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The study of the influence of micro-environmental signals on macrophage differentiation using a quantitative Petri net based model

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The complexity of many biological processes, which, thanks to the development of many fields of science, becomes for us more and more obvious, makes these processes extremely interesting for further analysis. In this paper a quantitative model of the process of macrophage differentiation, which is essential for many phenomena occurring in the human body, is proposed and analyzed. The model is expressed in the language of Petri net theory on the basis of one of the three hypotheses concerning macrophage differentiation existing in the literature. The performed analysis allowed to find an importance of individual factors in the studied phenomenon.

Key words: Petri nets, atherosclerosis, macrophages, t-invariants.

1. Introduction

The discovery of high independence between phenomena of macrophages differentiation and the T helper cells and the existence of macrophages subpopulations with different phenotype profiles M1 and M2 [19], opens new roads to better understanding the patomechanisms of many diseases and brings hope for their effective treatment in the future [28, 17].

The motivation for these studies were the results of recent research, which shed new light on immunological processes. Abnormal functioning of the immune system is the basis of many civilization-related diseases, inter alia the atherosclerosis. This disease ceased to be seen as a simple disorder caused by deposition of lipids in the arte-

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rial endothelium. Now we know that its etiology is highly complex and associated with multiple processes, such as inflammation, oxidative stress, immune disturbances and hyperlipidemia. It should be emphasized here that macrophage accumulation within the vascular wall is a hallmark of atherosclerosis [3]. Therefore, the role of macrophages to keep organism homeostasis is crucial.

Unfortunately, the mechanisms underlying macrophage differentiation are not fully known. The macrophages are very important cells for the development of many diseases, thus it is significant to determine how they are maturing and how the process of activation of particular phenotypic groups is going. In this paper macrophage differentiation process has been studied. There are three main hypotheses about this phenomenon in the literature. We have considered them and chosen one for analysis. Because of the aforementioned complexity of the studied phenomenon, for its modeling and analysis a systems approach based on the language of Petri nets theory has been used. Hence, an in-depth analysis has been made.

In this article an extended version of the model proposed in [25] is presented and analyzed. The model has been extensived by addition of the influence of macrophage-derived secretory products on the maturation of the monocytes.

The structure of the paper is as follows. In section 2 basic notions concerning Petri nets used in the other parts of this work are briefly presented. In section 3 biological aspects of the process of macrophage differentiation are described. In section 4 the Petri net based model of this process is presented, while in section 5 the results of its analysis are described. The paper ends with conclusions given in section 6.

2. Petri nets

Petri nets have been proposed in 1960s by Carl A. Petri as a formalism for modeling and analysis of concurrent computer systems [23]. For years they have been mainly used in the context of technical systems but in the mid 1990s it has been realized that nets of this type can be also used for describing and analysis of biological systems [24, 10].

A Petri net has a structure of a weighted directed bipartite graph. In such a graph a set of vertices can be divided into two disjoint subsets in such a way that there is no arc connecting vertices of the same subset. In Petri nets vertices from one of these subsets are called places while elements of the other subset are transitions. When a Petri net is a model of some biological system, places correspond to its passive components (e.g., substrates or products of reactions) and transitions are counterparts of some elementary subprocesses occurring in the system (e.g., chemical reactions). Hence, in Petri nets arcs connect places with transitions and transitions with places. There is also another, very important type of components of Petri nets, i.e., tokens. They bring dynamics to the net, which is one of its fundamental properties. Tokens flow between places through transitions, what corresponds to a flow of substances, information etc. in the modeled system. The flow of tokens is governed by a simple transition activation and firing rule, according to which a transition is active if in every place directly preceding this transition there

is a number of tokens which is equal to at least the weight of an arc connecting that place with the transition. An active transition can be fired what means that tokens flow from all places directly preceding it to places directly succeeding the transition and the number of flowing tokens is equal to the weight of an appropriate arc. There are two exceptions to this rule. A transition which is not preceded by any place, called an input transition, is continuously active (so, it can fire at any time). Moreover, a transition which is not succeeded by any place, called an output transition, does not generate tokens when fired. Input and output transitions often represent some interfaces between a modeled system and its environment. A distribution of tokens over the set of places, called marking, corresponds to a state of the modeled system [21].

There is a very intuitive graphical representation of Petri nets, where transitions are depicted as rectangles or bars, places as circles, arcs as arrows and tokens as dots or positive integer numbers within places. This representation is very helpful in understanding a structure of the model and in its simulation but it is not very well suited for an analysis of its formal properties. For this purpose another representation, called incidence matrix, is more suitable. In such matrix $A = (a_{ij})_{n\times m}$ rows correspond to places, columns correspond to transitions and entry a_{ij} is equal to the difference between the numbers of tokens residing in place p_i before and after firing transition t_j.

In the analysis of Petri net based models of biological systems especially important are transition invariants (t-invariants). An invariant of this type is vector x being a solution of the equation $A \cdot x = 0$. These vectors are dependent only on the structure of the network and the distribution of tokens does not affect them. For each Petri net minimal t-invariants [16] are looked for, i.e., those that are not linear combinations of other t-invariants. Algorithms for calculating t-invariants are described in several papers, e.g., in [4] (there are also freely available software tools for calculating invariants, e.g., INA [11], Charlie [9] and MonaLisa [5]). With t-invariant x there is associated set $s(x) = \{t_j : x_j > 0, j = 1,2,\ldots,m\}$ containing transitions corresponding to positive entries of vector x and is called a support of this invariant.

