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ORIGINAL ARTICLE

Features of monocyte-derived dendritic cells encompassing a rare subpopulation of cells that are capable of natural internalization of extracellular dsDNA

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ABSTRACT. The present study demonstrates that monocyte-derived dendritic cells (moDCs) produced *in vitro* using a GM-CSF and IFN-α differentiation protocol encompass a rare (~5%) subpopulation of cells showing classical dendritic cell morphology and capable of natural internalization of extracellular self-DNA. We established that DEFB, HMGB1, LL-37 and RAGE antigens, which mediate the process of DNA internalization, are expressed on the surface of moDCs similar to plasmacytoid dendritic cells. However, in constrast to the latter subpopulation, these cells do not produce interleukin (IL)-37. Nonetheless, the process of DNA internalization was not in direct relation to the presence of the above antigens on the surface of these cells. Dendritic cells were sorted into total and non-DNA-internalizing populations and cytokine production was analyzed at 24-48 hours post-DNA treatment. We show that massive secretion of cytokines by dendritic cells is associated with the dsDNA-internalizing subpopulation. A total pool of IFN-moDCs secrete pro-inflammatory "first-wave" cytokines (IL-2, IL-6, IL-8, TNF-α) at both 24 and 48 hours time points. The anti-inflammatory cytokines IL-4 and IL-10 were found to be modestly induced, whereas GM-CSF, G-CSF, and IFN-γ production was strongly induced. Treatment of moDCs with dsDNA results in the up-regulated transcription of IFN-α, IFN-β, IFN-γ, IL-8, IL-10, and VEGF by 6 hours. Combined dsDNA + chloroquine treatment has a synergistic effect on transcription of only one of the genes tested, with the pro-inflammatory cytokine IFN-β displaying the strongest fold induction by 24 hours.

Key words: cytokines, chloroquine, DNA internalization, receptor, TAMRA, TLR9

INTRODUCTION

Dendritic cells (DCs) belong to the class of antigen-presenting cells responsible for the development of an adaptive immune response and originate from the bone marrow resident CD34+ hematopoietic stem cells [1-5]. According to the most recently accepted classification based on differentiated expression of key transcription factors, such as interferon-regulating factors 8 and 4 (IRF8 and IRF4), three main types of DCs having different origin, functions, as well as the expression patterns of surface marker molecules and genes, are usually recognized [5]. They are plasmacytoid DC (pDC), conventional myeloid (classical) DC1 (cDC1), and conventional myeloid (classical) DC2 (cDC2), formerly known as CD141+ and CD1c+ DCs. The recent data, based on the "time of flight" cytometry and single-cell RNA sequencing, allow recognizing the sets of marker molecules that characterize the three indicated types of DC more specifically.

Plasmacytoid DCs are present in the blood as well as in peripheral lymph organs. These cells display low MHC expression, yet following activation they are capable of massive secretion of type I IFN molecules (IFN-α, β). These cells become highly activated following viral infection [2, 6-10]. This type of cells does not express the myeloid-specific antigens CD11c, CD33, CD11b, CD13, but demonstrates the expression of CD303 (CLEC4C; BDCA-2), CD304 (neiripilin; BDCA4),

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CD85K (IL3T), CD85g (ILT7), FsεR1, BTLA, DR6 (TNFRSF21; CB358), and CD300A [5, 11-15].

Human myeloid cDC1 display high level of CD141 (BDCA-3) expression. They share common marker antigens, such as CD13 and CD33, with cDC2, but differ in low expression of CD14, CD11c, CD11b, or CD172 (SIRPα). The expression of CLEC9A, CADM1 (NECL2), BTLA, and XCR1 antigens is also being specific for this DC subtype [16, 17].

The cDC2 subtype of myeloid DC expresses the CD1c, CB2b, FsεR1, SIRPA, CD11b, CD11c, CD13, and CD33 antigens, which along with the loss of cDC1-specific markers features this DC subtype. The data obtained from the analysis of the RNA expression profile (RNAseq) revealed additional specific markers of cDC2, namely CLEC10A (CD301a), VEGFA, FCGR2A (CD32A) [5, 18]. The feature of myeloid DCs is their strong surface expression of MHC molecules as well as participation in the initiation and boosting of the T cell response.

To study the properties of DCs, cells both isolated from the organism and generated *ex vivo* are used. Most of the data characterizing the molecular processes occurring in human DC have been obtained using DC cultures derived from blood monocytes. There are several protocols for generating human DCs. The most commonly used protocol involves the cultivation of blood monocytes in the presence of GM-CSF and IL-4, and is extensively described in the investigation [19]. Another protocol is characterized by the different sets of activating factors. In this case, the adherent fraction of blood monocytes is being cultured in the presence of GM-CSF and IFN-α. IFN-generated, monocyte-derived DCs (IFN-moDCs) were used in our investigation as the cellular model allowing us to analyze the molecular and cellular pathways in DCs. Hence, we summarize and compare the basic features of these cells.

Phenotypic analysis of IFN-moDCs showed the following expression pattern: monocyte marker CD14 – 22.2 ± 3.6%, DC maturation marker CD83 – 34.6 ± 3.7%, activation marker of mature DCs CD25 – 25.1 ± 3.5%. More than half of the generated IFN-moDCs (65.2 ± 4.2%) were positive for CD86. As for the expression of CD1a and CD123, these markers are not universal for IFN-moDCs and are found on 10.4 ± 2.0% and 40.1 ± 3.4% of IFN-moDCs that is in line with other studies. The tested population of DCs was free of CD3 expressing lymphocytes [20].

Phenotypic heterogeneity is a hallmark of IFN-moDCs, and there is no single unique marker to their identification. Moreover, these cells significantly differ from DCs generated in the presence of GM-CSF and IL-4. IFN-moDCs retained a higher CD14 expression after maturation but down-regulate CD1a to levels lower than those on mature IL-4-moDCs [21]. The IFN-induced differentiation is irreversible and, in contrast to that driven by GM-CSF and IL-4, persists upon the removal of the cytokines [22, 23]. Exposure of monocytes to GM-CSF plus type I IFNs leads, within 3 days, to loss of plastic adherence, and appearance of typical DC morphology [22-24]. These cells maintain CD14 expression, but are not macrophages, because they are presented as floating non-adherent cells with thin and long dendrites. IFN-moDCs express MHC molecules class I and II, co-stimulatory markers (CD25, CD40, CD80, and CD86), adhesion molecules (CD54, CD58) and cellular factors involved in antigen uptake and processing, CD8+ T cell cross-priming, and in priming of CD4+ T lymphocytes [22-27]. IFN-moDCs exhibit a combined phenotype as they display myeloid and plasmacytoid DC features associated with natural killer cell characteristics [25]. In addition to the expression of CD123, BDCA4, and low levels of CD209/DC-SIGN, these cells also express CD56 as well as cytotoxic effector molecules, like the granzymes B and M, TRAIL, and defensin-α1, which are important components of the cytotoxic arsenal of natural killer cells. Last, IFN-moDCs also express a large number of TLRs including TLR1, 2, 3, 4, 5, 6, and 8. Notably, in marked contrast to IL-4-moDCs, IFN-moDCs also express high levels of TLR7 [26], which is classically found in plasmacytoid DCs.

Despite their "partially" mature phenotype, IFN-moDCs proved to be fully susceptible to undergo activation/terminal differentiation after stimulation with TLR (*i.e.*, LPS, polyI-C, ssRNA) or CD40 ligands, as revealed by the enhanced expression of accessory molecules as well as by a massive CD83 induction [22, 23]. Upon TLR or CD40 triggering, IFN-moDCs release IL-12p70, IL-23, IL-27, IL-1β, IL-6, and TNF-α and increase the expression of IL-10, IL-15, and IL-18 [22, 26, 27]. Interestingly, it has been recognized that many of the stimuli-promoting DCs maturation, including TLR ligands, monocyte conditioned medium, HSV or imiquimod, also induce the production of large amounts of type I IFNs.

Comparative analysis has revealed that IL-4-DCs and IFN-moDCs did not differ in their key functional characteristics, *i.e.*, in their ability to stimulate proliferation of T-cells in response to alloantigens as well as to induce generation of T-regulatory cells in mixed leukocyte culture. IFN- and IL-4-induced DCs possess similar ability of boosting T-cells to produce Th1/pro-inflammatory (IFN-γ, IL-2, IL-1β, TNF-α, IL-12, IL-17) and Th2/anti-inflammatory cytokines (IL-4, IL-6, IL-10, IL-13), growth factors (G-CSF, GM-CSF, IL-7), and chemokines (IL-8, MIP-1β). Nevertheless, IFN-DCs have a more pronounced stimulatory effect upon the Th1 and Th2 cells, thus manifesting as a significantly higher IFN-γ, IL-5, and MIP-1β production. IFN-DCs were characterized by more prominent ability to activate Th1-cells, and by moderate Th2-stimulatory activity, which is absent in IL-4-DCs [28]. A direct comparative analysis of two *ex vivo* generated populations of DC characterizes them precisely as the cultures of DCs and testifies to their functional identity both among themselves and with above-described subtypes of DCs. Such an observation presumes the possibility to extrapolate the results obtained in experiments with the IFN-moDCs population to other types of DCs, both native and *ex vivo* generated.

To exert their antigen-presenting properties, DCs of any origin have to be activated. Major molecular players and pathways involved in DC activation are well-characterized. Such activators are exogenous

infectious ligands (pathogen-associated molecular patterns, PAMPs) and endogenous molecules that are released during host tissue injury/death (damage associated molecular patterns, DAMPs) [29-32].

To generate mature DCs *ex vivo,* standard maturation stimuli such as TNF-α in combination with IL-1β and prostaglandin E2 [33], LPS, polyI-C, ssRNA, CD40 ligands, monocyte conditioned medium, HSV or imiquimod [28] are commonly used.

