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(71) Applicant: **EPIONTIS GMBH** [DE/DE]; Rudower  
Chaussee 29, 12489 Berlin (DE).

(72) Inventor: **OLEK, Sven**; Boothstrasse 16, 12207 Berlin  
(DE).

(74) Agent: **KRAUSS, Jan**; BOEHMERT & BOEHMERT An-  
waltpartnerschaft mbB, Pettenkoferstrasse 22, 80336 Mu-  
nich (DE).

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CYTIC MYELOID-DERIVED SUPPRESSOR CELLS (mMDSCs)

(57) Abstract: The present invention relates to a method, in particular an *in vitro* method, for identifying specific immune cells, in particular MDSCs, comprising analyzing a modification, preferably the methylation status, of at least one CpG position in the mammalian gene region for endoplasmatic reticulum-golgi intermediate compartment 1 (ERGIC1), wherein a demethylation or lack of methylation or modification of said gene region is indicative for an MDSC, when compared to a non-MDSC. The analyses according to the invention can identify specific sub-populations of MDSCs, namely monocytic MDSCs (m MDSCs) on an epigenetic level and distinguish them from all other cells in complex samples, such as, for example, other blood or immune cells. The present invention furthermore provides an improved method for quantifying m MDSCs, in particular in complex samples. The method can be performed without a step of purifying and/or enriching cells, preferably in whole blood and/or non-trypsinized tissue.



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## **ERGIC1 as epigenetic marker for the identification of immune cells, in particular monocytic myeloid-derived suppressor cells (mMDSCs)**

The present invention relates to a method, in particular an *in vitro* method, for identifying specific immune cells, in particular MDSCs, comprising analyzing a modification, preferably the methylation status, of at least one CpG position in the mammalian gene region for endoplasmatic reticulum-golgi intermediate compartment 1 (ERGIC1), wherein a demethylation or lack of methylation or modification of said gene region is indicative for an MDSC, when compared to a non-MDSC. The analyses according to the invention can identify specific subpopulations of MDSCs, namely monocytic MDSCs (mMDSCs) on an epigenetic level and distinguish them from all other cells in complex samples, such as, for example, other blood or immune cells. The present invention furthermore provides an improved method for quantifying mMDSCs, in particular in complex samples. The method can be performed without a step of purifying and/or enriching cells, preferably in whole blood and/or non-trypsinized tissue.

Furthermore, the present invention relates to a kit for performing the above methods as well as respective uses thereof. It is one aim of this invention to provide a novel, more robust means to quantitatively detect and measure MDSCs of the blood within any solid organs or tissue or any other body fluid of a mammal.

### **Background of the invention**

MDSC (myeloid-derived suppressor cells) are a heterogenous group of immune cells from the myeloid lineage, a family of cells that originate from bone marrow stem cells. Certain subpopulations of MDSCs namely mMDSCs, strongly expand in pathological situations such as chronic infections and cancer, as a result of an altered hematopoiesis. MDSCs are discriminated from other myeloid cell types in that they possess strong immunosuppressive activities rather than immunostimulatory properties. Similar to other myeloid cells, MDSCs interact with other immune cell types including T cells, dendritic cells, macrophages and natural killer cells to regulate their functions. Although their mechanisms of action are not clear yet, clinical and experimental evidence suggests that cancer tissues with high infiltration of MDSCs are associated with poor patient prognosis and resistance to therapies.

Even though almost all cells in an individual contain the exact same complement of DNA code, higher organisms must impose and maintain different patterns of gene expression in the various types of tissue. Most gene regulation is transitory, depending on the current state of the cell and changes in external stimuli. Persistent regulation, on the other hand, is a primary role of epigenetics - heritable regulatory patterns that do not alter the basic genetic coding of the DNA. DNA methylation is the archetypical form of epigenetic regulation; it serves as the stable memory for cells and performs a crucial role in maintaining the long-term identity of various cell types. Recently, other forms of epigenetic regulation were discovered. In addition to the “fifth base” 5-methylcytosine (mC), a sixth (5-hydroxymethylcytosine, hmC), seventh (5-formylcytosine, fC) and eighth (5-carboxycytosine, cC) can be found (Michael J. Booth et al. Quantitative Sequencing of 5-Methylcytosine and 5-Hydroxymethylcytosine at Single-Base Resolution *Science* 18 May 2012, Vol. 336 no. 6083 pp. 934-937).

The primary target of mentioned DNA modifications is the two-nucleotide sequence Cytosine-Guanine (a 'CpG site'); within this context cytosine (C) can undergo a simple chemical modification to become formylated, methylated, hydroxymethylated, or carboxylated. In the human genome, the CG sequence is much rarer than expected, except in certain relatively dense clusters called 'CpG islands'. CpG islands are frequently associated with gene promoters, and it has been estimated that more than half of the human genes have CpG islands (Antequera and Bird, *Proc Natl Acad Sci USA* 90: 11995-9, 1993).

Aberrant methylation of DNA is frequently associated with the transformation from healthy to cancerous cells. Among the observed effects are genome-wide hypomethylation, increased methylation of tumor suppressor genes, and hypomethylation of many oncogenes (reviewed, for example, by Jones and Laird, *Nature Genetics* 21:163-167, 1999; Esteller, *Oncogene* 21:5427-5440, 2002; and Laird, *Nature Reviews/Cancer* 3:253-266, 2003). Methylation profiles have been recognized to be tumor specific (i.e., changes in the methylation pattern of particular genes or even individual CpGs are diagnostic of particular tumor types), and there is now an extensive collection of diagnostic markers for bladder, breast, colon, esophagus, stomach, liver, lung, and prostate cancers (summarized, for example, by Laird, *Nature Reviews/Cancer* 3:253-266, 2003).