Supports correspond to subprocesses which do not change a state of the modeled system. Hence, they are especially important and an analysis of dependencies among such subprocesses may lead to discoveries of some unknown properties of the system. Such an analysis can be done by searching for similarities among t-invariants. These similarities (properly defined) correspond to common parts of the supports. Transitions being elements of support intersections correspond to elementary processes composing subprocesses being counterparts of the supports. These subprocesses can interact with each other through the common elementary processes. In order to find similar t-invariants they are usually grouped into sets called t-clusters using standard clustering algorithms. Each of such clusters contains t-invariants similar to each other according to some similarity measure. Moreover, each of them corresponds to some subprocess of higher order [8, 7]. There are many clustering algorithms and similarity measures which may provide various clusterings (e.g., sets of clusters). Hence, in order to obtain a proper clustering (from the viewpoint of the analysis of the model of the biological system), the algorithm, the

similarity measure and also the number of clusters should be carefully chosen, what is not an easy task.

In addition, in the case where a Petri net contains a great number of transitions, they can be grouped into the so-called Maximum Common Transition sets (MCT sets). Each of these sets contains transitions being elements of supports of exactly the same minimal t-invariants and corresponds to some functional module of the modeled system [8, 7]. The formal definition of MCT sets and some considerations about their importance in the biological context can be found, e.g., in [26].

In figure 1 there is shown an example of a simple Petri net consisting of six transitions and four places. Among them there are two input transitions, representing input signals (named Input1 and Input2) and output transition, representing an output signal (named Output). The net is covered by two t-invariants. The subnets corresponding to these invariants are marked with red and green arrows. As can be seen, the support of the t-invariant marked in green does not contain transitions Input2 and Output. These transitions are in the support of the second t-invariant (the red one) but it does not contain transition Input1 and those ones forming the cycle in the net. It can be observed that in order to appear signal "Output" signal "Input2" is necessary but signal "Input1" is not. In addition, signal "Input2" (together with "Input1") is also necessary to start processes forming the cycle if in the initial marking there are no tokens in any of the places being elements of the cycle.

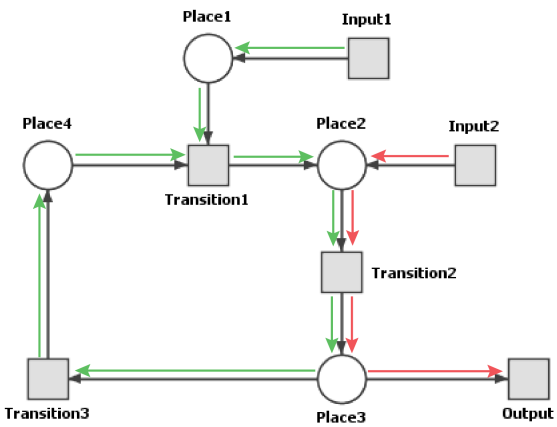

Figure 1: Simple Petri Net showing a cyclic process with two input and one output. It is covering by two t-invariants, marked by red and green arrows.

3. The process of macrophage differentiation

Macrophages, a type of white blood cells, are phylogenetically the oldest group of the immune cells. They are characterized by high heterogeneity and versatility of the immune response. Macrophages initiate and direct virtually all immune responses [20].

The main function of macrophages is phagocytosis, however, they are equipped with a number of other activities, which gives them the possibility to a comprehensive immune response. They produce, inter alia, a number of cytotoxic and effector molecules such as cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS). In addition, macrophages belong to antigen presenting cells (APC), so they can activate the T-cells and start the specific immune response. Diversity of macrophages function allows to divide them into resident tissue macrophages, tissue macrophages, and monocyte-derived macrophages [12].

Resident macrophages differentiate in the early stages of embryonic development and have ability to regenerate its population. Other macrophages arise mainly as a result of monocytes maturation. Monocytes are also not homogeneous group. There are two subpopulations of monocytes, classical and non-classical differing in the expression level of certain surface receptors. Both of these populations are formed in the bone marrow, from where they go into the bloodstream. Then they are recruited into the tissues and differentiate into various types of macrophages [12].

M1 macrophages, also called inflammatory macrophages, are activated in the presence of pathogens and inflammatory factors. They are characterized by the generation of cytotoxic compounds, such as ROS, nitric oxide (NO), citrulline, high levels of IL-12, and low levels of IL-10. Moreover, these macrophages produce plurality of pro-inflammatory cytokines, such as IL-23, IL-1, IL-6 and tumor necrosis factor (TNF). This phenotypic profile is capable of an antigen presentation (APCs). However, metabolism adapted to the production of toxic substances is not indifferent for the cells. Generally, the macrophages activated in this subtype, are able to self deactivation, when the cytotoxic functions are no longer required for the status of the human organism. However, if the process in M1 mode lasts too long, cells are not longer able to deactivate, and their destructive metabolism ultimately leads to apoptosis [19].