It is known that double-stranded DNA of various origins, in various forms and independently of the nucleotide sequence, is capable of activating DCs and macrophages [34-39]. Stimulating effect of DNA is being exerted as increased expression of MHC class II antigens and co-stimulatory molecules, as well as the synthesis of immunomodulatory cytokines, which largely determines the direction and severity of immune responses [34, 36-38, 40-42]. Many of the discrepancies between the studies of DNA immunogenicity may originate from the differences in the exact source of the DNA, the way it was delivered to the cells and the very cells that were analyzed. Bacterial DNA is known to be a potent immunostimulant due to its high content of non-methylated CpG dinucleotides, with immune reaction unfolding *via* a cytosolic TLR9 sensor. Similarly, mammalian DNA may also launch an immune response through its non-methylated CpG islands (for instance, those present in the telomeric sequences) interacting with TLR9. Yet, a number of additional cytosolic sensors have also possibly been implicated in immunomodulatory activity of self-DNA [43-45], which may indicate the presence of alternative pathways of DCs activation by the extracellular DNA in double-stranded form. Thus, it has been shown that cytosolic DNA sensor stimulator of interferon genes (STING) – but not classical Toll-like receptor (TLR)-myeloid differentiation primary response gene 88 (MyD88) pathway – is required for type I IFN production. DNA is released from apoptotic cells and taken up by DCs resulting in the activation of STING and the production of type I IFN, which activates DCs for antigen cross-presentation [46].

In order to activate a DC, the extracellular DNA have to get into its internal compartments. For example, bacterial DNA complexed with bacterial wall components (LPS) is delivered to the cell interior *via* TLR4 pathway where its unmethylated CpGs are sensed by TLR9. This leads to the activation of type I IFN production and launching of the immune response [10, 45, 47-53]. Relatively little is known about the molecular mechanisms that underlie the internalization of extracellular dsDNA, either exogenous non-bacterial or self-DNA, into DCs. Until recently, self-dsDNA was believed to become internalized by DCs *via* some form of endocytosis [43, 54-57]. Nonetheless, reports from the past several years argue that free dsDNA found in the blood plasma or interstitial space may become internalized by DCs *via* a receptor-mediated mechanism. Plasmacytoid DCs have been shown to engulf extracellular self-DNA *via* LL37, HMGB1, RAGE, or DEFB, which results in their further maturation [57-67]. And thus, the long-time existing paradigm of tolerance of antigen-presenting DCs to "self-DNA" can now be reconsidered.

The ability of extracellular dsDNA to activate antigen-presenting properties of DCs has long been studied in our group. Throughout our experiments, a standardized dsDNA preparation is used. This preparation referred to as Panagen has been extensively characterized in phase II clinical studies [68] and is a drug substance certified in both Russian and international regulatory agencies. We produced monocyte-derived DCs using a GM-CSF + IFN-α protocol (IFN-moDCs), which results in formation of myeloid-type DCs. It has been reported in multiple studies that adding fragmented dsDNA as a maturation stimulus boosts antigen-presenting properties of IFN-moDCs to the level comparable to that achieved with LPS or TNF-α [69, 70]. Furthermore, our *in vivo* experiments indicate that DCs activated this way displayed pronounced enhancement of antitumor immune response, as suggested by their significant suppression of tumor graft growth [71-73]. We asked whether DCs activation was dsDNA internalization-mediated or not. Our studies as well as literature data suggested that professional properties of DCs were indeed dependent on internalization of dsDNA into DC compartments [45, 49, 51, 70, 74-79].

In the present study, we explored the dynamics and the effects of TAMRA-labeled dsDNA probe internalization by a rare subpopulation of IFN-moDCs that display classical DC morphology. We observed no direct functional (receptor-ligand) connection between DNA internalization into IFN-moDCs and the surface expression of several internalization factors (DEFB, HMGB1, LL-37 and RAGE) that were reported to mediate DNA internalization into plasmacytoid DCs. We next characterized dsDNA-induced expression changes in a panel of cytokines at both the protein (17 Plex, 8 Plex) and the transcript (qPCR) levels. Up-regulated expression and secretion of cytokines in activated DCs are correlated with the presence of the cell subpopulation that internalizes dsDNA.

Finally, we show that co-treatment of DCs with chloroquine and dsDNA results in elevated expression of *IFN*-β only, not affecting the mRNA expression of other cytokines activated by chloroquine solely.

METHODS

In vitro *generation of IFN-moDCs*

Peripheral blood mononuclear cells (MNCs) were obtained by density gradient centrifugation (Ficoll-Paque, Sigma-Aldrich) of heparinized whole blood samples from 3 healthy volunteers. Informed consent was obtained from all donors. IFN-moDCs were generated by culturing plastic-adherent MNC fraction in RPMI-1640 medium (Sigma-Aldrich) supplemented with 0.3 g/l L-glutamine, 5×10^{-3} M HEPES buffer, 0,1 g/l gentamicin, and 2.5% fetal calf serum (FCS, Sigma-Aldrich) in the presence of rhGM-CSF (4×10^{-5} g/l, Sigma-Aldrich) and rhIFN-α (Roferon-A, 10^6 U/l, Roche, Switzerland) for 4 days at 37 °C and in a 5% CO_2 atmosphere [20]. The viability of obtained IFN-moDCs determined by Trypan blue exclusion

was above 85-95%. The cell yield was an average of $(0.1 \pm 0.009) \times 10^6$ IFN-moDCs/10^6 MNCs. All the experiments were performed with freshly generated DCs.

Internalization of TAMRA-DNA

DCs were incubated with Alu-TAMRA-DNA (3×10^{-7} g/1 $\times$ 10^6 cells) for 1 hour at room temperature in the dark as described [80]. Cells were washed, resuspended in PBS, and subjected to microscopy analysis.

Fluorescence microscopy

Slides were prepared using cytospin and a drop of DABCO antifade supplemented with 4×10^{-4} g/l DAPI was added. Fluorescence microscope Axioskop 2 plus (using AxioVision LE software) was used for imaging and analysis.

Quantification of pGFP plasmid DNA internalization

0.7 million DCs were incubated with $6{,}6 \times 10^{-7}$ g of HindIII-digested pGFP (4733 bp) for 1 hour at room temperature. The cells were washed three times and treated with DNase I (0.05 g/l) at 37 °C for 1 hour. The cells were washed three more times and treated with proteinase K (0.2 g/l) at 58 °C for 1 hour. After washing, the cells were lysed in 1% SDS, 0.1 M EDTA, and the lysate was subjected to phenol-chloroform extraction and isopropanol precipitation. DNA pellet was dissolved in 2×10^{-4} l water. Each rtPCR reaction used 5×10^{-6} l of DNA template. DNA quantification in the samples of interest was done using a standard curve approach.

Human DNA preparation

Human DNA preparation was manufactured from placentae of healthy women (negative for HIV, syphilis, hepatitis B and C). Phenol-free method was used to isolate total DNA. DNA was sonicated down to the fragments ranging 200-6000 bp and filtered through the 0.22 mkm filter. This preparation is a pharmacopeial drug (patented under the trademark Panagen) registered as LSR No. 004429/08 of 09.06.2008).

17 plex and 8 plex analysis

DCs were incubated with 3×10^{-7} g Alu-TAMRA-DNA/million cells in a serum-free medium for 1 hour at room temperature in the dark. Cells were then flow sorted on Cell Sorter SH800 (Sony Biotechnology, USA) into pools of 2×10^5 TAMRA− cells (*figure 3A*) per well. The total pool of untreated by the TAMRA-labeled DNA probe IFN-moDCs, consisting of cells both capable and unable of capturing the TAMRA-DNA probe, were subjected to "sort-mimicking" procedure on the FACS with following sampling by 2×10^5 cells per well.

Human DNA was added to the cells to the final concentration of 0.01 g/l, with control cells left untouched. The cells were incubated for 24 or 48 hours in the dark in CO_2 incubator, and cell supernatants were then aspirated for cytokine Bio-plex analysis. Prolonged incubation of sorted DCs with DNA preparation was required to assure that the cells received enough DNA, which is consistent with the protocols used in our previous studies.

All analyses were performed with a magnetic bead suspension array kit of Bio-Plex Pro Human Cytokine 17 and 8 Plex panels according to the manufacturer's instructions. The results were analyzed using the Luminex LX100/200 platform and Bio-PlexManager 4.0 software (Bio-Rad Laboratories). The 17 Plex included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP1, MIP-1β, and TNF-α. The 8 Plex kit included IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, and TNF-α. For statistical purposes, values that were below the lowest limit of detection were assigned the value of the lowest limit of detection.

Immunofluorescent detection of LL37, HMGB1, RAGE, and DEFB

DCs were obtained from 6 healthy volunteers. 1,5-2×10^6 DCs were fixed in a 4% paraformaldehyde in PBS for 1 hour at +4 °C. The cells were spun down for 10 minutes at 400 g, and resuspended in 10^{-3} l PBS supplemented with 10% FCS to block nonspecific binding. Following 10 minutes incubation at room temperature, the cells were centrifuged at 400 g for 10 minutes, resuspended in PBS, and split into 10^{-4} l aliquots. Primary antibodies specific for HMGB1, LL37, DEFB, and RAGE (Sino Biological or Sony Biotechnology) were added to the cells to a final concentration of 0.015 g/l, and incubation proceeded at room temperature for 1 hour. Following two PBS washes, secondary antibodies (FITC Goat anti-mouse or DyLight 488 Donkey anti-rabbit conjugates) were added to the final concentration of 0.01 g/l and incubated for 1 hour at room temperature. Cells were then washed twice with PBS and transferred onto glass slides. 500-2000 cells per slide were routinely scored to establish the percentage of positive cells.

DC treatment for cytokine expression profiling

DCs were produced from donor monocytes using the GM-CSF + IFN-α protocol. DCs from three donors were used: i) intact DCs (control); ii) DCs + chloroquine (10^{-4} M); iii) DCs +dsDNA (0.01 g/l); iv) DCs + chloroquine (10^{-4} M) + dsDNA (0.01 g/l). The cells were incubated for 6 and 24 hours, followed by mRNA isolation and cDNA preparation. The concentration of 10^{-4} M for chloroquine was chosen based on the reported experimental data [81, 82].

cDNA synthesis

PolyA mRNA was isolated using an appropriate kit (Medigen, Russia). This material was then used for

cDNA synthesis (GoTaq 2-Step RT-qPCR System (Promega, USA)). The RNA and cDNA samples were stored at −70 °C.

Real-time qPCR

Real-time qPCR was performed using SYBR® Green PCR Master Mix and the qPCR machine from Applied Biosystems®. Data were internally normalized for the expression of *RPLP0* [83]. The HPLC-purified oligonucleotides used in the qPCR experiment have been purchased from Biosset Ltd and their sequences are shown in *table S1* in the supplementary material. Relative expression values were measured using the ΔΔCt approach. REST 2009 software was used for statistical data processing.

