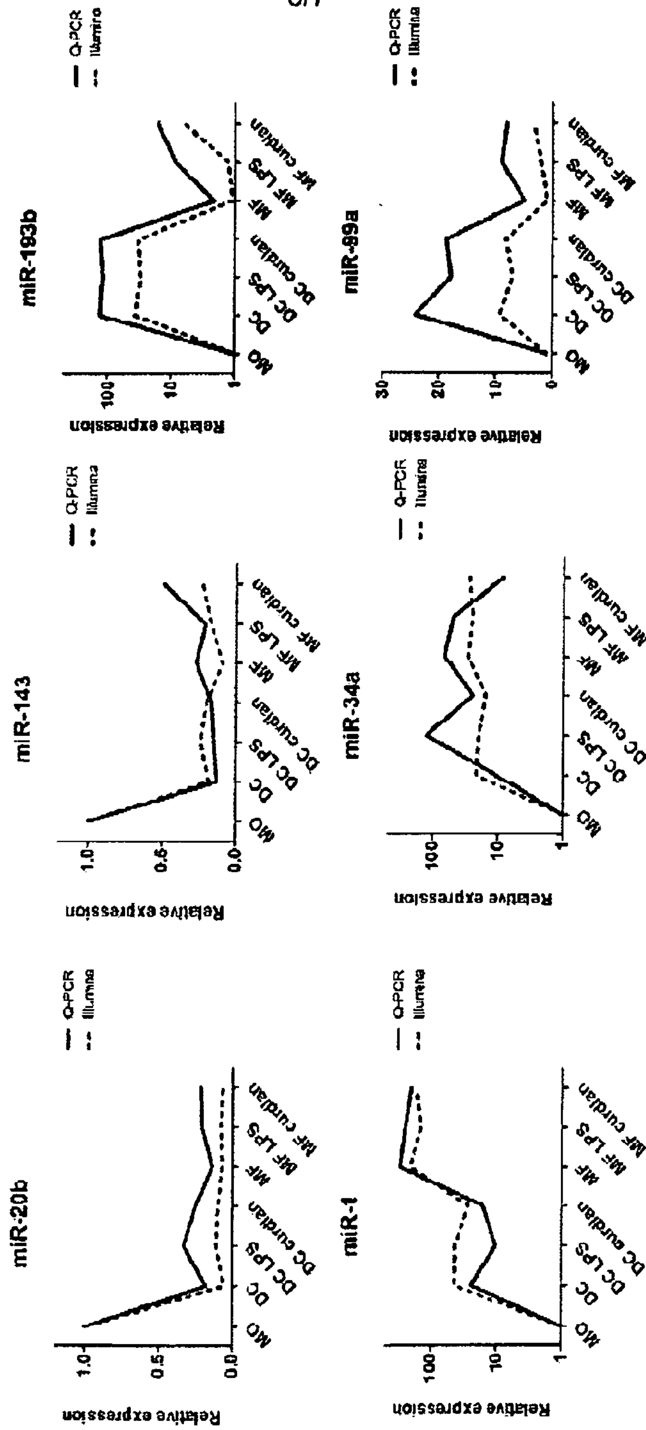

Fig. 3-2