For one of the recently described modification of cytosine, 5-hydroxymethylation, the utility

of oxidative bisulfite sequencing to map and quantify 5hmC at CpG islands was shown (Michael J. Booth et al. Quantitative Sequencing of 5-Methylcytosine and 5-Hydroxymethylcytosine at Single-Base Resolution *Science* 18 May 2012, Vol. 336 no. 6083 pp. 934-937). High levels of 5hmC were found in CpG islands associated with transcriptional regulators and in long interspersed nuclear elements. It is suggested that these regions might undergo epigenetic reprogramming in embryonic stem cells.

Kirby et al. (Genome-wide DNA methylation measurements in prostate tissues uncovers novel prostate cancer diagnostic biomarkers and transcription factor binding patterns. *BMC Cancer* 2017 (1): 273, pp 1-10) describes a region close to ERGIC1 as a diagnostic marker for prostate cancer.

Zhang et al. (Epigenetics in myeloid derived suppressor cells: a sheathed sword towards cancer. *Oncotarget* (2016) 7 (35): 57452-57463) summarizes data relating to epigenetic regulation in MDSCs.

US 2016-0237493 describes a method and apparatus for identifying biomarkers and in particular for identifying biomarkers for use in making clinical assessments, such as early diagnostic, diagnostic, disease stage, disease severity, disease subtype, response to therapy or prognostic assessments. In one particular example, the techniques are applied to allow assessments of patients suffering from, suspected of suffering from, or with clinical signs of SIRS (Systemic Inflammatory Response Syndrome) being either infection-negative SIRS or infection-positive SIRS. EPIC1 is mentioned as an IRS biomarker gene.

WO 2012/162660 describes methods using DNA methylation arrays are provided for identifying a cell or mixture of cells and for quantification of alterations in distribution of cells in blood or in tissues, and for diagnosing, prognosing and treating disease conditions, particularly cancer. The methods use fresh and archival samples.

In view of the above, it is an object of the present invention to provide an improved and in particular robust method based on DNA-methylation analysis as a superior tool in order to more conveniently and reliably detect, identify, discriminate, and quantify specific immune cells, in particular certain subgroups of MDSCs.

The present invention solves the above object by providing a method for identifying certain myeloid-derived suppressor cells (MDSCs) in a sample derived from a mammal, comprising analyzing a modification, preferably the methylation status, of at least one CpG position in the mammalian gene region for endoplasmatic reticulum-golgi intermediate compartment 1 (ERGIC1), wherein preferably said gene region as analyzed is a sequence according to SEQ ID No. 1, and wherein a demethylation or lack of methylation or modification of said gene region is indicative for monocytic MDSCs, when compared to a non-MDSC. The present method is able to identify monocytic myeloid-derived suppressor cells (mMDSC) which are an important component of the immunosuppressive milieu. In mice, monocytic MDSCs express high levels of the Ly6C surface marker with low or no expression of the Ly6G marker, while another group called granulocytic MDSCs express Ly6C and high levels of Ly6G. For the purpose of the present invention, human mMDSCs are defined as CD15-CD14+ CD33+ CD11b+ HLADR+. Granulocytic human MDSCs are CD15+. A third fraction seems to be CD15+ CD14+, and thus the present invention detects non-CD15 MDSCs.

The gene for human ERGIC1 is found on Ensembl. Ensembl-ID: ENSG00000113719.

In the context of the present invention, the gene region shall comprise all of the genomic regions relating to and encoding for ERGIC1. Thus, included are enhancer regions, promoter region(s), introns, exons, and non-coding regions (5'- and/or 3'-regions) that belong to ERGIC1. Preferred is thus a method according to the present invention, wherein the at least one CpG position is present in the 5' region upstream from the transcription start, promoter region, the 5' or 3' untranslated regions, exon, intron, exon/intron border and/or in the 3' region downstream of the transcriptional stop of the gene as analyzed. Preferably, said mammal is a mouse, goat, dog, pig, cat, cow rat, monkey or human. The samples can be suitably pooled, if required.

The present invention is further based on the surprising identification of a region of the ERGIC1 gene by the inventors, as specific epigenetic marker, allowing the identification of MDSCs as well as the clinical routine application of said analysis.

In the context of the present invention, the genomic region of ERGIC1, in particular according to SEQ ID No. 1 allows the identification of mMDSCs. Surprisingly, the discriminatory pattern of bisulfite convertible and non-convertible cytosine is particularly and

even exclusively limited to the genomic region according to SEQ ID No. 1 of mMDSCs as shown using the amplicon according to SEQ ID No. 1, and in particular in the bisulfite converted sequences according to SEQ ID No. 2 or 3.

The inventors could demonstrate that in mMDSCs the CpG motifs as disclosed are almost completely demethylated (i.e. to more than 70%, preferably 80%, preferably, more than 90% and most preferred more than 95%), whereas the same motifs are completely methylated in all other immune cells.

The differential methylation of the CpG motifs within the aforementioned regions is a valuable tool to identify mMDSCs, such as will be required/or at least of some value for identifying and quantifying said cells in autoimmune diseases, transplant rejections, cancer, allergy, primary and secondary immunodeficiencies, such as, for example, HIV infections and AIDS, Graft versus Host (GvH), hematologic malignancies, rheumatoid arthritis, multiple sclerosis, or a cytotoxic T cell related immune status in any envisionable diagnostic context. The assay allows measurement of mMDSCs without purification or any staining procedures.