RESULTS

Internalization of TAMRA-DNA probe by IFN-moDCs

Previously, we used TAMRA-labeled DNA probe as a tool to dissect the process of DNA internalization by various stem- and stem-like cell populations [80, 84-87]. In the present study, we resorted to the standard and well-characterized technique of dsDNA internalization by IFN-moDCs. We show that 2-5% of cultured monocyte-derived IFN-moDCs become TAMRA-positive and that two modes of internalization exist (*figure 1A, B*). The first mode results in the cytoplasmic localization of the probe as dispersed spots. In the second case, labeled DNA is confined to vacuole-like structures. Judging from the signal intensity, the vacuole-like structures harbor much more of labeled DNA molecules. The fact that exactly the target DNA is being internalized is further supported by our qPCR experiments measuring the uptake of linearized plasmid DNA (pGFP) by DCs (*figure 1C*). We estimate that about 3×10^4 plasmid copies were present in each DNA-internalizing cell (which translates into ~1% of the haploid genome size), given that such cells constitute only 2-5% of the total cell population.

Surface profiling of IFN-moDCs for the presence of DEFB, HMGB1, RAGE, and LL37 specific factors implicated in internalization of extracellular DNA

Immune tolerance to self-DNA has long remained a paradigm; however, recent evidence suggests that extracellular DNA of non-bacterial origin may well reach the DCs in a specific antigene-mediated fashion thereby stimulating their antigen-presenting properties [58, 61-65]. In the context of plasmacytoid DCs, four proteins responsible for DNA internalization are known. These include anti-inflammatory peptides (LL37, DEFB), HMGB1, and RAGE. We performed immunostaining of monocyte-derived IFN-moDCs (n=6) using antibodies specific for these molecules. All examined antigens are detectable on the surface of IFN-moDCs (*figure 2*).

Next, we asked whether these antigens and TAMRA-DNA probe may colocalize (*table 1*).

Only the minority of RAGE-, HMGB1-, and DEFB-positive cells are capable of internalizing extracellular DNA both antigen-positive and antigen-negative cells were shown to internalize DNA (*table 1*). In assays on co-detection of RAGE and HMGB1 surface antigens and internalized DNA, both antigen-positive and antigen-negative cells were shown to internalize DNA. In the similar assays on co-detection of DEFB antigen and internalized DNA, only DEFB-positive cells were found to internalize DNA.

Cytokine secretion is mediated by TAMRA+ IFN-moDCs

One of the salient features of professional DCs is their expression and secretion of a specific set of cytokines. Given that dsDNA internalization can be used as a convenient marker, we sought to compare cytokine production and secretion in IFN-moDCs contained TAMRA+ cells and TAMRA− subpopulations of IFN-moDCs.

In our initial experiments we treated IFN-moDC cultures with TAMRA-DNA with following FACS-sorting. As well as in previous similar experiments [85], it turned out that cells that have captured DNA lose their "cell durability factors", and thus DCs having a large surface are being destroyed upon centrifugation even at small G values. Consequently, whole DCs were extremely rare on cytological preparations. In this connection, and to standardize the conditions, the FACS-sorting procedure was used both to isolate the TAMRA− cells and to collect the total pool of IFN-moDCs consisting of cells both capable and unable to internalize TAMRA-labeled DNA probe, and that were not subjected to pretreatment by the TAMRA-labeled DNA (*figure 3A*). In this approach, it was important that the cells of both the samples were subjected to "sorting" procedure.

The cells were further incubated with the DNA preparation for different times and the level of cytokines was measured in a Plex assay. Using this approach with the total pool of IFN-moDCs, we estimated the integral response of the culture to activation by the dsDNA preparation. It is quite obvious that DNA-activated DCs of the minor subpopulation through secreted cytokines will affect the DCs unable to internalize the extracellular DNA fragments. Nevertheless, the only question had to be addressed in this work: is naked extracellular DNA able to activate the antigen-presenting properties of DCs without additional internalization factors? The production of cytokines in the population of IFN-moDCs would mean that the DCs had been activated. In the context of this task, it would not matter whether the major population of the DCs was paracrinically activated or not. Since up to now it is believed that DCs can be activated only upon internalization of extracellular DNA, the fact of initial activation can be related only to the minor subpopulation of IFN-moDCs, capable of internalizing extracellular dsDNA fragments by a natural way.

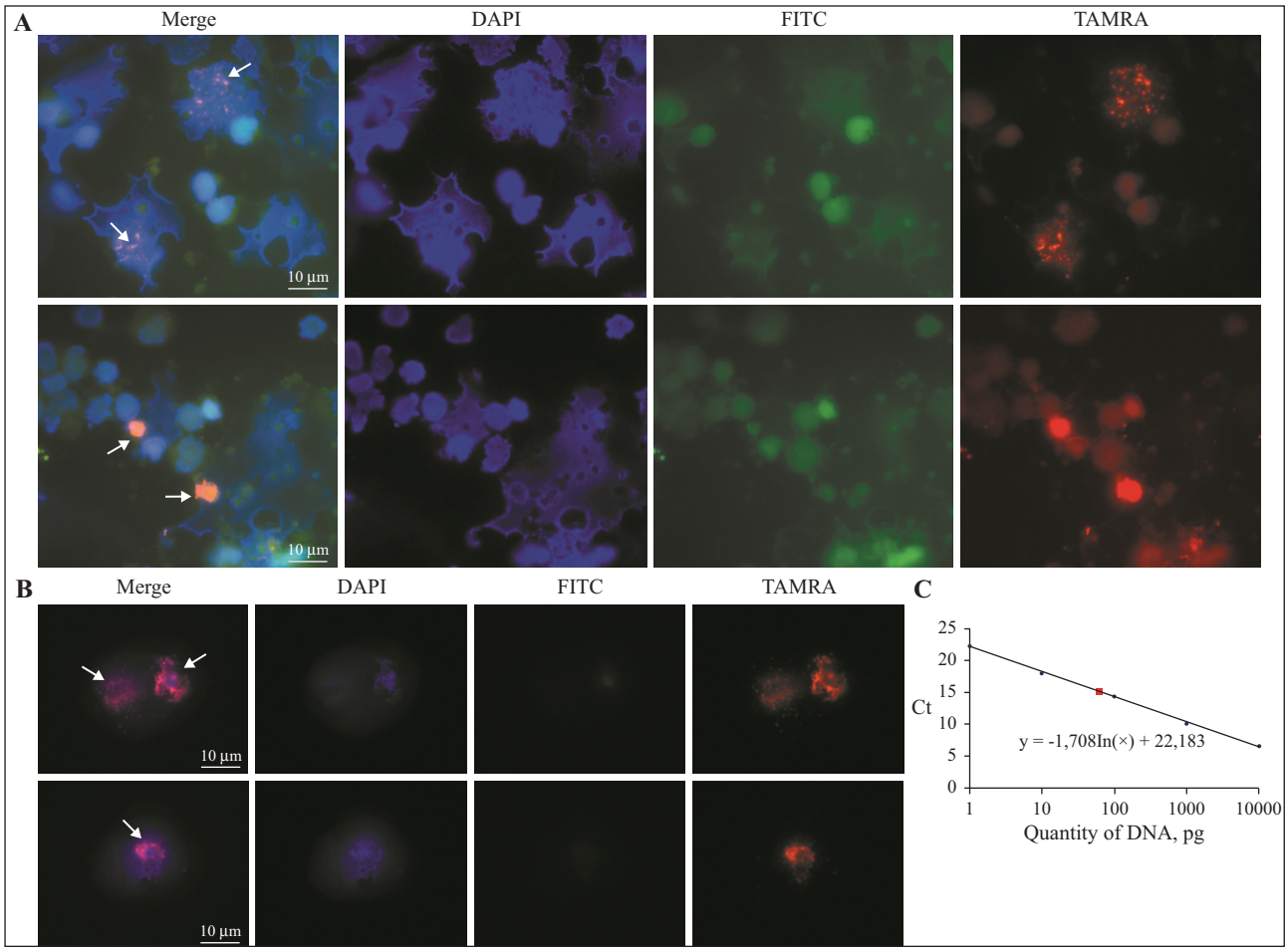

Figure 1

Internalization of TAMRA-labeled AluI PCR probe into DCs and monocytes. **A)** Mature DCs, DNA probe is detectable as either diffuse spots or in vacuole-like cytoplasmic structures; **B)** Immature monocytes. The arrows indicate TAMRA signal. The images were taken using the blue, green, and red filters to show that TAMRA signal is specific; **C)** qPCR analysis of pGFP internalization by DCs. Red square on the calibration plot indicates the qPCR datapoint that was used to calculate the number of plasmid molecules internalized by DCs. As a control, the plasmid DNA isolated both from the incubation medium as well as from DNase-treated wash supernatant was used. Specific PCR products were detected in samples of DNA isolated both from the incubation medium and from the DC. In the sample of DNA isolated from the DNase-treated wash buffer, no specific PCR products were detected (not shown).

On assessing the activation of the TAMRA− cells, it was necessary to take into account the fact that this population of DCs could be partially activated by the earlier activated DCs of the minor population during incubation time with TAMRA-labeled DNA and subsequent sorting procedure, which usually took about 3 hours.

Following the sorting, the cells were incubated for 24 or 48 hours with dsDNA preparation and supernatants were analyzed using 17 Plex or 8 Plex. The experiment was repeated twice using the cells from the same donor, and the data are presented in *figure 3*. Cytokine levels values (in pg/ml) are in *tables S2 and S3* in the supplementary material.

Elevated cytokine secretion was observed in the total pool of IFN-moDC population that contained TAMRA+ cells. Data on cytokine quantification performed on both Plex platforms are consistent and indicate that a total pool of IFN-moDCs secrete pro-inflammatory "first-wave" cytokines (IL-2, IL-6, IL-8, TNF-α) at both 24 and 48 hours timepoints. Anti-inflammatory cytokines IL-4 and IL-10 were found to be modestly induced, whereas GM-CSF, G-CSF, and IFN-γ showed pronounced stimulation.

Our analysis indicates that TAMRA− IFN-moDCs are capable of secreting IL-2, IL-6, IL-8, TNF-α, IL-12 upon induction with dsDNA.

mRNA expression profiling of cytokine- and CD-encoding genes in human IFN-moDCs pretreated with chloroquine or not, and exposed to dsDNA for 6 and 24 hours

Having established the correlation between cytokine production and the presence of a rare dsDNA-internalizing DCs subpopulation in the sample, we focused on a more detailed analysis of the ability of IFN-moDCs to produce specific cytokines and CDs upon incubation with extracellular dsDNA. We expected that the dsDNA-activated rare subpopulation of IFN-moDCs would serve as the major contributing factor into cytokine gene expression profile of total pool of IFN-moDCs.