M2 macrophages, called anti-inflammatory macrophages, create the second phenotype class. This is the basic activation program. Cells of these type are responsible for the maintenance of tissue homeostasis, remodeling, growth and regeneration of cells, damaged by injury or inflammation. Among this class, three subtypes are distinguished, i.e., M2a, M2b and M2c. They differ in the expression and their function in the organism. Attraction M2a induces Th2 response, stimulates the type II inflammatory response (cytotoxic) and combats parasites. It is characterized by secretion of IL-10, IL-1ra, polyamides, and decoy IL-1RII. In turn, the M2b program has immunological response regulatory activity and is distinguished by TNF, IL-1, IL-6 secretion, high levels of IL-10, and low levels of IL-12 secretion. These types of cells are also involved in the activation of Th lymphocytes and inhibition of tumor growth. M2c, a third of these programs, is referred as immunosuppressant and is designed to inhibit inflammatory reactions and is involved in reconstruction and recomposing of the tissues. M2c produce, among others, IL-10 and transforming growth factor beta (TGF-β). They have a unique metabolic machinery (plasticity) that allows them to switch from M1 to M2 [20]. However, these populations are much more heterogeneous, meaning that the M1(routine heal mode) and M2 (inhibit mode) are extrema of the spectrum of intermediate phenoty-

phes. A multitude of factors influencing the phenotype of these versatile cells makes that macrophages may take mixed activity.

There are three main hypotheses of macrophage differentiation. The first one assumes that each one of subpopulations of monocytes can differentiate into specific macrophages phenotype. According to this, classical monocytes and monocyte-derived tissue macrophages can differentiate into M1 macrophages, while M2 macrophages are formed from non-classical monocytes and resident macrophages [12]. According to the second hypothesis, macrophages phenotype depends on factors affecting differentiation of monocytes in a tissue. Micro-environmental signals and cytokines present in the tissue are different for various inflammatory conditions. The resultant of these factors affect to the expression of the macrophages involved in the immune response. Usually local population of macrophages include both subtypes. However, the percentage of any fraction is different and affected by many factors and circumstances. At the beginning of the inflammation an amount of M1 macrophages is much larger than M2 fraction, the number of which increases with time and reaches the largest share in the post-inflammatory phase [12, 1]. According to the third hypothesis, mature macrophages have ability to change their phenotype from pro-inflammatory (M1/inhibit mode) to anti-inflammatory (M2/heal mode), and vice versa, depending on the different conditions in the tissues [13, 22, 27].

In vitro studies have demonstrated that macrophage activation into M1 phenotype takes place under the influence of infectious agents such as lipopolysaccharide (LPS), granulocyte macrophage colony stimulating factor (GM-CSF), and pro-inflammatory cytokines: tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). Activation of the M2 program is dependent on the presence of certain cytokines (IL-4 and IL-13), anti-inflammatory agents (IL-10, TGF-β) and activity Fc and TL receptors. Influence of IL-4 and IL-13 gives rise to a subpopulation M2a of macrophages. Activation of phenotype M2b depends on the simultaneous launch of an immune complex receptors Fcγ and TL. M2C - the third of the programs, is stimulated by IL-10 and TGF-β.

The activity of particular classes of macrophage phenotypes stimulates the differentiation and activity of another monocytes. M2b activity leads to a local increase in the level of IL-10, which in turn leads to the formation of increased number of macrophages M2c. In contrast, the IL-12 secretion by M1 macrophages activates T-cells, which produce IFN-γ, which, as stated above, is a major factor inducing the classic macrophage activation M1. The share of particular subpopulation of macrophages in the tissue has an impact on the subsequent differentiation of macrophages [18].

For better understanding the intricacies of the discussed biological process, it has been schematically depicted in the figure 2. According to the accepted hypothesis, the possibility of differentiation all types of macrophages to all pehonotypic classes, depending on the microenviroment conditions was considered. Some of macrophages tend to differentiate into a specific phenotype more than others. It has been presented by the lines thickness. Inflammatory factors, and their effect on the macrophage differentiation to type M1 and stimulation of recrutiement of classical monoctets to the tissue, are highlighted in red. In green, yellow and blue color are marked anti-inflammatory factors

which promote the formation M2a, M2b and M2c macrophages, respectively. Each phenotypic class has a different expression profile and level of interleukins secretion. All types of macrophages secrete IL-10 but M1 secrete it in very limited amount, what is signify on the figure by dotted lines.

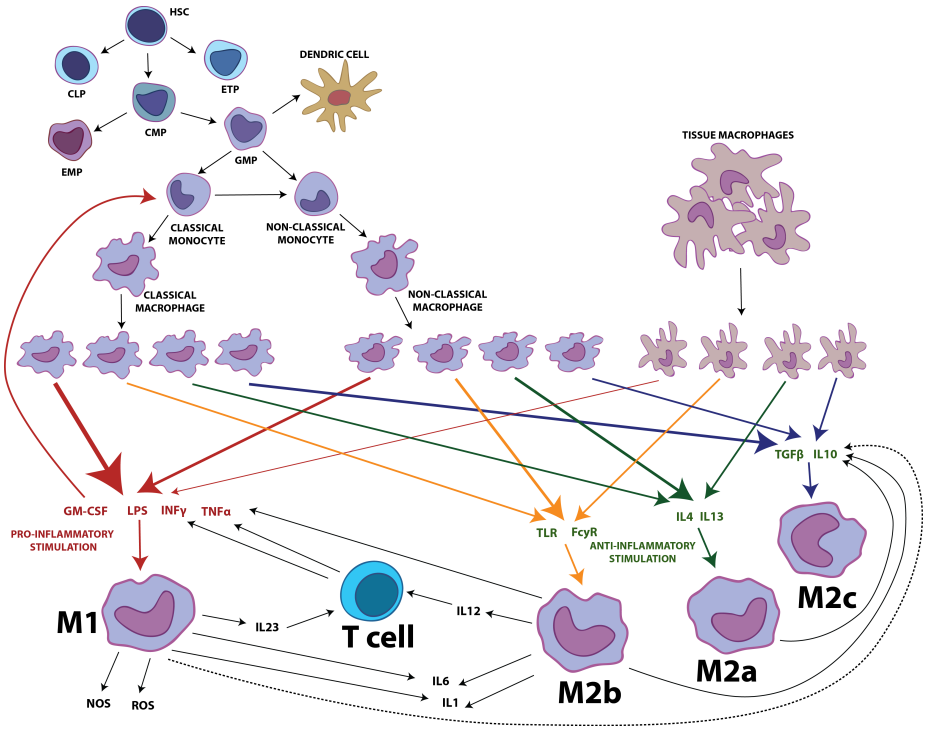

Figure 2: The analyzed process of macrophages polarization.