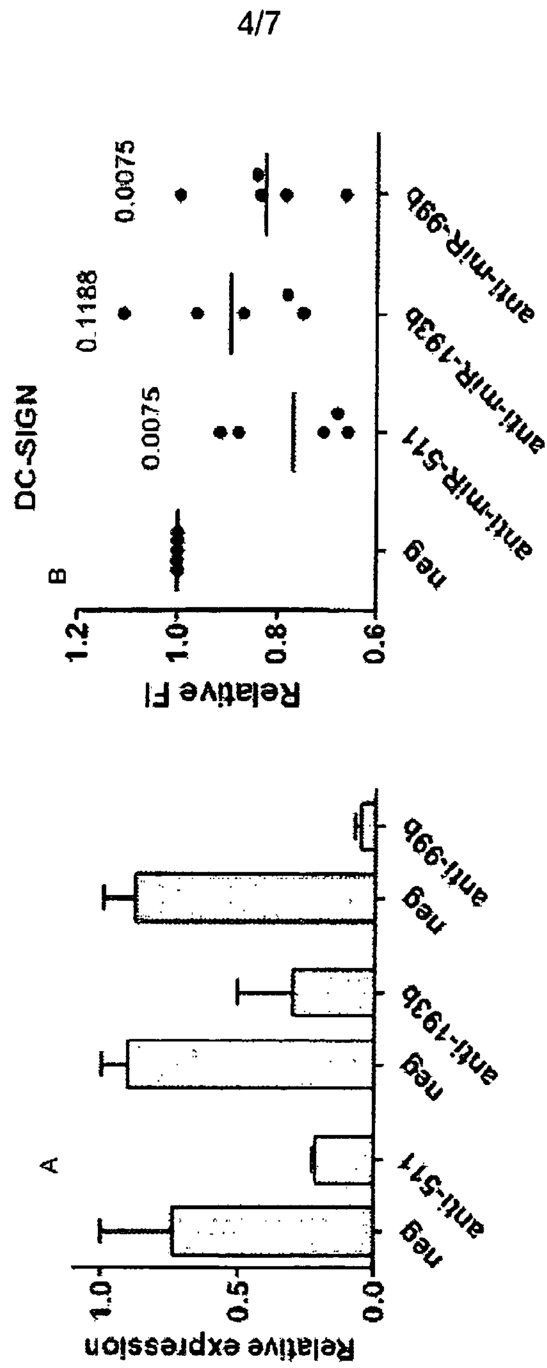

Fig. 4

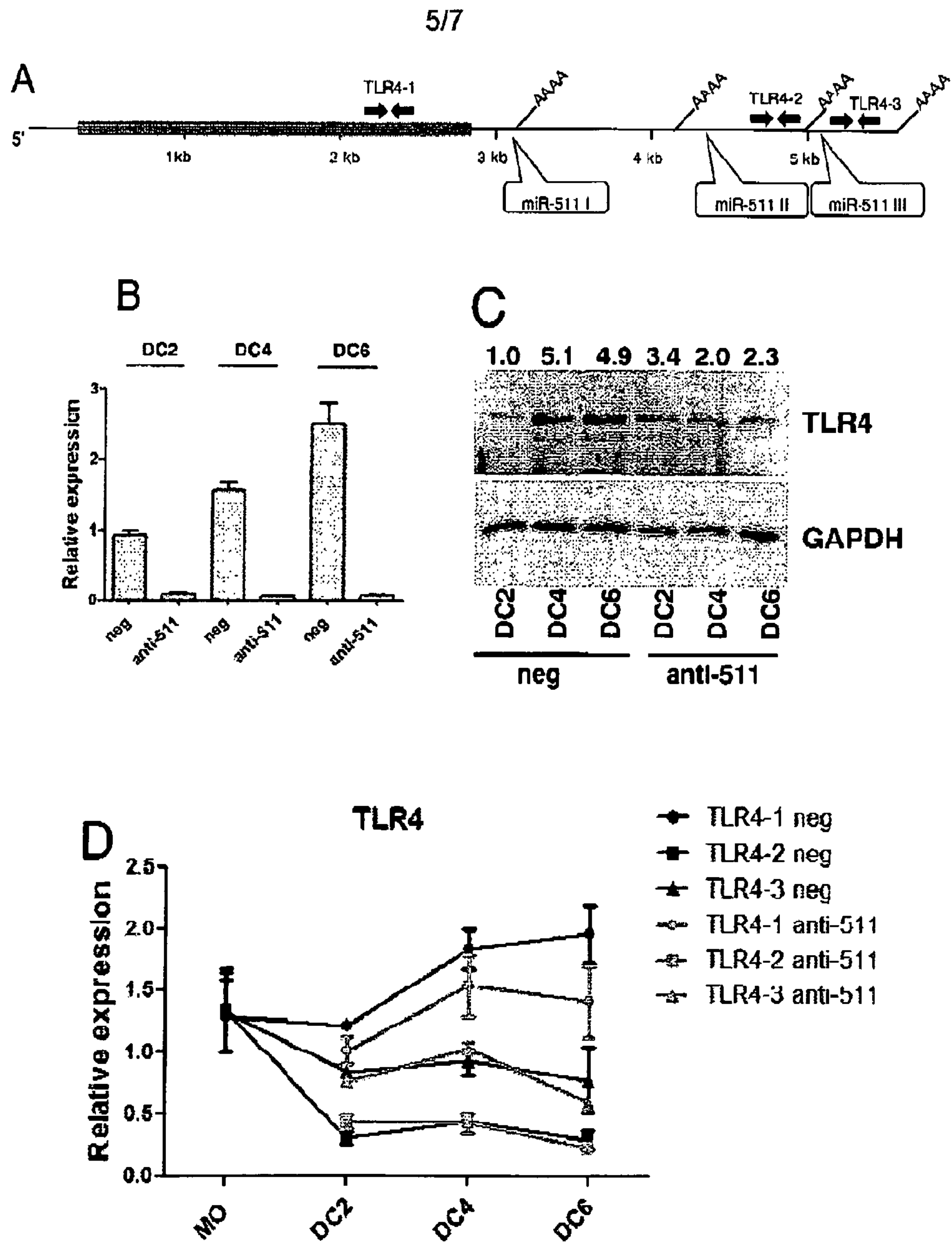