We proceeded to the analysis of mRNA expression changes in selected genes (*IFN*-α, *IFN*-β, *IFN*-γ, *IL-8*, *IL-10*, *MCP1*, *VEGF*, *CD25*, and *CD83*) in human IFN-moDCs pretreated with chloroquine or not and exposed to dsDNA for 6 and 24 hours in the absence of

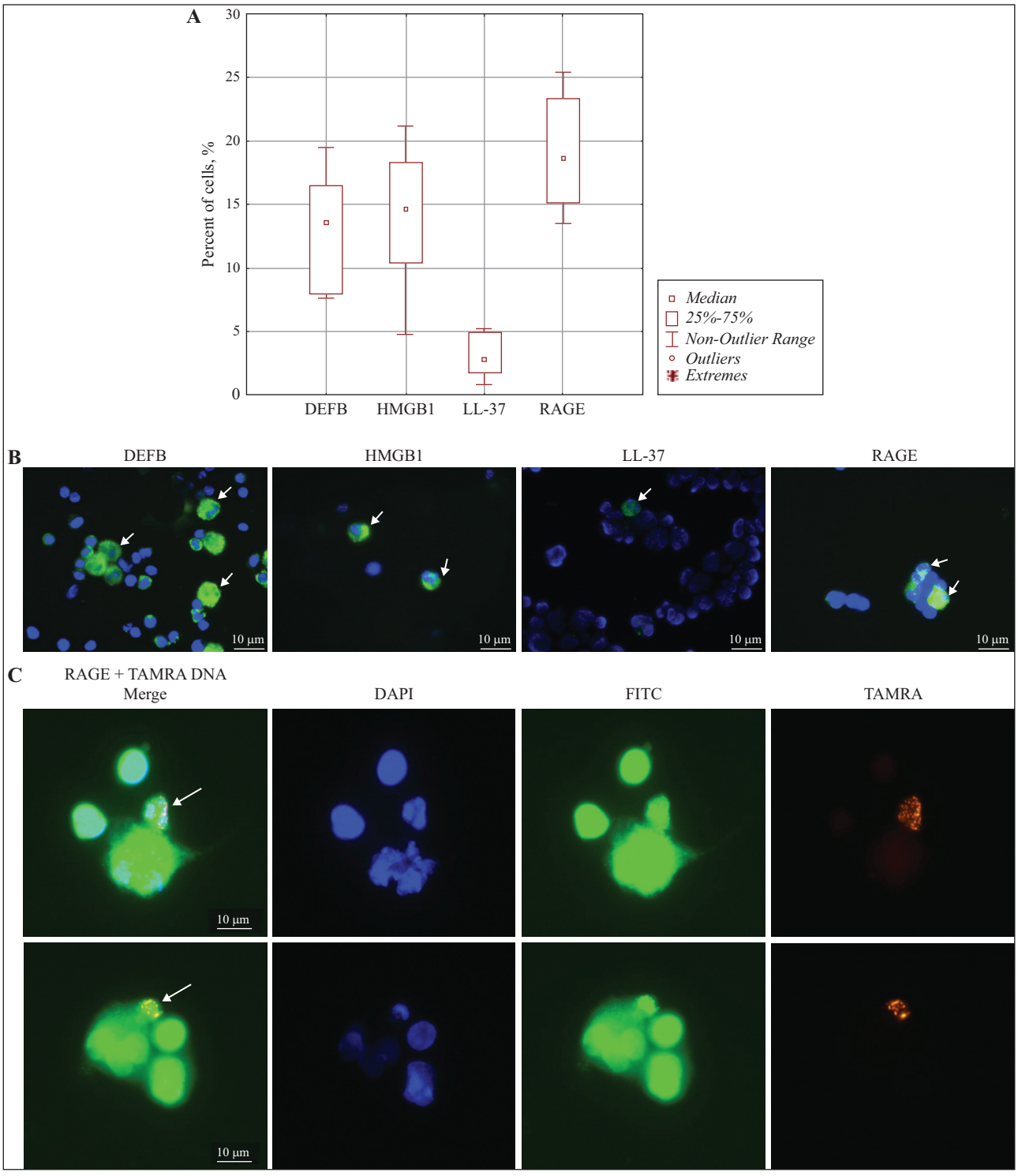

Figure 2
Surface profiling of IFN-moDCs for the presence of DEFB, HMGB1, RAGE, and LL37 antigens. **A)** Quantitative determination of the percent of cells carring antigens. The data are medians and interquartile diapasons for six different donors; **B)** colocalization of TAMRA-DNA (red) and RAGE-specific (green) signals in IFN-moDCs.

Table 1
Percentages of IFN-DCs that are positive for the surface markers of interest and internalizing Alu-TAMRA-DNA probe

Surface marker	DEFB	HMGB1	RAGE
%, Alu-TAMRA-DNA-internalizing cells	5.1	4.3	3.3
Of which receptor-positive cells, %	36	33	21
Of which receptor-negative cells, %	64	67	79

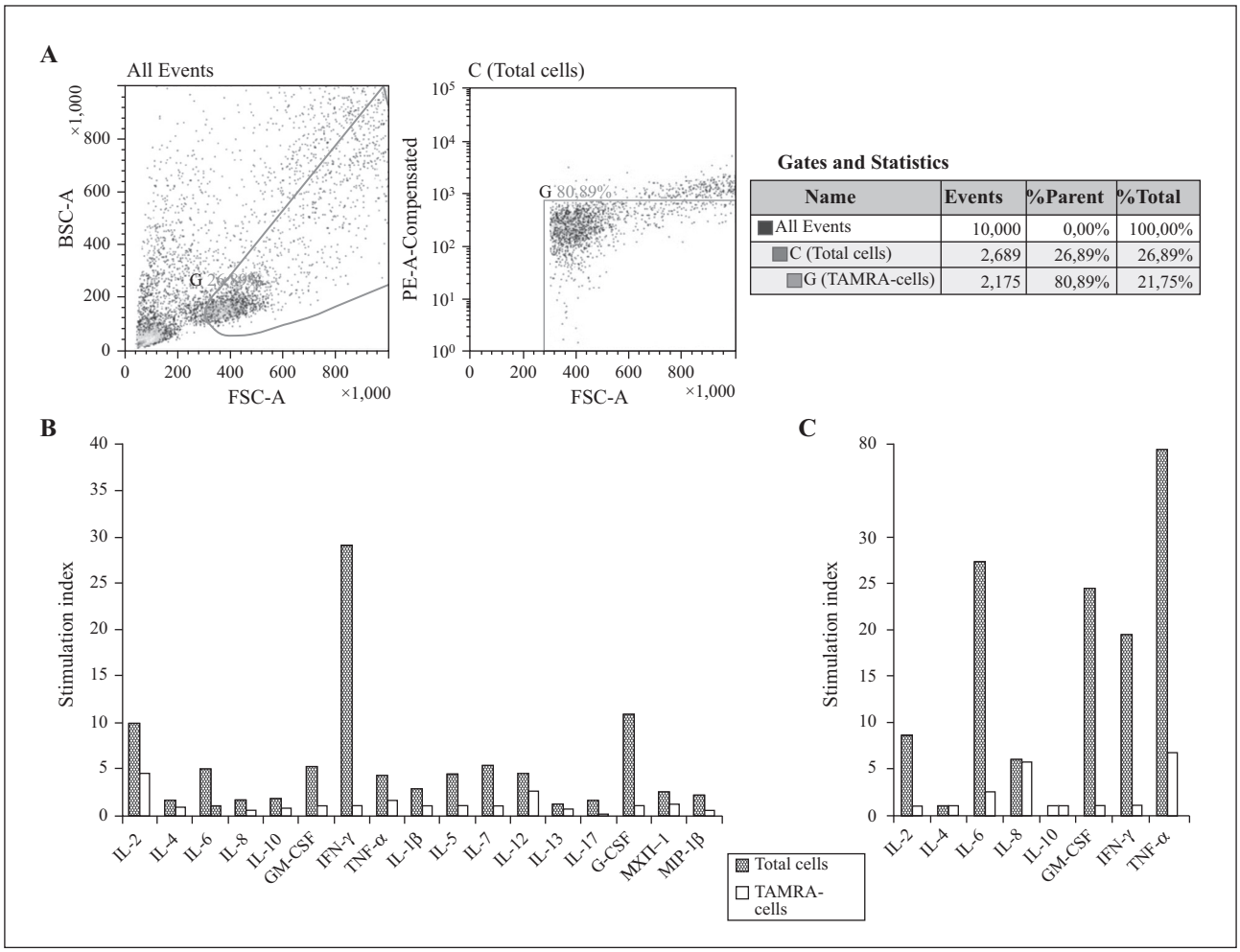

Figure 3
Comparison of cytokine fold induction in total cells (TAMRA+ and TAMRA− cells) *versus* TAMRA− cell population. **A)** gating strategy applied to IFN-moDCs incubated with TAMRA-DNA; **B)** 24 hours after incubation with human dsDNA; **C)** 48 hours after incubation with human dsDNA. Stimulation index-fold induction of cytokine levels in DNA-stimulated *versus* non-stimulated cells. The level of cytokines production (pg/ml) was measured in 17 or 8 Plex analysis.

transfection reagents. The results obtained are summarized in *figure 4*.

First, chloroquine treatment itself was found to up-regulate expression of several cytokine genes: at 6 hours timepoint, two transcripts, *IL-8* and *CD83,* were induced, and by 24 hours all the genes analyzed showed increased expression (*figure 4*).

When treated with dsDNA preparation along, IFN-moDCs displayed up-regulated expression of *IFN*-α, *IFN*-β, *IFN*-γ, *IL-8*, *IL-10*, and *VEGF* at 6 hours timepoint, with only two transcripts, *IFN*-α and *IL-8*, remaining up-regulated upon 24 hours (*figure 4*).

Upon joint chloroquine + dsDNA treatment, transcription of only one of the genes tested, with pro-inflammatory cytokines *IFN*-β displaying the strongest fold induction by 24 hours (*figure 4*).