4. The model

The model created for the purpose of this study has been based on the second hypothesis of macrophage differentiation, according to which the macrophage phenotype depends on the micro-environmental signals. Accordingly, the model is limited to the differentiation and maturation of monocytes into macrophages, without taking into account the possibility of changing phenotype of already mature macrophages. Moreover, the ability to inactivate macrophages and the presence of memory macrophages has not been taken into account. The model contains the activity of selected cytokines, directly related with the macrophages differentiation process. In addition to monocyte derived macrophages, four main types of resident tissue macrophages are included in the model: Langerhans cells, microglial cells, Kupffer cells and Alveolar macrophages. The Petri

net based model consists of 67 transitions and 41 places described in Tables 1 and 2 and it is shown in Fig 3.

With each of the transitions there is associated a literature reference, mentioned in Table 3. The analyzed process is not autonomous, the individual elements are involved in other reactions in the organism. This fact, likewise the formal requirements of the Petri net, made it necessary to include output transitions to the model, that signify the contribution of some factors in other physiological processes, whose specification was not important for this study. For example, only part of IL-23 produced by M1 macrophages is involved in stimulating T-cells, other molecules of IL-23 may participate in other processes not included in the model. All similar phenomena are indicated in column "Biological meaning" of Table 2 by word "output".

The model described in this paper is available in SBML and SPEED formats on the website http://www.cs.put.poznan.pl/krzosinska/research.html

Table 1: List of places

Place	**Biological meaning**	**Place**	**Biological meaning**
p_0	Langerhans cells (LC)	p_{21}	lipopolysaccharides (LPS)
p_1	microglial cells	p_{22}	toll-like receptors (TLR)
p_2	Kupffer cells	p_{23}	receptors for the Fc region of IgG (FcγR)
p_3	Alveolar macrophage	p_{24}	IL-4
p_4	hematopoietic stem cells (HSC)	p_{25}	IL-13
p_5	common lymphocyte progenitor (CLP)	p_{26}	IL-10
p_6	common myeloid progenitor (CMP)	p_{27}	transforming growth factor β (TGF-β)
p_7	early T-lineage progenitor (ETP)	p_{28}	M2a
p_8	granulocyte and monocyte progenitor(GMP)	p_{29}	M2b
p_9	megakaryocyte/erythrocyte progenitors(MEP)	p_{30}	M2c
p_{10}	macrophage/dendritic cells progenitors (MDP)	p_{31}	M1
p_{11}	common monocyte progenitor(cMoP)	p_{32}	nonclassical monocyte
p_{12}	common dendritic progenitors (CDP)	p_{33}	classical monocyte
p_{13}	dendritic cells	p_{34}	nitric oxide (NO)
p_{14}	non-classical monocyte in blood	p_{35}	ROS
p_{15}	classical monocyte in blood	p_{36}	IL-6
p_{16}	monocyte derived macrophages	p_{37}	IL-12
p_{17}	non-classical macrophage	p_{38}	IL-23
p_{18}	tumor necrosis factor alpha (TNF-α)	p_{39}	IL-1
p_{19}	interferon γ (IFN-γ)	p_{40}	T cells
p_{20}	granulocyte-macrophage colony-stimulating factor (GM-CSF)		