Another preferred aspect of the method according to the present invention then further comprises a quantification of the relative amount of mMDSCs based on comparing relative amounts of said methylation frequency in the region as analyzed with relative amounts of the methylation frequency in a control gene, such as, for example, GAPDH. Said quantification is thus achieved based on the ratio of the bisulfite convertible DNA to non-convertible DNA in the genetic region of ERGIC1 (e.g. of SEQ ID No. 1) as described and analyzed herein. Most preferred is a quantification of the relative amount of mMDSCs is based on an (preferably parallel or simultaneous) analysis of the relative amount of bisulfite convertible DNA of cell-specific region for ERGIC1, and of the relative amount of bisulfite convertible DNA of cell-unspecific genes (preferably designated "control genes" or "control regions", such as, for example, the gene for GAPDH).

In a further preferred embodiment of the method according to the present invention, said analysis of bisulfite convertibility comprises amplification with at least one primer of suitable primer pairs that can be suitably designed based on SEQ ID No. 1, preferably oligomers according to any of SEQ ID No. 4 to 11.

In contrast to FACS and mRNA measurements, using the methods according to the present invention, the measurement(s) and analyses can be done independent of purification, storage - and to quite some extent - also of tissue quality.

Preferably, the amplification involves a polymerase enzyme, a PCR or chemical amplification reaction, or other amplification methods as known to the person of skill as described below, e.g. in the context of MSP, HeavyMethyl, Scorpion, MS-SNUPE, MethylLight, bisulfite sequencing, methyl specific restriction assays and/or digital PCR (see, for example Kristensen and Hansen PCR-Based Methods for Detecting Single-Locus DNA Methylation Biomarkers in Cancer Diagnostics, Prognostics, and Response to Treatment *Clinical Chemistry* 55:8 1471–1483 (2009)).

With the amplification, an amplicon of the ERGIC1 gene region is produced that is a particularly preferred “tool” for performing the method(s) according to the present invention. Consequently, oligomers according to any of SEQ ID No. 4 to 11, or an amplicon as amplified by a primer pair based on SEQ ID No. 4 and 5, 6 and 7, or 9 and 10 as mentioned herein constitute preferred embodiments of the present invention. Thus, the sequences of SEQ ID No. 1 to 3 (and, if needed, the complementary sequences thereto) can be used to design primers for amplifications, i.e. serve as “beacons” in the sequence as relevant. Similarly, additional primers and probes can be designed based on the amplicon according to SEQ ID No. 1. Amplification can take place either in the genomic and/or bisulfite (i.e. “converted”) DNA sequence.

The person of skill will furthermore be able to select specific subsets of CpG positions in order to minimize the amount of sites to be analyzed, for example at least one of CpG position selected from a CpG position in an amplicon according to SEQ ID No. 1, and is preferably selected from CpG positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 in the amplicon No. 2505 according to SEQ ID No. 1. The positions are numerically counted from the 5'-end of an amplicon as generated and analyzed, and are designated in Figure 1 as AMP2505:37, 56, 62, 71, 121, 170, 227, 234, 247, 264, 271, 389, and 421. Preferred are combinations of 3, 4, 5, 6, 7, 8, 9, or 10 positions, the analysis of which produces sufficient data and/or information in order to be informative in the context of the present invention.

The person of skill will furthermore be able to select specific subsets of CpG positions in

order to minimize the amount of sites to be analyzed, for example at least one of CpG position 62 in the amplicon No. 2505 of the ERGIC1 specific bisulfite convertible region (SEQ ID No. 1), or all sites as present on the bisulfite convertible region according to SEQ ID No 1. One or more of positions 234, and/or 37 may be excluded.

In order to analyze the bisulfite convertibility of CpG positions, any known method to analyze DNA methylation can be used. In a preferred embodiment of the method according to the present invention, the analysis of the methylation status comprises a method selected from methylation specific enzymatic digests, bisulphite sequencing, analysis selected from promoter methylation, CpG island methylation, MSP, HeavyMethyl, MethyLight, Ms-SNuPE or other methods relying on a detection of amplified DNA. These methods are well known to the person of skill, and can be found in the respective literature.

In a preferred embodiment of the method according to the present invention, said method is suitable for routine application, for example on a DNA-chip. Based on the above information and the respective literature, the person of skill will be able to adjust the method as above to such settings.

In yet another preferred embodiment of the methods according to the present invention, said method is performed without a step of purifying and/or enriching said cells to be identified, preferably using whole blood and/or non-trypsinized tissue.

In another preferred embodiment of the method according to the present invention, the identification comprises a distinction of said MDSCs from all major peripheral blood cell types and/or non-blood cells, preferably, but not limited to, from CD19+ B cells; CD8+ cytotoxic T cells; CD15+ granulocytes; various MDSC fractions, including CD14+ only, CD14+CD124+, CD14+HLADR-, and CD14CD15 double positive cells; CD56+NK cells; and CD4+ T helper cells.

In yet another preferred embodiment of the method according to the present invention, the sample is selected from a mammalian body fluid, including mammalian blood samples, or a tissue, organ or a sample of leukocytes or a purified or separated fraction of such tissue, organ or leukocytes or a cell type sample. The samples can be suitably pooled, if required.

Another preferred aspect of the method according to the present invention then further comprises the step of concluding on the immune status of said mammal based on said MDSCs. MDSCs can be quantified and be used as a benchmark to relatively quantify further detailed subpopulations, or it can be used as a predictive and/or screening and/or diagnostic and/or prognostic and/or adverse events detecting factor, or it can be used to finally detect this population to determine the overall immune activity status.