DISCUSSION

Internalization of TAMRA-labeled DNA by IFN-moDCs

The focus of the present study was on understanding whether fragmented dsDNA may be internalized into monocyte-derived DCs and activating them directly, in the absence of transfection. We for the first time demonstrate that the monocyte-derived DCs encompass a rare (<5%) subpopulation of DCs that are indeed capable of naturally internalizing dsDNA. TAMRA-DNA is detected in such cells either in vacuoles or appears as spots unrelated to any subcellular organelles.

In our early works, it has been multiply demonstrated that it is the extracellular fragments of DNA, but not the TAMRA precursor that fall into the internal compartments of the cell. It has been proved that the radioactive label found in the nucleus refers to that incorporated into the internalized DNA and is not an artifact of S phase-related synthesis [87]. It has also been established that the internalized linear DNA undergoes partial boundary hydrolysis followed by a ligation into the ring [84].

In this study, the standardized internalization protocol we have previously developed, and which includes exhaustive DNase treatment, was used. A series of experiments on internalization was carried out. To control the internalization of TAMRA-labeled DNA probe into the DCs, the RT PCR analysis was performed, where the synthesis of a specific PCR product in the culture medium, in the last washing

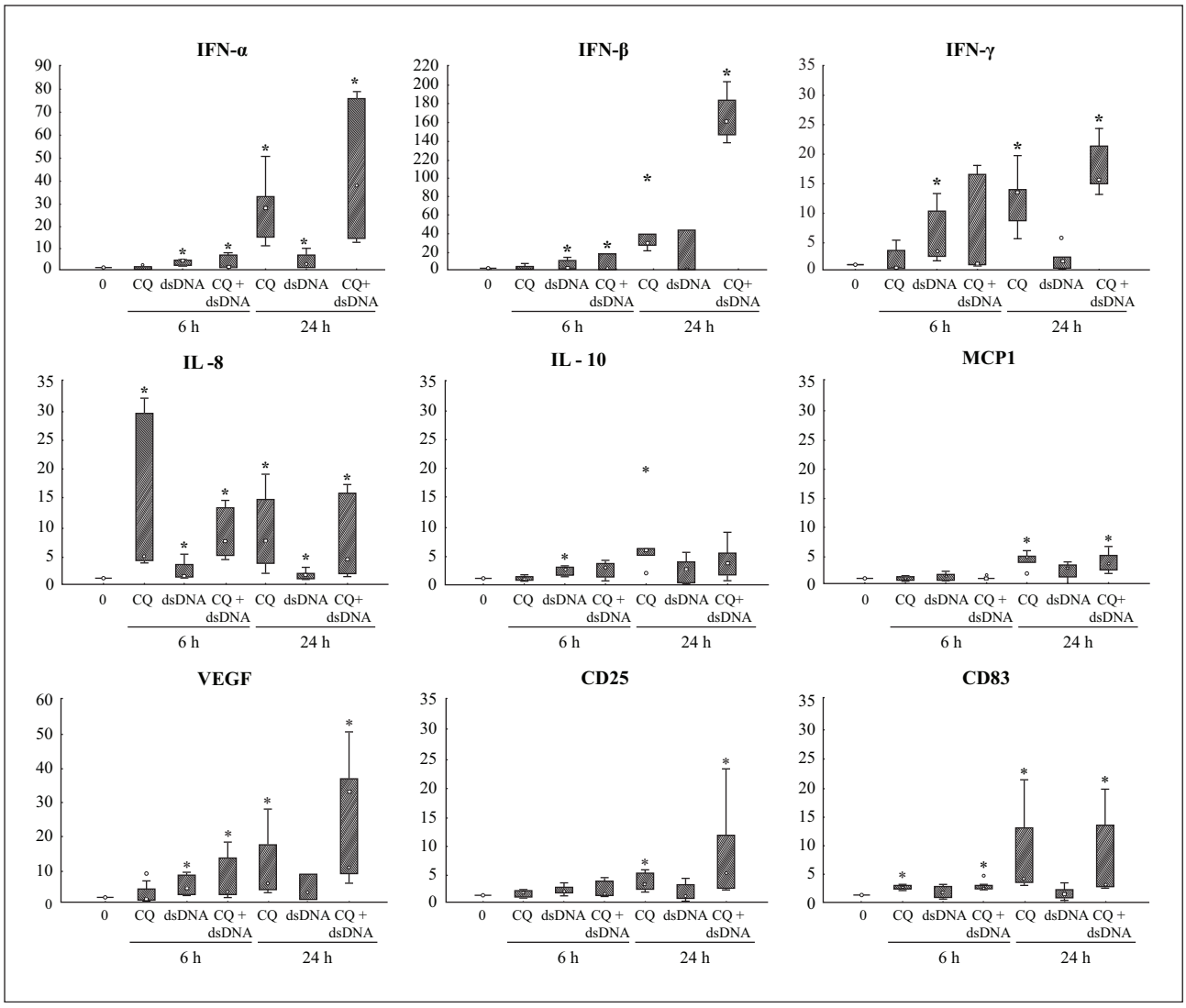

Figure 4

Changes in mRNA expression of cytokine- and CD-encoding genes in human IFN-moDCs treated with an appropriate preparation for 6 or 24 hours normalized to the basal expression of the corresponding genes in intact DCs, detected in Real-time PCR experiments. 0: intact DCs (control); CQ: chloroquine-pretreated cells; dsDNA: cells incubated with dsDNA; CQ+ dsDNA: chloroquine-pretreated cells incubated with dsDNA. Data have been normalized to the control datapoint. Data are shown in accordance with the results of Real-time PCR performed as triple tests for each of three donors. Quartile range (25-75%) and median values for each datapoint are shown. * $P < 0.05$, significant differences relatively to the control, Mann-Whitney U-test.

supernatant, and directly in DNA isolated from the DCs, was monitored. The detectable specific TAMRA signal as well as the accumulation of a specific PCR product indicate the presence of extracellular DNA in the internal compartments of IFN-moDCs.

There is a paradigm concerning the immunological tolerance of DCs to extracellular self-DNA. It is known that to activate DCs, extracellular DNA must be internalized into the cell. Under normal conditions, the amount of DNA in plasma is controlled by specific DNases and does not exceed the quantitative threshold that is supposed to destroy this tolerance [65]. Nevertheless, DCs are always being activated upon large-scale destruction of tissues. At this, it is almost impossible to determine the cause of activation of the DCs: does it relate to the excess of DNA that resulted from the necrotic destruction of cells in sterile injury, or is it the consequence of the presence of pathogens, the appearance of which always accompanies the destruction of tissues.

Recently, a number of works that testify to the existence of both a mechanism and factors for delivery of fragments of extracellular self-dsDNA into DCs of both plasmacytoid and myeloid types have appeared [88].

In the context of plasmacytoid DCs, four factors have been reported to mediate internalization of extracellular DNA [58, 61-65]. Two mechanisms of internalization have been described. In the first case, it is the capture by immune cells of complexes of DNA with antimicrobial peptides or HMGB1 that were formed in the extracellular space. Upon inflammation, antimicrobial peptides are in mass being produced and secreted by neutrophils as well as by many other types of cells including macrophages, NK cells (LL37), skin epithelial cells (LL37, DEFB) leukocytes, etc. [62, 89-92]. HMGB1 is released both upon cells destruction and under inflammation [58]. In the second case, DNA internalization is mediated by the transmembrane RAGE receptor capable of

binding both the DNA-HMGB1 complex and pure DNA itself [65].

It was suggested that IFN-moDCs could internalize extracellular dsDNA using the described factors. We performed immunostaining of IFN-moDCs with antibodies specific to these antigens, and found DEFB, HMGB1, LL-37 and RAGE present on the surface of IFN-moDCs (*figure 2*A, B). The percentage of cells carrying the corresponding antigens was for DEFB: 7-20%, for the protein HMGB1: 5-21%, for LL-37: 1-5%, and for RAGE: 13-25%.

From the co-detection of the surface antigens and internalized DNA, we draw the following conclusions. First, only the minority of RAGE-, HMGB1-, and DEFB-positive cells are able to internalize extracellular DNA. Second, both antigen-positive and antigen-negative cells were shown to internalize DNA. If DNA internalization were mediated by RAGE, HMGB1 and DEFB, all the cells positive for these antigens would be TAMRA+. All the mentioned above presumes no direct functional relations, which can be described in terms of receptor-ligand interaction, between the tested markers (antigen-DNA).

This makes us speculate that some DNA material may be delivered to the interior of DCs *via* yet undescribed internalization mechanism that is not directly related to the presence of the dsDNA-internalizing factors described for plasmacytoid DCs.

Activation of IFN-moDCs by dsDNA preparation

The goal of this study was to explore the induction of professional activity of monocyte-derived dendritic cells by extracellular DNA, and we performed protein secretion measurements as well as RNA expression profiling for a panel of cytokines.

So, initially, we asked whether increased cytokine production would be in any way related to the presence of the rare dsDNA-internalizing subpopulation of IFN-moDCs. To address this question, we compared cytokine secretion by the total DCs population composed of TAMRA+ and TAMRA− cells and by the TAMRA− cells performing 17 and 8 Plex assays at 24 hours and 48 hours timepoints, respectively. Fold induction was expressed as the ratio of the cytokine concentration in the samples incubated with dsDNA *versus* control untreated samples (*figure 3*, *tables S2, S3* in the supplementary material). Our data reveal the association between induced cytokine production by IFN-moDCs and the presence of dsDNA-internalizing cells (TAMRA+). Some stimulated secretion is also observed in TAMRA− cell subpopulation. It is demonstrative that after 24 hours of incubation the most actively secreted cytokine turned out to be IFN-γ. It is known that gene network activated by *IFN*-γ is extremely broad and, in turn, induces multiple immunity-related factors [93, 94].

Early expression of IFN-γ is considered to trigger the very onset of immunostimulatory reaction. We suggest that self-DNA in our experiments drives transcription of *IFN*-γ followed by protein expression and secretion. Next, this induces autocrine or paracrine activation coupled with the activation of other immunity-related genes in the same or neighboring cells.

The confirmation of such a scenario is the characteristic profile of cytokine production after 48 hours of incubation of IFN-moDCs with DNA. A total pool of IFN-moDCs secrete pro-inflammatory "first-wave" cytokines (IL-2, IL-6, IL-8, TNF-α). GM-CSF, G-CSF also showed pronounced stimulation.