Fig. 5

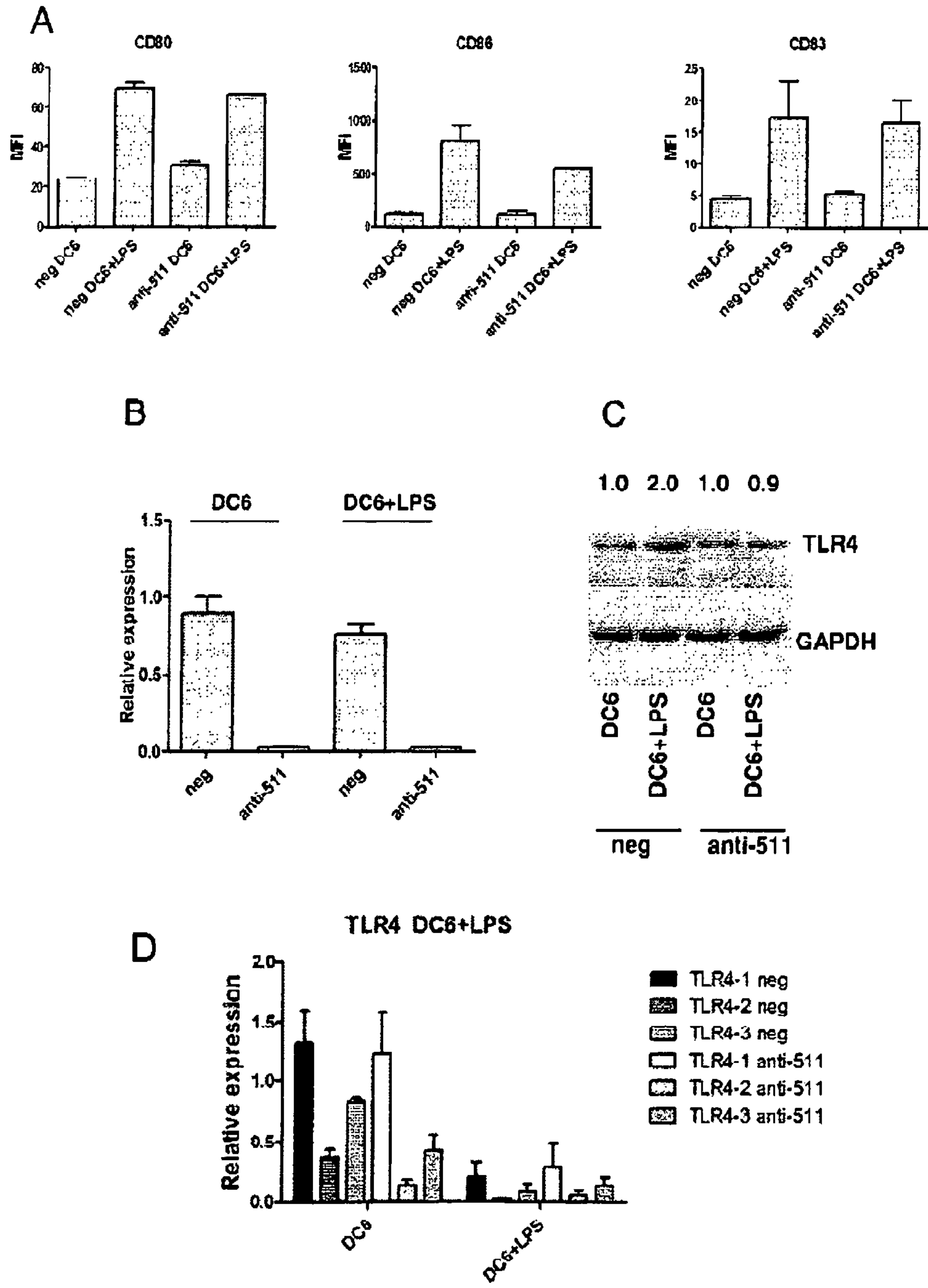