In yet another preferred embodiment of the methods according to the present invention, the mammal suffers from or is likely to suffer from autoimmune diseases, transplant rejections, infection diseases, cancer, and/or allergy as but not limited to *Trypanosoma cruzi*-infection, Malaria and HIV infection; Hematologic Malignancies as but not limited to chronic Myelogenous Leukemia, Multiple Myeloma, Non Hodgkin's Lymphoma, Hodgkin's Disease, chronic Lymphocytic Leukemia, Graft versus Host and Host versus Graft Disease, Mycosis fungoides, Extranodal T cell lymphoma, Cutaneous T cell lymphomas, Anaplastic large cell lymphoma, Angioimmunoblastic T cell lymphoma and other T-cell, B-cell and NK cell neoplasms, T cell deficiencies such as but not limited to lymphocytopenia, severe combined immunodeficiency (SCID), Omenn syndrome, Cartilage-hair hypoplasia, acquired immune deficiency syndrome (AIDS), and hereditary conditions such as DiGeorge syndrome (DGS), chromosomal breakage syndromes (CBSs), multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, systemic sclerosis, dermatomyositis, primary biliary cirrhosis, primary sclerosing cholangitis, ulcerative colitis, Crohn's disease, psoriasis, vitiligo, bullous pemphigoid, alopecia areata, idiopathic dilated cardiomyopathy, type 1 diabetes mellitus, Graves' disease, Hashimoto's thyroiditis, myasthenia gravis, IgA nephropathy, membranous nephropathy, and pernicious anemia; and B-cell and T-cell combined disorders such as but not limited to ataxia telangiectasia (AT) and Wiskott-Aldrich syndrome (WAS); and carcinomas such as but not limited to breast cancer, colorectal cancer, gastric cancer, pancreatic cancer, hepatocellular carcinoma, cholangiocarcinoma, melanoma, and head and neck cancer.

Another preferred aspect of the method according to the present invention then relates to a method as above, further comprising measuring and/or monitoring the amount of mMDSCs in response to chemical and/or biological substances that are provided to said mammal, i.e. in response to a treatment of said patient. Said method comprises the steps as above, and comparing said relative amount of said cells as identified to a sample taken earlier or in

parallel from the same mammal, and/or to a control sample. Based on the results as provided by the method(s) of the invention, the attending physician will be able to conclude on the immune status of the patient, and adjust a treatment of the underlying disease accordingly.

Preferably, said method is performed without a step of purifying and/or enriching cells, preferably in whole blood and/or non-trypsinized tissue, or any other biological sample potentially containing said mMDSCs as e.g. a sample for cell transfer into a patient.

Another preferred aspect of the method according to the present invention then relates to a method as above, further comprising formulating said mMDSCs as identified for transplantation into a patient. Pharmaceutical preparations for these purposes and methods for their production are performed according to methods known in the art of transplantation medicine.

Another preferred aspect of the method according to the present invention relates to an oligomer according to any of SEQ ID No. 4 to 11, or an amplicon according to SEQ ID No. 1 to 3.

Yet another preferred aspect of the present invention then relates to a kit for identifying, quantifying, and/or monitoring MDSCs in a mammal based on the analysis of the bisulfite accessibility of CpG positions in the gene region of ERGIC1, comprising components for performing a method according to any of claims 1 to 12, in particular a kit comprising a) a bisulfite reagent, and b) materials for the analysis of the methylation status of CpG positions selected from the CpG positions in the region according to SEQ ID NO: 1, such as an oligomer selected from the sequences according to SEQ ID NOs: 4 to 11.

The present invention also encompasses the use of oligomers or amplicon or a kit according to the present invention for identifying and/or for monitoring MDSCs in a mammal as described herein.

As mentioned above, recently three new cytosine modifications were discovered. Therefore, it is expected that future scientific findings will correct epigenetic patterns of modification described in the past. These past patterns of cytosine modification encompass bisulfite convertible (non-methylated, non-modified) and non-convertible (methylated, modified)

cytosine. Both termini need to be corrected, as described. According to the novel scientific findings (i) non-bisulfite convertible cytosine encompasses 5-methylcytosine (mC) and 5-hydroxymethylcytosine (hmC), and (ii) bisulfite convertible (i.e. the “bisulfite convertibility”) cytosine encompasses 5-formylcytosine (fC), 5-carboxycytosine (cC), as well as non-modified cytosine.

Additionally, past inventions are based on (i) the ratio of bisulfite convertible cytosine to whole amount of chromatin (cell-type independent, 100% bisulfite convertible DNA locus) or (ii) on the ratio of bisulfite convertible cytosine (fC, cC, non-modified cytosine) to non-bisulfite convertible cytosine (hmC and mC). These ratios characterize cell type, cell differentiation, cell stage as well as pathological cell stages. Therefore, new techniques will result in novel, more specific ratios and might supplement current cell specific, cell state specific as well as pathological patterns of epigenetic modifications and therefore, define potential novel biomarkers. Novel ratios to be discovered as biomarkers can be defined as:

$$\text{Biomarker Ratio} = a/b$$

$$a = \sum (\text{C and/or mC and/or hmC and/or fC and/or cC})$$

$$b = \sum (\text{C and/or mC and/or hmC and/or fC and/or cC}),$$

whereby a and b differs from each other by one to four kinds of modifications. Discovery of novel DNA modifications will enlarge this enumeration.