Also, in the present study, the mRNA synthesis of various factors produced by DCs and indicating to their mature state has been estimated. Under a DNA-only regimen and in the absence of transfecting agents, we observed strong induction of the "first-wave" cytokines. Three interferon genes, IFN-α/β/γ, as well as chemokine IL-8 are actively expressed, particularly upon 24 hours. Both IFN-α and IL-8 function as the main inducers of innate immune responses by launching the differentiation, maturation, and migration of DCs to the damaged tissues or sites of inflammation [95]. LPS, bacterial DNA, and CpG demonstrate exactly the similar effect on DCs, which may testify to that molecular pathways activating the cytokine genes are exactly the same both for conventional ligands and for self-DNA [45, 49, 51, 78, 79, 96]. The data obtained are in agreement with the results of the Plex analysis of secreted cytokines in the supernatant of IFN-moDCs activated by dsDNA. Of note the dsDNA-stimulated expression of IFN-γ mRNA during the first 6 hours is correlated with the kinetics of mature cytokine production, the peak of which is being observed after 24 hours of incubation of IFN-moDCs with the dsDNA preparation. Very similar observations were made by Huang *et al*. [97], who showed that response to DNA (present in HKBA heat-killed *Brucella abortus*) reaches its maximum in treated animals at 6-8 hours post-induction.

Summarizing the results of the analysis of the expression of cytokine genes and their products, it is necessary to emphasize once again that the DNA preparation mainly affects the synthesis of mediators of inflammation of the first-wave. It must be noted that the "first-wave" mediators (IFN-α/β, TNF-α, IL-6, IL-8) are known to directly induce innate immunity reactions (TNF-α, IFN-α/β), to recruit neutrophils into the sites of damage/inflammation (IL-8), as well as to induce expression of acute-phase proteins in hepatocytes (IL-6). IFN-α is also one of the key inducers of adaptive Th1-type immune response. IFN-α was shown to protect T-cells from apoptosis, whereas IL-6 can inhibit the activity of CD4+ CD25 + T-regs. Importantly, IFN-α and IL-6 also boost the differentiation and maturation of DCs from hematopoietic progenitors [98], thereby providing the replenishment of antigen-presenting cells in the sites of damage or inflammation.

Finally, we included chloroquine, a nonspecific TLR9 inhibitor, as a pretreatment to modulate the possible effects of dsDNA on IFN-moDCs. Our experiments indicate that dsDNA + chloroquine combination synergistically affected the *IFN*-β gene transcription only. Chloroquine is broadly known as an antimalarial drug [99]. It is a small lypophilic weak base that can freely diffuse across the cell membrane in a deprotonated form [99]. Once in the cytoplasm, it reaches acidic cellular compartments – endosome and lysosomes, where it becomes protonated and accumulated. This is

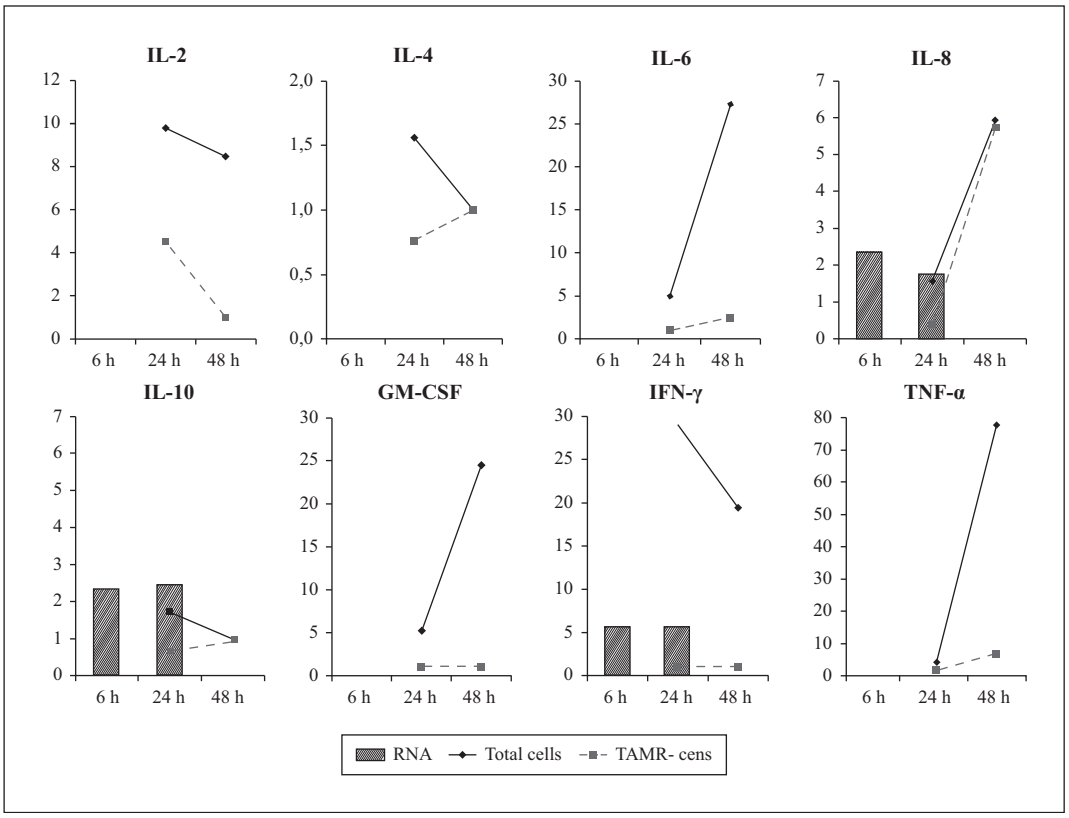

Figure 5
Comparisons of protein and transcript fold induction levels for total (TAMRA+ and TAMRA− cells) *versus* TAMRA− cells, 24 and 48 hours post-incubation with human dsDNA. The *y*-axis shows fold induction (ratio of the cytokine concentration (dots) or average cytokine transcript levels (columns) in treated *versus* untreated samples); incubation time is shown on the *x*-axis.

accompanied by increase in pH and so prevents endosomal acidification [100].

Chloroquine inhibits many molecules in the cell, related to reduction of lysosomal acidification, including TLR9.

This inactivation results in the failure of pro-inflammatory cytokine gene network to become activated in response to internalization of bacterial DNA enriched with non-methylated CpGs [101]. This property of chloroquine is frequently used in experiments when the TLR9 signaling pathway needs to be turned off [10, 95, 97, 102, 103]. Chloroquine displays yet another striking property. It was demonstrated that at 100-fold lower concentrations, chloroquine also abrogates TLR9-dependent signaling, and this occurs under unperturbed pH in the endosomes. In this case, chloroquine directly interacts with foreign internalized dsDNA thereby outcompeting TLR9 [104, 105]. In our assay, we found that chloroquine exposure directly stimulated expression of *IFN*-α/β, *IFN*-γ, *IL-10*, *VEGF*, *IL-8*, *MCP1*, as well as of the marker of mature DCs *CD83*, which was particularly pronounced upon 24-h long incubation. We speculate that chloroquine acts in this case *via* ROS-mediated mechanism (by activating NOX and ultimately NFkB) and so it induces a broad range of interferons, cytokines, and chemokines [106-108].

The observed synergistic effect of chloroquine and dsDNA preparation only on the *IFN*-β gene transcription could be due to two facts. First, chloroquine abrogates the activation of pro-inflammatory cytokines by destroying acidified cytoplasmic compartments. Second, the internalized DNA, *via* the cytoplasmic sensors, activates the transcription of *IFN*-β [77, 109].

We established the correlation between the dynamics of cytokine transcripts and protein levels. In our assays, the induction of three cytokines (IL-8, IL-10, IFN-γ) was measured at both RNA and protein levels (*figure 5*), and expectedly the changes in the transcript levels were consistent with and preceded those of the protein products.

CONCLUSIONS

At present, much attention is paid to the issues of the ontological relationships between the multitude of subpopulations within the mononuclear phagocyte system and especially the DCs. The use of modern ontogenetic, transcriptional, and proteomic research approaches has led to the identification of numerous new subpopulations of mature DCs and their bone marrow precursors with inevitable subsequent erosion of clearly established ontogenetic and functional differences between conventional DCs types either obtained from *in vivo* or generated *ex vivo* [4, 5, 110]. The data obtained in this study suggest that in the mononuclear phagocyte system there may additionally be a small population of trigger DCs capable of being activated by the extracellular dsDNA fragments to lead to the expansion and increasing of the activating signal for various DCs populations.

Further, to fully characterize the newly identified DCs population, it is necessary to perform its molecular

typing, and comparative analysis with already known and characterized ones.

Disclosure. Authors declare that there is no conflict of interest. ***Acknowledgments:*** This study was funded by the Russian state project [0324-2019-0042] and grant from the Russian Fund for Basic Research [16-34-00007].

SUPPLEMENTARY DATA

Supplementary data (tables S1, S2 and S3) associated with this article can be found, in the online version, at doi:10.1684/ecn.2019.0427.

Supplementary data

Table S1
Sequences of oligonucleotides used in the qPCR experiment.

Gene name	Primer name	Length, b	Sequence 5‘- 3‘	Optimal annealing temperature, °C	PCR product size, bp
IFN-α	for	23	CAGAGTCACCCATCTCAGCAAGC	60	118
	rev	22	CAGCCCAGAGAGCAGCTTGACT		
IFN-β	for	21	CCTTTGCTCTGGCACAACAGG	58	189
	rev	23	CATTCAATTGCCACAGGAGCTTC		
IFN-γ	for	23	GACTTGAATGTCCAACGCAAAGC	58	140
	rev	23	CAGGACAACCATTACTGGGATGC		
IL-8	for	22	GCCAAGGGCCAAGAGAATATCC	58-60	177
	rev	22	GGCTAGCAGACTAGGGTTGCCA		
IL-10	for	23	ACGAAACTGAGACATCAGGGTGG	60	165
	rev	22	AATGGGGGTTGAGGTATCAGAGG		
MCP1	for	23	GCAGATGGTGGAGCTGAATATGC	60	174
	rev	23	GCTAAGCCACAGTTGCACTCATG		
VEGF	for	22	GAAGGAGCCTCCCTCAGGGTTT	60	161
	rev	23	GCGCAGAGTCTCCTCTTCCTTCA		
CD-25	for	23	GAATTTCTTGGTAAGAAGCCGGG	58	116
	rev	20	CTTCCAAAACGCAGGCAAGC		
CD-83	for	22	AAGGGGCAAAATGGTTCTTTCG	60	96
	rev	19	GCACCTGTATGTCCCCGAG		
RPLP0	for	23	AGGCCTTCTTGGCTGATCCATCT	58-60	135
	rev	22	TATCCTCGTCCGACTCCTCCGA		

Table S2
Induced cytokine production by total (TAMRA+ and TAMRA− cells) *versus* TAMRA− cells 24 hours after incubation with dsDNA relatively to control untreated cells.