Fig. 6

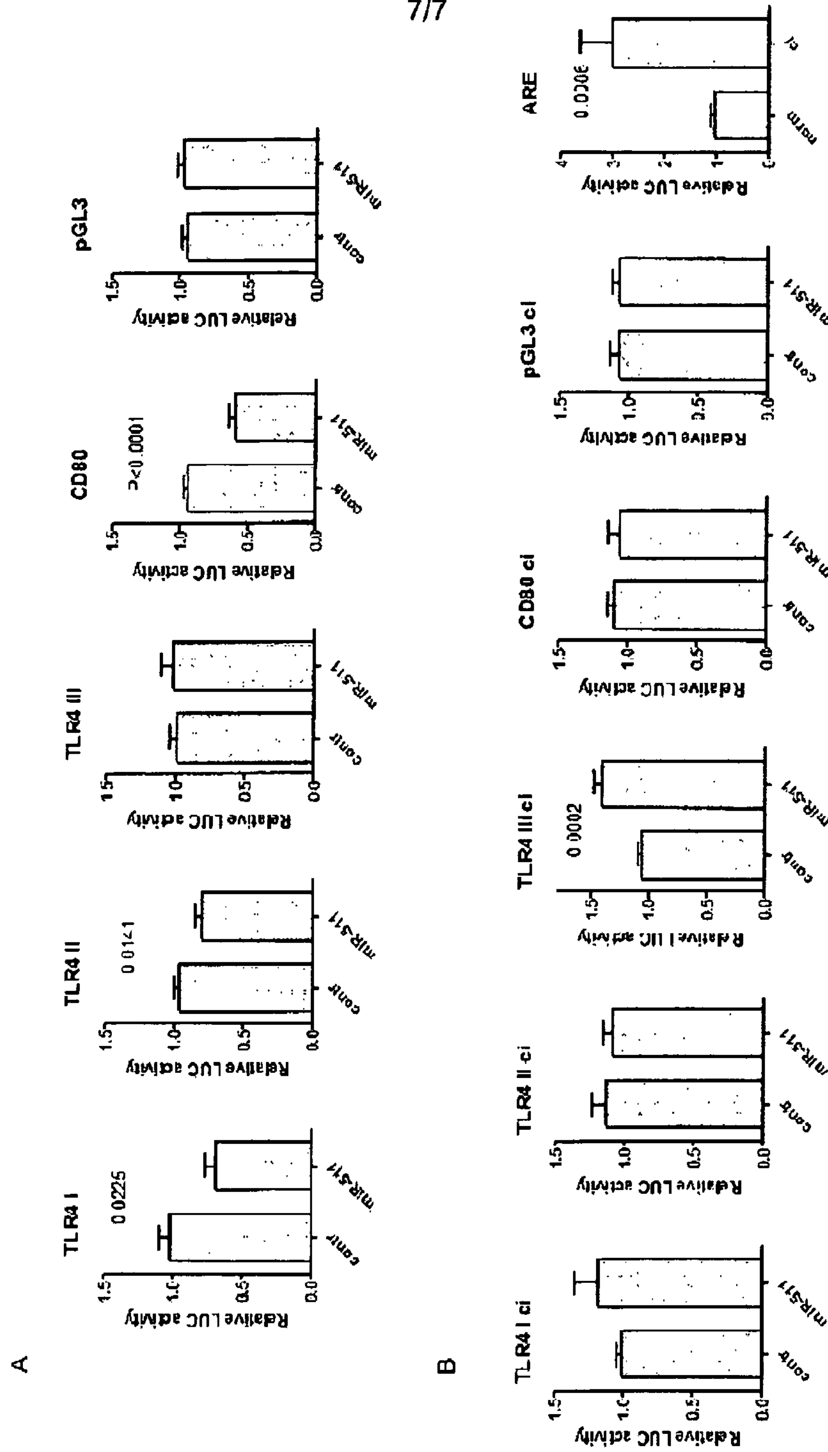

Fig. 7