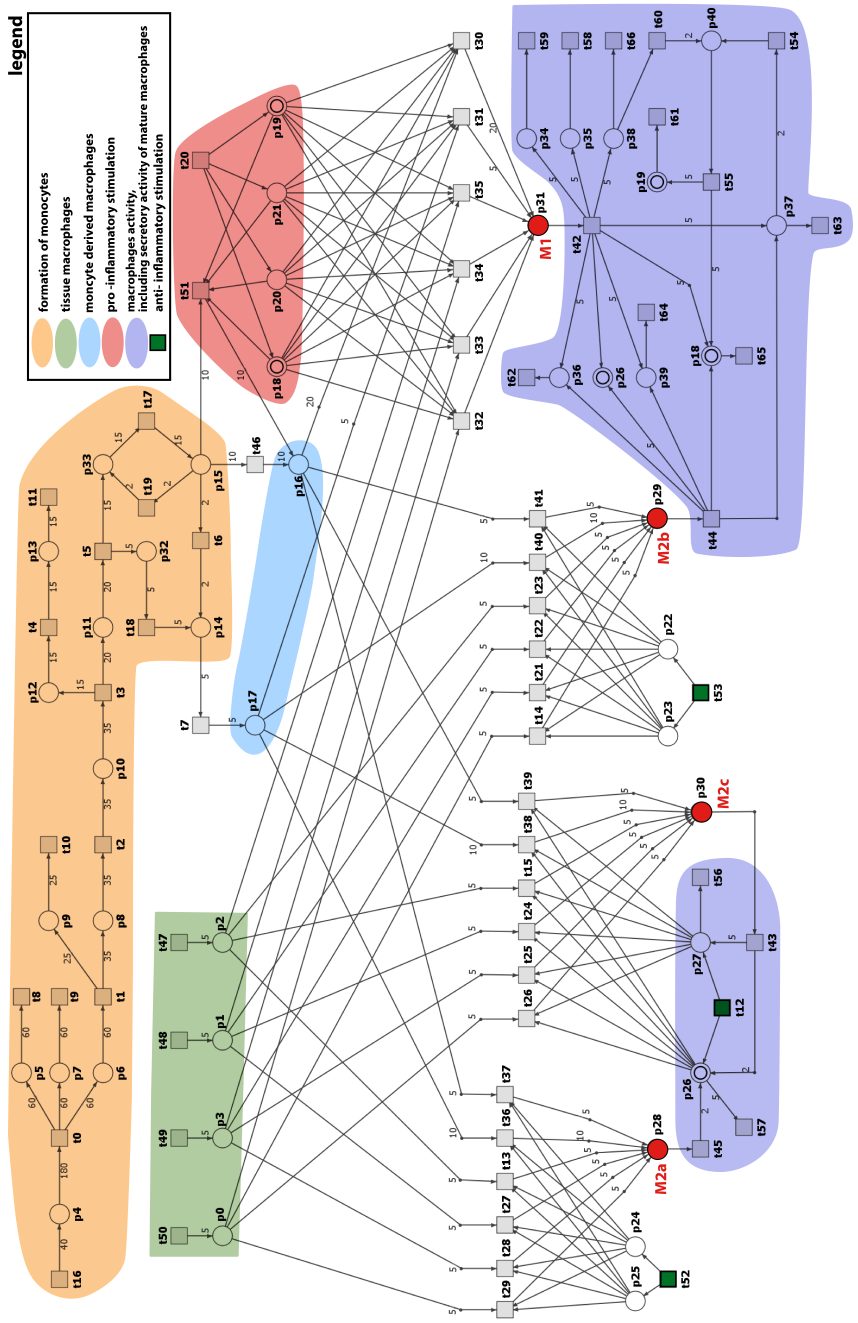

Figure 3: Macrophage differentiation Petri net based model, with biological descriptions of the most important parts of the net.

Table 2: List of transitions

Transition	Biological meaning	Transition	Biological meaning
t_0	HSC differentiation	t_{34}	microglial to M1 activation
t_1	CMP differentiation	t_{35}	Kupffer cells to M1 activation
t_2	GMP differentiation	t_{36}	non-classical monocytes to M2a activation
t_3	MDP differentiation	t_{37}	classical monocytes to M2a activation
t_4	maturation of dendritic cells (DC)	t_{38}	non-classical monocytes to M2c activation
t_5	exit from the bone marrow	t_{39}	classical monocytes to M2c activation
t_6	loss of expression of Ly6C	t_{40}	non-classical monocytes to M2b activation
t_7	non-classical monocytes maturation	t_{41}	classical monocytes to M2b activation
t_8	lymphoid line	t_{42}	M1 activity
t_9	formation of T-cells	t_{43}	M2c activity
t_{10}	erythrogenesis	t_{44}	M2b activity
t_{11}	presenting antigens	t_{45}	M2a activity
t_{12}	anti-inflammatory stimulation	t_{46}	recruitment of classical monocytes into tissue
t_{13}	Kupffer cells M2a activation	t_{47}	Kupffer cells self-renewal
t_{14}	LC to M2b activation	t_{48}	microglial self-renewal
t_{15}	Kupffer cells M2c activation	t_{49}	Alveolar macrophage self-renewal
t_{16}	HSCs self-renewal	t_{50}	LC self-renewal
t_{17}	transport to blood classical monocytes	t_{51}	increase in the recruitment of classical monocytes into tissue
t_{18}	transport to blood non-classical monocytes	t_{52}	IL-13 i IL-4 input
t_{19}	return to the marrow of classical monocytes	t_{53}	Fc*gamma*R and TLR input
t_{20}	proinflammatory stimulation	t_{54}	T-cells activation by IL-12
t_{21}	alveolar macrophage to M2b activation	t_{55}	T-cells activity
t_{22}	microglial to M2b activation	t_{56}	TGF-β activity
t_{23}	Kupffer cells to M2b activation	t_{57}	IL-10 activity
t_{24}	microglial to M2c activation	t_{58}	ROS output
t_{25}	alveolar macrophage to M2c activation	t_{59}	NO output
t_{26}	LC to M2c activation	t_{60}	T-cells activation by IL-23
t_{27}	microglial to M2a activation	t_{61}	IFN output

Transition	Biological meaning	Transition	Biological meaning
t_{28}	alveolar macrophage to M2a activation	t_{62}	IL-6 output
t_{29}	LC to M2a activation	t_{63}	IL-12 output
t_{30}	classical monocytes to M1 activation	t_{64}	IL-1 output
t_{31}	non-classical monocytes to M1 activation	t_{65}	TNF output
t_{32}	LC to M1 activation	t_{66}	IL-23 output
t_{33}	alveolar macrophage to M1 activation		

Table 3: The references to transitions

References	Transition
[6]	$t_0, t_1, t_2, t_3, t_4, t_8, t_{10}, t_{11}, t_{16}, t_{52}, t_{53}, t_{54}, t_{58}, t_{59}, t_{61}, t_{62}, t_{63}, t_{64}, t_{65}, t_{66}$
[12]	$t_0, t_1, t_2, t_3, t_4, t_5, t_6, t_7, t_{13}, t_{14}, t_{15}, t_{16}, t_{17}, t_{18}, t_{19}, t_{20}, t_{21}, t_{22}, t_{23}, t_{24}, t_{25}, t_{26}, t_{27}, t_{28}, t_{29}, t_{30}, t_{31}, t_{32}, t_{33}$
[18]	$t_{42}, t_{43}, t_{44}, t_{45}, t_{51}$
[19]	$t_{12}, t_{42}, t_{43}, t_{44}, t_{45}, t_{46}$

5. Analysis of the model

The net is covered by 126 t-invariants. Moreover, there are six multi-element MCT sets and 42 trivial (i.e., single-element) MCT sets.