For the purpose of definition for the present application, “epigenetic modifications” in the DNA sequence is referred to by the terminology of (i) bisulfite convertible cytosine (5-formylcytosine, (fC) and/or 5-carboxycytosine (cC)) and (ii) non-bisulfite convertible cytosine ((including 5-methylcytosine (mC), 5-hydroxymethylcytosine, (hmC)). As both kinds of methylation, mC and hmC, are not bisulfite convertible, it is not possible to distinguish between these two. Likewise, fC, cC as well as non-modified cytosine are bisulfite convertible and can also not be distinguished from each other as well. The term “methylated” DNA encompasses mC as well as hmC. The term “non-methylated” DNA encompasses fC, cC, and non-modified DNA. It is expected that novel variants of DNA modifications will be discovered in future. Each type of modification will be either bisulfite convertible or not. However, since the present method reliably distinguishes between the two groups, these novel modifications will also be usable as markers.

Furthermore, apart from the modifications of DNA, also histones undergo posttranslational

modifications that alter their interaction with DNA and nuclear proteins. Modifications include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP-ribosylation. The core of the histones H2A, H2B, and H3 can also be modified. Histone modifications act in diverse biological processes such as gene regulation, DNA repair, chromosome condensation (mitosis) and spermatogenesis (meiosis). Also for these modifications a specific pattern of modification is specific for different cell types, cell stages, differentiation status and such a pattern can be analyzed for bisulfite convertibility or similar methods in order to identify certain cells and cell stages. The present invention also encompasses a use of these modifications.

In summary, using the ERGIC1 genetic region and in particular the amplicon as described herein as a marker, the inventors very specifically identified, quantified and particularly differentiated MDSCs, and in their relation to other cell types in a sample, for example to other blood cells.

The invention will now be further described based on the following examples and with reference to the accompanying figures and the sequence listing, without being limited thereto. For the purposes of the present invention, all references as cited herein are incorporated by reference in their entireties.

Figure 1 shows the analysis of CpG sites on amplicon No. 2505 (SEQ ID No. 1) according to the invention. The horizontal boxes in the table correspond to the CpG positions in the amplicon as analyzed (e.g. CpG 2, 3, 4, ... etc.) with the positions indicated (37, 56, 62, 71, 121, 170, 227, 234, 247, 264, 271, 389, and 421 corresponding to CpG 2, 3, ...etc.), and the columns correspond to the cell types as analyzed. Positions 1 (24), and 15 (431) as also analyzed are not shown. The vertical boxes (columns) correspond to the cellular fractions as analyzed.

Figure 2 shows data regarding the linearity and specificity of the TpG -specific PCR-system according to the present invention.

SEQ ID No: 1 represents the genomic sequence of amplicon AMP2505 according to the present invention.

SEQ ID No: 2 represents the bisulfite converted (b2F), previously modified (CpG) sequence with conversion resistant CpG residues.

SEQ ID No: 3 represents the bisulfite converted (b2F), previously unmodified (TpG) sequence with no conversion resistant CpG residues.

SEQ ID No: 4 to 11 show oligomers (primers and probes) as used in the present invention.

## EXAMPLES

### Example 1

In order to identify the target mMDSC subpopulation, qPCR was performed on bisulphite converted samples stemming from the human genomic region according to sequence AMP2505, SEQ ID No. 1. Relevant CpGs are indicated bold and underlined.

GAGGGAATGAGTAGGTGAACAAA**CG**AGTGAGTTTT**CG**CCTCACTCCCTGGCAC  
**T****CG**GAC**CG**GAGGGCT**CG**ACAGGCTGGTGGCCAGGGCTGACCAGCCAGAGTTTC  
 ATCTAAAAGCCT**CG**ATGGCTATTTTTGGGACAAATGACCCACCACTTCCAATGAC  
 TTGCT**CG**AAATGGCATCTAATTTTCATTAAGCCTTGGAAACAGTGACATCACAAG  
 TGCTGCC**CG**GTAG**CG**CCACATATGG**CG**AACCACAGAGTGAT**CG**GAGATT**CG**  
 TCTGCAAGAACAGGGGAGAACTAAGGTCCCAAGCAGCAAAAAGTTAAAATAGCAA  
 AGCTGAGGCATTCTGCTATGAAAAGAAATTACAGATGAAATCCATTAGTCAGACA  
 TTCCAG**CG**GCAGTTTCCTTGCTTGGGAGCAGGGGAAG**CG**TGTTTATT**CG**GGGT  
 TGGGCTCTGCAGCCATTCC

SEQ ID No. 2 represents the bisulfite converted (b2F), previously modified (CpG) sequence with conversion resistant CpG residues. Relevant CpGs are indicated bold and underlined.

AAAAAATAAATAAATAAACAAA**CG**AATAAATTTTT**CG**CCTCACTCCCTAACAC  
**T****CG**AC**CG**AAA**CG**AACT**CG**ACAACTAATAACCAAACTAACCAACCAAAATTTTC  
 ATCTAAAACCT**CG**ATAACTATTTTTTAAAACAAATAACCCACCACTTCCAATAAC  
 TACT**CG**AAATAACATCTAATTTTCATTAACCTTAAAAACAATAACATCACAAA  
 TACCTAC**CG**ATAAC**CG**CCACATATA**CG**AACCACAAAATAAT**CG**AAATT**CG**  
 TCTACAAAAACAAAAAAACTAAAATCCCAAACAACAAAAATTAATAACAA  
 AACTAAAACATTCTACTATAAAAAAAATTACAAATAAAATCCATTAATCAAACA  
 TTTCCA**CG**ACAATTTCTTACTTAAAAACAAAAAA**CG**TATTTATT**CG**AAAT  
 TAAACTCTACAACCATTCC

SEQ ID No. 3 represents the bisulfite converted (b2F), previously unmodified (TpG) sequence with no conversion resistant CpG residues. Relevant positions are indicated bold and underlined.