		IL-2	IL-4	IL-6	IL-8	IL-10	GM-CSF	IFN-γ	TNF-α	IL-1β	IL-5	IL-7	IL-12	IL-13	IL-17	G-CSF	MCP-1	MIP-1β
Total cells	**+DNA**	36.3	26.6	33.8	8186	10.1	20.8	900.4	16.8	5.2	7.5	23.2	21.3	32.1	27.1	75.7	188	3428
	0	<3.7	17.1	<6.8	5209	5.9	<4.0	<31.0	<4.0	<1.9	<1.7	<4.4	4.8	28.5	17.6	<7.0	<78	1697
Fold induction		**9,8**	**1.6**	**5.0**	**1.6**	**1.7**	**5.2**	**29.0**	**4.2**	**2.7**	**4.4**	**5.3**	**4.4**	**1.1**	**1.5**	**10.8**	**2.4**	**2.0**
TAMRA– cells	**+DNA**	16.7	7.0	<6.8	5090	2.5	<4.0	<31.0	6.4	<1.9	<1.7	<4.4	13.2	7.1	12.0	<7.0	92	1320
	0	<3.7	9.3	<6.8	13135	3.6	<4.0	<31.0	<4.0	<1.9	<1.7	<4.4	5.2	11.8	152.7	<7.0	<78	3101
Fold induction		**4,5**	**0.8**	**1.0**	**0.4**	**0.7**	**1.0**	**1.0**	**1.6**	**1.0**	**1.0**	**1.0**	**2.5**	**0.6**	**0.1**	**1.0**	**1.2**	**0.4**

Note: Values are given in pg/ml after 17 Plex analysis. +DNA – human dsDNA preparation was added to the cells grown in serum-free medium to the final concentration of 10 mkg/ml. 0 – control cells (no dsDNA added). < indicates that the cytokine level was below the minimum detection level (indicated). Fold induction is a ratio between the cytokine levels found in the treated sample to those observed in the control. Whenever the cytokine levels measured were below the minimum detection level, the latter value was used to calculate fold induction.

Table S3
Induced cytokine production by total (TAMRA+ and TAMRA− cells) *versus* TAMRA− cells 48 hours after incubation with dsDNA relatively to control untreated cells.

		IL-2	IL-4	IL-6	IL-8	IL-10	GM-CSF	IFN-γ	TNF-α
Total cells	**+DNA**	34	<5	327	7343	<15	611	971	310
	0	<4	<5	<12	1238	<15	<25	<50	<4
Fold induction		**8.5**	**1.0**	**27.3**	**5.9**	**1.0**	**24.4**	**19.4**	**77.5**
TAMRA– cells	**+DNA**	<4	<5	30	723	<15	<25	<50	27
	0	<4	<5	<12	126	<15	<25	<50	<4
Fold induction		**1.0**	**1.0**	**2.5**	**5.7**	**1.0**	**1.0**	**1.0**	**6.8**

Note: Values are given in pg/ml after 8 Plex analysis. +DNA – human dsDNA preparation was added to the cells grown in serum-free medium to the final concentration of 10 mkg/ml. 0 – control cells (no dsDNA added). < indicates that the cytokine level was below the minimum detection level (indicated). Fold induction is a ratio between the cytokine levels found in the treated sample to those observed in the control. Whenever the cytokine levels measured were below the minimum detection level, the latter value was used to calculate fold induction.

REFERENCES

1. Boltjes A, van Wijk F. Human dendritic cell functional specialization in steady-state and inflammation. *Front Immunol* 2014; 5 : 131.
2. Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. *Immunology* 2013; 140 : 22-30.
3. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 2013; 31 : 563-604.
4. Kushwah R, Hu J. Complexity of dendritic cell subsets and their function in the host immune system. *Immunology* 2011; 133 : 409-19.
5. Collin M, Bigley V. Human dendritic cell subsets: an update. *Immunology* 2018; 154 : 3-20.
6. Paludan S, Bowie A. Immune sensing of DNA. *Immunity* 2013; 38 : 870-80.
7. Paludan SR. Activation and regulation of DNA-driven immune responses. *Microbiol Mol Biol Rev* 2015; 79 : 225-41.
8. Schmidt SV, Nino-Castro AC, Schultze JL. Regulatory dendritic cells: there is more than just immune activation. *Front Immunol* 2012; 3 : 274.
9. Hammer GE, Ma A. Molecular control of steady-state dendritic cell maturation and immune homeostasis. *Annu Rev Immunol* 2013; 31 : 743-91.
10. Amorim KNS, Chagas DCG, Sulczewski FB, Boscardin SB. Dendritic cells and their multiple roles during malaria infection. *J Immunol Res* 2016; 2016 : 2926436.
11. Dzionek A, Fuchs A, Schmidt P, *et al*. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* 2000; 165 : 6037-46.
12. Dzionek A, Sohma Y, Nagafune J, *et al*. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med* 2001; 194 : 1823-34.
13. MacDonald KPA, Munster DJ, Clark GJ, Dzionek A, Schmitz J, Hart DNJ. Characterization of human blood dendritic cell subsets. *Blood* 2002; 100 : 4512-20.
14. Ju X, Zenke M, Hart DNJ, Clark GJ. CD300a/c regulate type I interferon and TNF-alpha secretion by human plasmacytoid dendritic cells stimulated with TLR7 and TLR9 ligands. *Blood* 2008; 112 : 1184-94.
15. Bao M, Liu Y-J. Regulation of TLR7/9 signaling in plasmacytoid dendritic cells. *Protein Cell* 2013; 4 : 40-52.
16. Ahrens S, Zelenay S, Sancho D, *et al*. F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity* 2012; 36 : 635-45.
17. Zhang J-G, Czabotar PE, Policheni AN, *et al*. The dendritic cell receptor Clec9A binds damaged cells *via* exposed actin filaments. *Immunity* 2012; 36 : 646-57.
18. Heidkamp GF, Sander J, Lehmann CHK, *et al*. Human lymphoid organ dendritic cell identity is predominantly dictated by ontogeny, not tissue microenvironment. *Sci Immunol* 2016; 1 : eaai7677-17.
19. Nair S, Archer GE, Tedder TF. Isolation and generation of human dendritic cells. *Curr Protoc Immunol* 2012;. chapter 7: unit 7.32.
20. Leplina OY, Tyrinova TV, Tikhonova MA, Ostanin AA, Chernykh ER. Interferon alpha induces generation of semi-mature dendritic cells with high pro-inflammatory and cytotoxic potential. *Cytokine* 2015; 71 : 1-7.
21. Ruben JM, Bontkes HJ, Westers TM, *et al*. Differential capacity of human interleukin-4 and interferon-α monocyte-derived dendritic cells for cross-presentation of free *versus* cell-associated antigen. *Cancer Immunol Immunother* 2015; 64 : 1419-27.
22. Santini SM, Lapenta C, Logozzi M, *et al*. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity *in vitro* and in Hu-PBL-SCID mice. *J Exp Med* 2000; 191 : 1777-88.
23. Della Bella S, Nicola S, Riva A, Biasin M, Clerici M, Villa ML. Functional repertoire of dendritic cells generated in granulocyte macrophage-colony stimulating factor and interferon-alpha. *J Leukoc Biol* 2004; 75 : 106-16.
24. Paquette RL, Hsu NC, Kiertscher SM, *et al*. Interferon-alpha and granulocyte-macrophage colony-stimulating factor differentiate peripheral blood monocytes into potent antigen-presenting cells. *J Leukoc Biol* 1998; 64 : 358-67.
25. Korthals M, Safaian N, Kronenwett R, *et al*. Monocyte derived dendritic cells generated by IFN-alpha acquire mature dendritic and natural killer cell properties as shown by gene expression analysis. *J Transl Med* 2007; 5 : 46.
26. Mohty M, Vialle-Castellano A, Nunes JA, Isnardon D, Olive D, Gaugler B. IFN-alpha skews monocyte differentiation into Toll-like receptor 7-expressing dendritic cells with potent functional activities. *J Immunol* 2003; 171 : 3385-93.

27. Carbonneil C, Aouba A, Burgard M, *et al*. Dendritic cells generated in the presence of granulocyte-macrophage colony-stimulating factor and IFN-alpha are potent inducers of HIV-specific CD8 T cells. *AIDS* 2003; 17 : 1731-40.

28. Leplina OY, Tikhonova MA, Tyrinova TV, *et al*. Functional activity of IFNα- and IL-4-induced human dendritic cells: a comparative study. *Med Immunol* 2014; 16 : 43-52.

29. Mifsud EJ, Tan ACL, Jackson DC. TLR agonists as modulators of the innate immune response and their potential as agents against infectious disease. *Front Immunol* 2014; 5 : 79.

30. Tan RST, Ho B, Leung BP, Ding JL. TLR cross-talk confers specificity to innate immunity. *Int Rev Immunol* 2014; 33 : 443-53.

31. Leifer CA, Medvedev AE. Molecular mechanisms of regulation of Toll-like receptor signaling. *J Leukoc Biol* 2016; 100 : 927-41.

32. Goulopoulou S, McCarthy CG, Webb RC. Toll-like receptors in the vascular system: sensing the dangers within. *Pharmacol Rev* 2015; 68 : 142-67.

33. Jonuleit H, Kühn U, Müller G, *et al*. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 1997; 27 : 3135-42.

34. Decker P, Singh-Jasuja H, Haager S, Kötter I, Rammensee H-G. Nucleosome, the main autoantigen in systemic lupus erythematosus, induces direct dendritic cell activation *via* a MyD88-independent pathway: consequences on inflammation. *J Immunol* 2005; 174 : 3326-34.

35. Yasuda K, Ogawa Y, Yamane I, Nishikawa M, Takakura Y. Macrophage activation by a DNA/cationic liposome complex requires endosomal acidification and TLR9-dependent and -independent pathways. *J Leukoc Biol* 2005; 77 : 71-9.

36. Martin DA, Elkon KB. Intracellular mammalian DNA stimulates myeloid dendritic cells to produce type I interferons predominantly through a Toll-like receptor 9-independent pathway. *Arthritis Rheum* 2006; 54 : 951-62.