The biological meaning of t-invariants has been established on the basis of the analysis of the significance of the transitions from the support of particular t-invariants. Covered the entire Petri Net by t-invariants, and determining the biological significance for each of them, is a key step in the analysis of biological Petri Net models.

Invariant x_1 corresponds to flow of classical monocytes between the bone marrow and blood. Invariant x_6 describes outside activity of IL-10 and TGF-β caused by anti-inflammatory stimulation. The Tables 4 and 5 describe the remaining invariants that reflect the tissue macrophages differentiation and bone marrow-derived macrophages differentiation to all phenotypic classes, as well as their activity, including the ability to stimulate T cells. Table 4 shows the bone marrow derived macrophages and the resident macrophages, differentiating only into one of specific phenotypes. In Table 5 there are described those t-invariants which depict the formation of mixed populations. In this group a major phenotype class arises from non-classical monocyte, and the second phenotype from classical monocytes. There do not exist invariants indicating that tissue macrophages are involved in the formation of mixed populations. Also not found any

t-invariant, which suggests formation of a mixed population consisting more than two phenotypes.

In the model there exist six multielement MCT sets shown in the Table 6 and 42 Trivial MCT-transitions shown in the Table 7. MCT m_1 corresponds to monocytes formation in the bone marrow and maturation of non-classical monocytes. The set of m_2 presents M1 macrophages activity for the formation of cytotoxic compounds like ROS and RNS. Set m_3 comprises three output transitions corresponding to outflow of TNF-α, IL-1 and IL-6 from the system. m_4 and m_5 are two-element sets of transitions, corresponding to stimulation of macrophages to differentiation to M2a and M2b and the activity of these macrophages. Set m_6 also includes only two transitions - they correspond to synthesis and outflow of IFN-γ from the system.

To find additional biological links between elements of the model, clusters analysis was performed. The t-invariants have been clustered, using several algorithms: UPGMA, Centroid method, complete linkage, McQuitty's method, median method, single linkage and Ward's method, including the following metrics: Correlation, Pearson, Minkowski distance, Maximum distance, Canberra distance, Manhattan distance, Euclidean distance and binary distance. Calinski-Harabasz index [2] and Mean Split Silhuette (MSS) [14] were used to appoint the best algorithm and the best similarity measure with the optimal numbers of clusters. The MMS allow to determine the best clustering method, by checking how each t-invariants is matched to given class. The high CH value means the number of clusters generated by tested clustering method is better, than clustering with lower CH value.

The high values of both coefficients showed clustering using the Pearson and Correlation measures. Finally, clustering by UPGMA algorithm with Pearson similarity measure has been chosen. It grouped t-invariants into six t-clusters (see in Table 8). In this case, the coefficient values were high on mean for the entire clustering, as well as for each cluster separately. In addition, this clustering contains only one single transition clusters.

Single-element cluster c_1 corresponds to circulation of classic monocytes between bone marrow and bloodstream (invariant x_1). All other clusters contain invariants corresponding to formation of monocytes in the bone marrow and their transport into a blood and transformation of classical monocytes into non-classical ones, by the loss of expression, and increasing a recruitment of classical monocytes into the tissue by proinflammatory factors. Clusters c_2, c_3 and c_6 contain t-invariants that represent T-cells activation, wherein in c_2 and c_3 clusters T-cells are activated by IL-12, while in cluster c_6 activation is influenced by two interleukins, i.e., IL-12 and IL-23. In clusters c_4 and c_5, there is no corresponding t-invariants to the T cells activation. In turn, the input transitions corresponding to the proinflammatory and anti-inflammatory factors that stimulate macrophages to activate the relevant phenotypes, i.e., M1, M2a and M2b are present in all the clusters. However, an input transition for the external stimulation for differentiating of macrophages to the M2c type occurs only in the cluster c_3. The obtained clusters demonstrate differences in the presence of t-invariants corresponding to the differentia-

tion of macrophages (tissue and the bone marrow-derived) to the different phenotypes, and activity of the differentiated macrophages:

- Cluster c_2 (11 t-invariants): contains invariants corresponding to the differentiation of tissue macrophages to M2a, non-classical monocytes to M2a and M2b and classical monocytes to M2a.
- Cluster c_3 (18 t-invariants): contains invariants corresponding to the differentiation of tissue macrophages to M2c, non-classical monocytes to all classes of phenotypes and classical monocytes to M2a and M2c.
- Cluster c_4 (11 t-invariants): contains invariants corresponding to the differentiation of tissue macrophages to M2b, non-classical monocytes to M2a, M2a, M2c and classical monocytes to M2b.
- Cluster c_5 (17 t-invariants): contains invariants corresponding to the differentiation of tissue macrophages to M1, non-classical monocytes to all classes of phenotypes and classical monocytes to M2a, M2b and M1.
- Cluster c_6 (68 t-invariants): contains invariants corresponding the differentiation of tissue macrophages M1 and M2b, and differentiation of both non-classical and classical monocytes to all classes of phenotypes.