AAAAAATAAATAAATAAACAAA**CA**AATAAATTTTT**CA**CCTCACTCCCTAACACT  
**CA**AC**CA**AAA**CA**AACT**CA**ACAACTAATAACCAAACTAACCAACCAAAATTTCA  
 TCTAAAACCT**CA**ATAACTATTTTTTAAAACAAATAACCCACCACTTCCAATAACT

TACTCCCAAAATAACATCTAATTTTCATTAACCTTAAAAACAATAACATCACAAAT  
 ACCTACCCCATAAACCCACCCACATATAACAAACCACAAAATAATCCCAAAATTCAT  
 CTACAAAAACAAAAAAACTAAAATCCCAAACAACAAAAATTTAAAATAACAAA  
 ACTAAAACATTCTACTATAAAAAAAATTACAAATAAAATCCATTAATCAAACAT  
 TTCCAACCAACAATTTCTTACTTAAAAACAAAAAACATATTTATTCAAAATT  
 AAACTCTACAACCATTCC

For the actual epigenetic profiling of the amplicon region in blood cell subtypes, the immune cell populations as analyzed were as follows (see Figure 1)

1. As target population: CD15- CD14+ CD33+ CD11b+, HLADR+
2. CD19+ B cells; CD8+ cytotoxic T cells; CD15+ granulocytes; various MDSC fractions, including CD14+ only, CD14+CD124+, CD14+HLADR-, CD14CD15 double positive; CD56+NK cells; and CD4+ T helper cells.

The following primers and probe were used for the qPCR (TpG variant; demethyl-specific; CpG variant; methyl-specific):

|                              |              |                                                  |
|------------------------------|--------------|--------------------------------------------------|
| Forward amplification primer | 2505-fwd     | GAGGGAATGAGTAGGTGAATAA (SEQ ID No. 4)            |
| Reverse amplification primer | 2505-rev     | AAAATAACTACAAAACCCAACC (SEQ ID No. 5)            |
| Forward primer TpG-specific  | 2505_TpG-fwd | CCCTAACACTCAACACCA (SEQ ID No. 6)                |
| Reverse primer TpG-specific  | 2505_TpG-rev | GGGTTATTTGTTTTAAAATAGTTATTG (SEQ ID No. 7)       |
| Probe TpG-specific           | 2505_TpG-pro | TGGTTATTAGTTTGTGGAGTTTTTGGT (SEQ ID No. 8)       |
| Forward primer CpG-specific  | 2505_CpG-fwd | CCCTAACACTCGACACCG (SEQ ID No. 9)                |
| Reverse primer CpG-specific  | 2505_CpG-rev | GGGTTATTTGTTTTAAAATAGTTATC (SEQ ID No. 10)       |
| Probe CpG-specific           | 2505_CpG-pro | TTTTGGTTATTAGTTTGTCTGGAGTTTTCTGG (SEQ ID No. 11) |

The linearity and specificity of the TpG -specific PCR-system was demonstrated using test-templates (plasmid-DNA) containing either CpG or TpG variants of the in silico converted sequence. The corresponding values between the actually measured qPCR results (calculated methylation) compared to the prepared dilutions (estimated methylation) are shown in Figure 2. The cell type specificity (as measured by qPCR) was found to be as follows:

| <b>Type of immune cell</b>    | <b>qPCR-Detection [%]</b> |
|-------------------------------|---------------------------|
| Target subpopulation of MDSCs | 68.5                      |
| CD8+ T-cells                  | 0.9                       |
| CD56+ NK-cells                | 0.1                       |
| B-cells                       | 0.1                       |
| CD14+ monocytes               | 0.1                       |
| CD4+ T-cells                  | 0.5                       |
| CD15+ granulocytes            | 0.1                       |

## CLAIMS

1. A method for identifying monocytic myeloid-derived suppressor cells (mMDSCs) in a sample derived from a mammal, comprising analyzing a modification, preferably the methylation status, of at least one CpG position in the mammalian gene region for endoplasmatic reticulum-golgi intermediate compartment 1 (ERGIC1), wherein preferably said gene region as analyzed comprises the sequence according to SEQ ID No. 1, wherein a demethylation or lack of methylation or modification of said gene region is indicative for an mMDSC, when compared to a non-mMDSC.
2. The method according to claim 1, wherein said at least one CpG position is present in the 5' region upstream from the transcription start, promoter region, the 5' or 3' untranslated regions, exon, intron, exon/intron border and/or in the 3' region downstream of the transcriptional stop of said gene region as analyzed.
3. The method according to claim 1 or 2, wherein said at least one CpG position is selected from a CpG selected from the CpG positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 in the amplicon according to SEQ ID No. 1, and is preferably selected from CpG positions 3, 4, 5, 6, 7, 8, and 9 in a fragment of the amplicon No. 2505, or in a fragment of the bisulfite-converted sequence according to SEQ ID No. 2 or 3.
4. The method according to any one of claims 1 to 3, wherein said analysis of the bisulfite convertibility comprises a method selected from a methylation specific enzymatic digest, bisulfite sequencing, an analysis selected from promoter methylation, CpG island methylation, MSP, HeavyMethyl, MethyLight, Ms-SNuPE, and other methods relying on a detection of amplified DNA.
5. The method according to any one of claims 1 to 4, further comprising a quantification of the relative amount of mMDSCs based on comparing relative amounts of said methylation frequency in the region as analyzed with relative amounts of the methylation frequency in a control gene, such as, for example, GAPDH.

6. The method according to any one of claims 1 to 5, wherein said sample is selected from a mammalian body fluid, including human blood samples, or a tissue, organ, bone marrow, or cell type blood sample, a sample of blood lymphocytes or a fraction thereof.
7. The method according to any one of claims 1 to 6, further comprising a distinguishing of said mMDSC from all or at least one of the cell types selected from CD19+ B cells; CD8+ cytotoxic T cells; CD15+ granulocytes; various MDSC fractions, including CD14+ only, CD14+CD124+, CD14+HLADR-, and CD14CD15 double positive cells; CD56+NK cells; and CD4+ T helper cells.
8. The method according to any one of claims 1 to 7, wherein said method is performed without a step of purifying and/or enriching said cells to be identified, preferably using whole blood and/or non-trypsinized tissue.
9. The method according to any one of claims 1 to 8, further comprising the step of concluding on the immune status of said mammal based on said mMDSC as identified.
10. A method for monitoring the level of mMDSCs in a mammal, comprising performing the method according to any one of claims 5 to 9, and furthermore comparing said relative amount of said cells as identified to a sample taken earlier or in parallel from the same mammal, and/or to a control sample.
11. The method according to any one of claims 1 to 10, further comprising measuring and/or monitoring the amount of said mMDSCs in response to chemical and/or biological substances that are provided to said mammal.
12. The method according to any one of claims 1 to 11, wherein said mammal suffers from or is likely to suffer from autoimmune diseases, transplant rejections, infection diseases, cancer, and/or allergy.
13. A kit for identifying, quantifying, and/or monitoring mMDSCs in a mammal based on the analysis of the bisulfite accessibility of CpG positions in the gene region of endoplasmatic reticulum-golgi intermediate compartment 1 (ERGIC1), comprising components for performing a method according to any of claims 1 to 12, in particular a kit comprising a)

bisulfite reagent, and b) materials for the analysis of the methylation status of CpG positions selected from the CpG positions in the region according to SEQ ID NO: 1, such as an oligomer selected from the sequences according to SEQ ID NOs: 4 to 11.

14. An oligomer according to any of SEQ ID No. 4 to 11, or the amplicon according to SEQ ID No. 1 to 3.

15. Use of the kit according to claim 13, or of the oligomer or amplicon according to claim 14 for identifying, quantifying, and/or monitoring mMDSCs in a mammal.

Figure 1

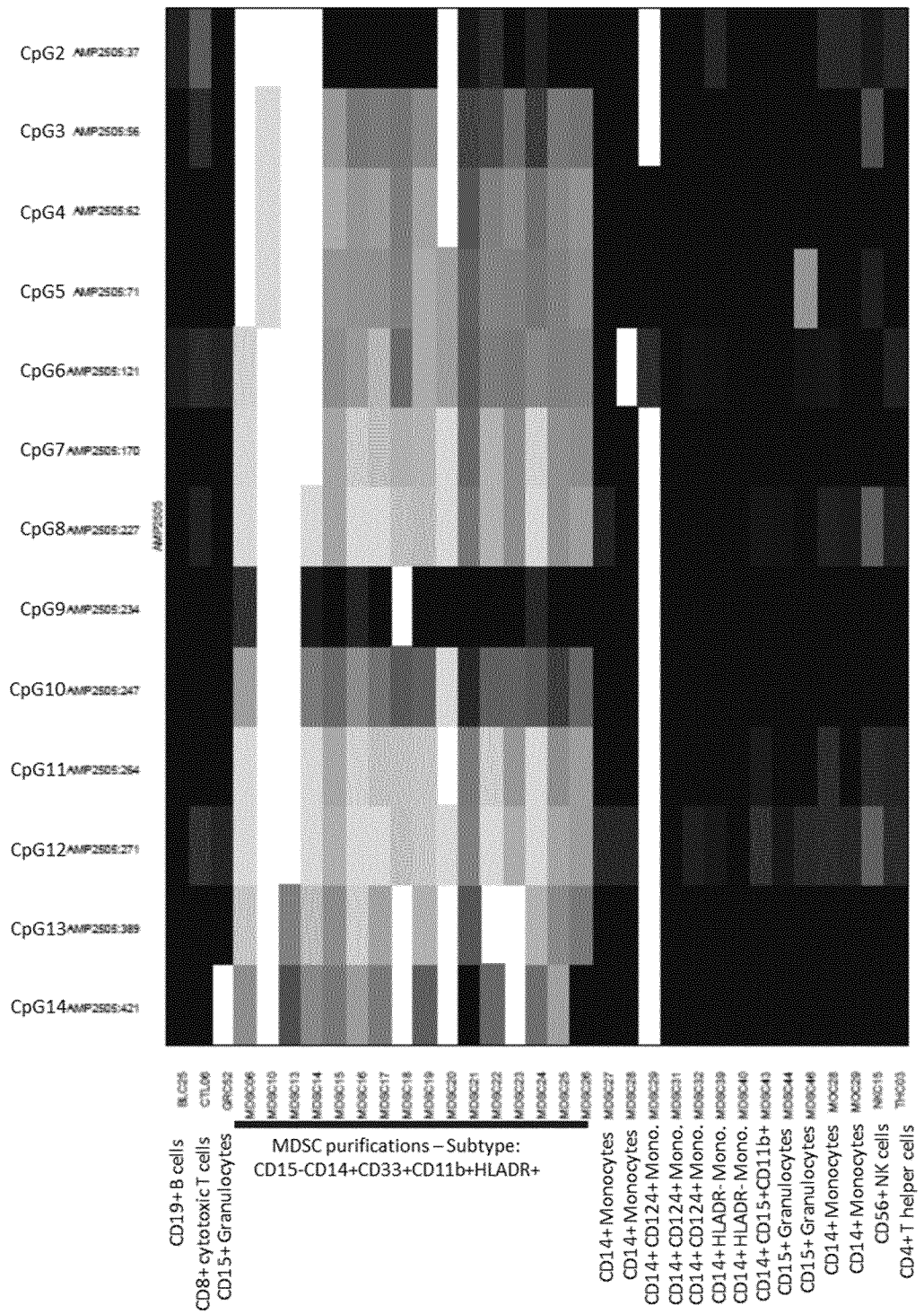
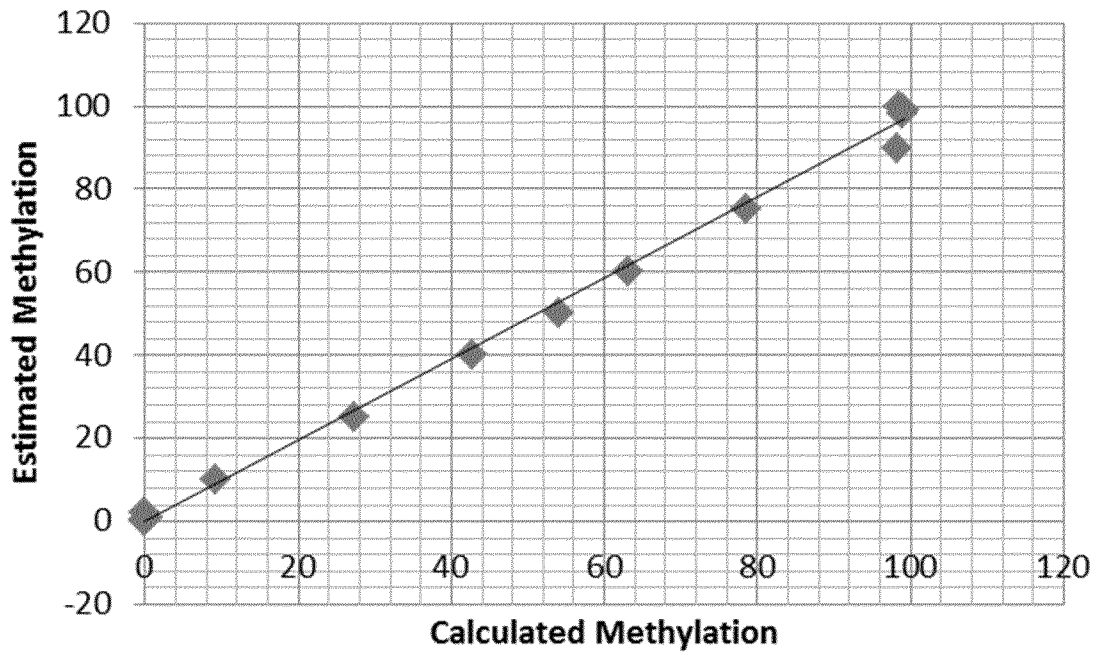