Table 4: t-invariants corresponding to various ways of T-cell activation. The homogeneous population of macrophages.

M1	tissue macrophages	x_{77}	x_{78}	x_{79}	x_{80}	x_{81}	x_{82}	x_{83}	x_{84}
		x_{85}	x_{86}	x_{87}	x_{88}	x_{89}	x_{90}	x_{91}	x_{92}
	non-classical monocytes	x_{73}	x_{74}	x_{75}	x_{76}				
M2a	tissue macrophages	x_2	x_3	x_4	x_5				
	non-classical monocytes	x_{35}							
M2b	tissue macrophages	x_{11}	x_{12}	x_{13}	x_{14}	x_{23}	x_{24}	x_{25}	x_{26}
	non-classical monocytes	x_{69}	x_{72}						
M2c	tissue macrophages	x_7	x_8	x_9	x_{10}				
	non-classical monocytes	x_{52}							

The table shows t-invariants corresponding to differentiation of tissue and non-classical macrophages to only one of phenotypic population. t-invariants corresponding to T-cell activation by IL-23 are marked in blue, those corresponding to activation of T-cells by IL-12 are marked in green while in yellow are marked t-invariants corresponding to activation of T-cells by both of these interleukins. The t-invariants which are not marked correspond to the cases, where the path of T-cell stimulation is not active.

Table 5: t-invariants corresponding to various ways of T-cell activation. The mixed population of macrophages.

M1	M1	x_{65}	$x_{66}\uparrow$	x_{67}	$x_{68}\uparrow$	x_{69}	$x_{70}\uparrow$	x_{71}	$x_{72}\uparrow$	
	M2a	x_{76}	$x_{82}\uparrow$	x_{79}	$x_{85}\uparrow$	x_{77}	$x_{83}\uparrow$	x_{80}	$x_{86}\uparrow$	
	M2b	x_{105}	$x_{107}\uparrow$	$x_{116}\uparrow$	x_{119}	$x_{121}\uparrow$	x_{106}	$x_{108}\uparrow$	x_{120}	$x_{122}\uparrow$
	M2c	x_{88}	$x_{94}\uparrow$	x_{91}	$x_{97}\uparrow$	x_{89}	$x_{95}\uparrow$	x_{92}	$x_{98}\uparrow$	
M2a	M1	x_{42}	$x_{43}\uparrow$	x_{48}	$x_{49}\uparrow$	x_{44}	$x_{45}\uparrow$	x_{50}	$x_{51}\uparrow$	
	M2a	x_{36}	$x_{37}\uparrow$							
	M2b	x_{40}	$x_{41}\uparrow$	x_{46}	$x_{47}\uparrow$					
	M2c	x_{38}	$x_{39}\uparrow$							
M2b	M1	x_{101}	$x_{102}\uparrow$	x_{115}	x_{103}	$x_{104}\uparrow$	x_{117}	$x_{118}\uparrow$		
	M2a	x_{75}	$x_{81}\uparrow$	x_{78}	$x_{84}\uparrow$					
	M2b	x_{99}	$x_{100}\uparrow$	x_{113}	$x_{114}\uparrow$					
	M2c	x_{87}	$x_{93}\uparrow$	x_{90}	$x_{96}\uparrow$					
M2c	M1	x_{59}	$x_{60}\uparrow$	x_{65}	$x_{66}\uparrow$	x_{61}	$x_{62}\uparrow$	x_{67}	$x_{68}\uparrow$	
	M2a	x_{53}	$x_{54}\uparrow$							
	M2b	x_{57}	$x_{58}\uparrow$	x_{63}	$x_{64}\uparrow$					
	M2c	x_{55}	$x_{56}\uparrow$							

The table shows t-invariants corresponding to macrophage differentiation leading to the formation of mixed populations. The first column shows populations of macrophages resulting from the non-classical monocytes. In the second column there are subpopulations arising from classical monocytes. As in Table 4 t-invariants corresponding to T-cell activation by IL-23 are marked in blue, those corresponding to activation of T-cells by IL-12 are marked in green while in yellow are marked t-invariants corresponding to activation of T-cells by both of these interleukins. The t-invariants which are not marked correspond to the cases, where the path of T-cell stimulation is not active. The arrow at an invariant indicates the occurrence of an increase in the recruitment of classical monocytes by inflammatory agents.

Table 6: List of non-trivial MCT sets

MCT set	**Contained transitions**	**Biological meaning**
m_1	$t_0, t_1, t_2, t_3, t_4, t_5, t_7, t_8, t_9, t_{10}, t_{11}, t_{16}, t_{18}$	monocytes formation in the bone marrow and maturation of non-classical monocytes
m_2	t_{42}, t_{58}, t_{59}	M1 macrophages activity for the manufacture of cytotoxic compounds like ROS and RNS.