Figure 2



INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2018/080235

A. CLASSIFICATION OF SUBJECT MATTER  
INV. C12Q1/6883  
ADD.  
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED  
Minimum documentation searched (classification system followed by classification symbols)  
C12Q  
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
EPO-Internal, BIOSIS, EMBASE, WPI Data

| C. DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                              |                       |
|----------------------------------------|------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                              | Citation of document, with indication, where appropriate, of the relevant passages                                           | Relevant to claim No. |
| X                                      | WO 2014/170497 A2 (EPIONTIS GMBH [DE])<br>23 October 2014 (2014-10-23)<br>abstract; claims 1-17, 26,27; table 4I<br>page 101 | 1-15                  |
| X                                      | -----<br>WO 2015/159292 A2 (YISSUM RES DEV CO [IL];<br>HADASIT MED RES SERVICE [IL])<br>22 October 2015 (2015-10-22)         | 13,14                 |
| A                                      | abstract; claims 31,37; sequence 309<br>-----<br>-/--                                                                        | 1-12                  |

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

|                                                                                                                                                                      |                                                                      |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|
| Date of the actual completion of the international search<br><br>7 December 2018                                                                                     | Date of mailing of the international search report<br><br>04/01/2019 |
| Name and mailing address of the ISA/<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Authorized officer<br><br>Costa Roldán, Nuria                        |

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2018/080235

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                           |                       |
|------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                        | Relevant to claim No. |
| A                                                    | <p>CHAO ZHANG ET AL: "Epigenetics in myeloid derived suppressor cells: a sheathed sword towards cancer",<br/>ONCOTARGET,<br/>vol. 7, no. 35,<br/>30 August 2016 (2016-08-30), pages<br/>57542-57463, XP55489801,<br/>DOI: 10.18632/oncotarget.10767<br/>the whole document</p> <p style="text-align: center;">-----</p>                                                                                   | 1-15                  |
| A                                                    | <p>KALLINTERIS N L ET AL: "Results of epigenetic-based quantitative PCR assisted immune cell counting analysis in bavituximab SUNRISE trial subgroup",<br/>JOURNAL FOR IMMUNOTHERAPY OF CANCER<br/>20171101 BIOMED CENTRAL LTD. NLD,<br/>vol. 5, no. Supplement 2,<br/>1 November 2017 (2017-11-01), XP002787211,<br/>ISSN: 2051-1426<br/>the whole document</p> <p style="text-align: center;">-----</p> | 1-15                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/080235

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |            |
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