MCT set	Contained transitions	Biological meaning
m_3	t_{62}, t_{64}, t_{65}	outflow of TNF-α, IL-1 and IL-6 from the system
m_4	t_{44}, t_{53}	stimulate macrophages to differentiate to M2b and the M2b activity
m_5	t_{45}, t_{52}	stimulate macrophages to differentiate to M2a and the M2a activity
m_6	t_{55}, t_{61}	production and outflow IFN-γ from the model

Table 7: Trivial MCT sets

Transitions composing trivial MCT sets
$t_6, t_{12}, t_{13}, t_{14}, t_{15}, t_{17}, t_{19}, t_{20}, t_{21}, t_{22}, t_{23}, t_{24}, t_{25}, t_{26}, t_{27}, t_{28}, t_{29}, t_{30}, t_{31}, t_{32}, t_{33}, t_{34}, t_{35}, t_{36}, t_{37}, t_{38}, t_{39}, t_{40}, t_{41}, t_{43}, t_{46}, t_{47}, t_{48}, t_{49}, t_{50}, t_{51}, t_{54}, t_{56}, t_{57}, t_{60}, t_{63}, t_{66}$

Table 8: Clusters composition

t-cluster	t-invariants
c_1	x_1
c_2	$x_2, x_3, x_4, x_5, x_{35}, x_{36}, x_{37}, x_{75}, x_{78}, x_{81}, x_{84}$
c_3	$x_6, x_7, x_8, x_9, x_{10}, x_{38}, x_{39}, x_{52}, x_{53}, x_{54}, x_{55}, x_{56}, x_{87}, x_{88}, x_{90}, x_{93}, x_{94}, x_{96}$
c_4	$x_{11}, x_{12}, x_{13}, x_{14}, x_{40}, x_{41}, x_{57}, x_{58}, x_{69}, x_{99}, x_{100}$
c_5	$x_{15}, x_{16}, x_{17}, x_{18}, x_{42}, x_{43}, x_{59}, x_{60}, x_{70}, x_{76}, x_{82}, x_{101}, x_{102}, x_{105}, x_{107}, x_{109}, x_{110}$
c_6	$x_{19}, x_{20}, x_{21}, x_{22}, x_{23}, x_{24}, x_{25}, x_{26}, x_{27}, x_{28}, x_{29}, x_{30}, x_{31}, x_{32}, x_{33}, x_{34}, x_{44}, x_{45}, x_{46}, x_{47}, x_{48}, x_{49}, x_{50}, x_{51}, x_{61}, x_{62}, x_{63}, x_{64}, x_{65}, x_{66}, x_{67}, x_{68}, x_{71}, x_{72}, x_{73}, x_{74}, x_{77}, x_{79}, x_{80}, x_{83}, x_{85}, x_{86}, x_{89}, x_{91}, x_{92}, x_{95}, x_{97}, x_{98}, x_{103}, x_{104}, x_{106}, x_{108}, x_{111}, x_{112}, x_{113}, x_{114}, x_{115}, x_{116}, x_{117}, x_{118}, x_{119}, x_{120}, x_{121}, x_{122}, x_{123}, x_{124}, x_{125}, x_{126}$

6. Conclusions

The previously published [25] our basic model focused mainly on the influence of the micro-environment on the macrophage differentiation process. The main problem that occurred during the building of the model was the discrepancy between knowledge and the availability of accurate, quantitative and qualitative data, which are required by the formalism of Petri nets. The model that has been build and then analyzed in this paper has been extended (as compared to the previous one) by mutual macrophages interactions. It should be underlined that the activity of the already differentiated macrophages to the particular phenotype is very important, because it affects next differentiating

macrophages. The products of the activity of every phenotype are factors that directly affect the subsequent differentiation of macrophages or indirectly affect this differentiation, inter alia by the activation of T-cells. The analysis of the extended model allowed to observe new relationships between the cells.

The assumption of the model was based on the possibility of differentiation of all types of macrophages to both of the phenotypic classes. The origin of macrophages and an influence of micro-environmental factors affect a likelihood of the acquisition of the specific phenotypic activity by them. Proinflammatory factors, contributing to the activation of phenotype M1, stimulate the recruitment of classical monocytes from the bloodstream to the target tissues, in order to enhance macrophages population that is involved in the local inflammation.

From the analysis of the model there have been drawn several conclusions. It has been revealed that macrophages recruited into tissue, by inflammatory stimulation, can differentiate into all phenotypic classes, not only to the proinflammatory one (M1). This means that the proinflammatory factors can initiate the anti-inflammatory response of macrophages M2, by increasing the pool of macrophages in a tissue. In the model there are also t-invariants suggesting that the stimulation of monocytes migration by proinflammatory factors leads to the increased maturation of macrophages (to the anti-inflammatory phenotypes) from the M2 macrophages without leading to the activation of the proinflammatory M1 phenotype. Furthermore, due to high inflows of classical monocytes into a tissues it is possible the rise of a mixed population of macrophages in the immune response by simultaneous activation of a part of the macrophages to the different phenotypes. In the model mixed populations of macrophages contain only two phenotypes in which classical macrophages play only auxiliary roles and independently does not differentiate into a homogeneous population.

Although various types of macrophages differ in their activity considerably and also in their secreted substances, IL-10 was always produced in the analyzed network. For this reason an external stimulation of macrophages for differentiation to the phenotype M2c has no real effect on the formation of such macrophages. Macrophages M2c are formed only as a result of macrophage activity of other classes of phenotypes, which produce IL-10.

M1 and M2b macrophages can activate T-cells, but it does not always happen. Macrophages M1, regardless of the origin, produce IL-12 and IL-23, however, it does not always lead to the activation of T-cells. Such activation may only take place with the participation of one of the cytokines or both of them. Similarly, macrophages M2b always produce IL-12, but this does not always lead to activation of the T-cells.

The above findings, formulated on the basis of t-invariants, MCT sets and clustering analyzes, could be interesting starting points for further study of macrophage differentiation mechanisms. A further work on modeling and analysis of macrophage differentiation will focus on the impact of other immune system cells on the studied phenomenon and mutual impact of interleukins and cytokines on macrophages.

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