37. Shirota H, Ishii KJ, Takakuwa H, Klinman DM. Contribution of interferon-beta to the immune activation induced by double-stranded DNA. *Immunology* 2006; 118 : 302-10.

38. Suzuki K, Mori A, Ishii KJ, *et al*. Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc Natl Acad Sci U S A* 1999; 96 : 2285-90.

39. Krieg AM. CpG motifs: the active ingredient in bacterial extracts? *Nat Med* 2003; 9 : 831-5.

40. Ishii KJ, Suzuki K, Coban C, *et al*. Genomic DNA released by dying cells induces the maturation of APCs. *J Immunol* 2001; 167 : 2602-7.

41. Zhu F-G, Reich CF, Pisetsky DS. Effect of cytofectins on the immune response of murine macrophages to mammalian DNA. *Immunology* 2003; 109 : 255-62.

42. Jiang W, , Reich III CF, Pisetsky DS. Mechanisms of activation of the RAW264.7 macrophage cell line by transfected mammalian DNA. *Cell Immunol* 2004; 229 : 31-40.

43. Yasuda K, Richez C, Uccellini MB, *et al*. Requirement for DNA CpG content in TLR9-dependent dendritic cell activation induced by DNA-containing immune complexes. *J Immunol* 2009; 183 : 3109-17.

44. Lande R, Ganguly D, Facchinetti V, *et al*. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med* 2011; 3 : 73ra19.

45. Pisetsky DS. The origin and properties of extracellular DNA: from PAMP to DAMP. *Clin Immunol* 2012; 144 : 32-40.

46. Liu X, Pu Y, Cron K, *et al*. CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. *Nat Med* 2015; 21 : 1209-15.

47. Takeshita F, Ishii KJ. Intracellular DNA sensors in immunity. *Curr Opin Immunol* 2008; 20 : 383-8.

48. Barbalat R, Ewald SE, Mouchess ML, Barton GM. Nucleic acid recognition by the innate immune system. *Annu Rev Immunol* 2011; 29 : 185-214.

49. Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines* 2011; 10 : 499-511.

50. Barber GN. Cytoplasmic DNA innate immune pathways. *Immunol Rev* 2011; 243 : 99-108.

51. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. *Science* 2010; 327 : 291-5.

52. Song J, Guan M, Zhao Z, Zhang J. Type I interferons function as autocrine and paracrine factors to induce autotaxin in response to TLR activation. *PLoS One* 2015; 10 : e0136629.

53. Gordon JR, Ma Y, Churchman L, Gordon SA, Dawicki W. Regulatory dendritic cells for immunotherapy in immunologic diseases. *Front Immunol* 2014; 5 : 7.

54. Tissot B, Daniel R, Place C. Interaction of the C1 complex of complement with sulfated polysaccharide and DNA probed by single molecule fluorescence microscopy. *Eur J Biochem* 2003; 270 : 4714-20.

55. Garlatti V, Martin L, Lacroix M, *et al*. Structural insights into the recognition properties of human ficolins. *J Innate Immun* 2010; 2 : 17-23.

56. Uccellini MB, Busto P, Debatis M, Marshak-Rothstein A, Viglianti GA. Selective binding of anti-DNA antibodies to native dsDNA fragments of differing sequence. *Immunol Lett* 2012; 143 : 85-91.

57. Pisetsky D. The expression of HMGB1 on microparticles released during cell activation and cell death *in vitro* and *in vivo*. *Mol Med* 2014; 20 : 158-63.

58. Kang R, Chen R, Zhang Q, *et al*. HMGB1 in health and disease. *Mol Aspects Med* 2014; 40 : 1-116.

59. Kauffman WB, Fuselier T, He J, Wimley WC. Mechanism matters: a taxonomy of cell penetrating peptides. *Trends Biochem Sci* 2015; 40 : 749-64.

60. Lande R, Gregorio J, Facchinetti V, *et al*. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 2007; 449 : 564-9.

61. Kahlenberg JM, Kaplan MJ. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol* 2013; 191 : 4895-901.

62. Tewary P, de la Rosa G, Sharma N, *et al*. β-defensin 2 and 3 promote the uptake of self or CpG DNA, enhance IFN-α production by human plasmacytoid dendritic cells, and promote inflammation. *J Immunol* 2013; 191 : 865-74.

63. Chamilos G, Gregorio J, Meller S, *et al*. Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL37. *Blood* 2012; 120 : 3699-707.

64. Seil M, Nagant C, Dehaye J-P, Vandenbranden M, Lensink MF. Spotlight on human LL-37, an immunomodulatory peptide with promising cell-penetrating properties. *Pharmaceuticals* 2010; 3 : 3435-60.

65. Sirois CM, Jin T, Miller AL, *et al*. RAGE is a nucleic acid receptor that promotes inflammatory responses to DNA. *J Exp Med* 2013; 210 : 2447-63.

66. Pilzweger C, Holdenrieder S. Circulating HMGB1 and RAGE as clinical biomarkers in malignant and autoimmune diseases. *Diagnostics (Basel)* 2015; 5 : 219-53.

67. Barton GM. A calculated response: control of inflammation by the innate immune system. *J Clin Invest* 2008; 118 : 413-20.

68. Proskurina AS, Gvozdeva TS, Alyamkina EA, *et al*. Results of multicenter double-blind placebo-controlled phase II clinical trial of Panagen preparation to evaluate its leukostimulatory activity and formation of the adaptive immune response in patients with stage II-IV breast cancer. *BMC Cancer* 2015; 15 : 122.

69. Alyamkina EA, Leplina OY, Sakhno LV, *et al*. Effect of double-stranded DNA on maturation of dendritic cells *in vitro*. *Cell Immunol* 2010; 266 : 46-51.

70. Orishchenko KE, Ryzhikova SL, Druzhinina YG, *et al*. Effect of human double-stranded DNA preparation on the production of cytokines by dendritic cells and peripheral blood cells from relatively healthy donors. *Cancer Ther* 2013; 8 : 191-205.

71. Alyamkina EA, Nikolin VP, Popova NA, *et al*. A strategy of tumor treatment in mice with doxorubicin-cyclophosphamide combination based on dendritic cell activation by human double-stranded DNA preparation. *Genet Vaccines Ther* 2010; 8 : 7.

72. Alyamkina EA, Dolgova EV, Likhacheva AS, *et al*. Exogenous allogenic fragmented double-stranded DNA is internalized into human dendritic cells and enhances their allostimulatory activity. *Cell Immunol* 2010; 262 : 120-6.

73. Alyamkina EA, Leplina OY, Ostanin AA, *et al*. Effects of human exogenous DNA on production of perforin-containing CD8+ cytotoxic lymphocytes in laboratory setting and clinical practice. *Cell Immunol* 2012; 276 : 59-66.

74. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001; 413 : 732-8.

75. Moseman EA, Liang X, Dawson AJ, *et al*. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+ CD25+ regulatory T cells. *J Immunol* 2004; 173 : 4433-42.

76. Yasuda K, Yu P, Kirschning CJ, *et al*. Endosomal translocation of vertebrate DNA activates dendritic cells *via* TLR9-dependent and -independent pathways. *J Immunol* 2005; 174 : 6129-36.

77. Kis-Toth K, Szanto A, Thai T-H, Tsokos GC. Cytosolic DNA-activated human dendritic cells are potent activators of the adaptive immune response. *J Immunol* 2011; 187 : 1222-34.

78. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002; 20 : 709-60.

79. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; 392 : 245-52.

80. Dolgova EV, Efremov YR, Orishchenko KE, *et al*. Delivery and processing of exogenous double-stranded DNA in mouse CD34+ hematopoietic progenitor cells and their cell cycle changes upon combined treatment with cyclophosphamide and double-stranded DNA. *Gene* 2013; 528 : 74-83.

81. Ma J-P, Xia H-J, Zhang G-H, Han J-B, Zhang L-G, Zheng Y-T. Inhibitory effects of chloroquine on the activation of plasmacytoid dendritic cells in SIVmac239-infected Chinese rhesus macaques. *Cell Mol Immunol* 2012; 9 : 410-6.

82. Martinson JA, Montoya CJ, Usuga X, Ronquillo R, Landay AL, Desai SN. Chloroquine modulates HIV-1-induced plasmacytoid dendritic cell alpha interferon: implication for T-cell activation. *Antimicrob Agents Chemother* 2010; 54 : 871-81.

83. Wang T, Liang Z-A, Sandford AJ, *et al*. Selection of suitable housekeeping genes for real-time quantitative PCR in CD4+ lymphocytes from asthmatics with or without depression. *PLoS One* 2012; 7 : e48367.

84. Dolgova EV, Nikolin VP, Popova NA, *et al*. Internalization of exogenous DNA into internal compartments of murine bone marrow cells. *Russ J Genet Appl Res* 2012; 2 : 440-52.

85. Dolgova EV, Shevela EY, Tyrinova TV, *et al*. Nonadherent spheres with multiple myeloma surface markers contain cells that contribute to sphere formation and are capable of internalizing extracellular double-stranded DNA. *Clin Lymphoma Myeloma Leuk* 2016; 16 : 563-76.

86. Dolgova EV, Potter EA, Proskurina AS, *et al*. Properties of internalization factors contributing to the uptake of extracellular DNA into tumor-initiating stem cells of mouse Krebs-2 cell line. *Stem Cell Res Ther* 2016; 7 : 76.

87. Dolgova EV, Alyamkina EA, Efremov YR, *et al*. Identification of cancer stem cells and a strategy for their elimination. *Cancer Biol Ther* 2014; 15 : 1378-94.

88. Brown KL, Poon GFT, Birkenhead D, *et al*. Host defense peptide LL-37 selectively reduces proinflammatory macrophage responses. *J Immunol* 2011; 186 : 5497-505.

89. Chamorro CI, Weber G, Grönberg A, Pivarcsi A, Ståhle M. The human antimicrobial peptide LL-37 suppresses apoptosis in keratinocytes. *J Invest Dermatol* 2009; 129 : 937-44.

90. Doring Y, Drechsler M, Wantha S, *et al*. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res* 2012; 110 : 1052-6.

91. Ruangsri J, Kitani Y, Kiron V, *et al*. A novel beta-defensin antimicrobial peptide in Atlantic cod with stimulatory effect on phagocytic activity. *PLoS One* 2013; 8 : e62302.

92. Mahlapuu M, Håkansson J, Ringstad L, Björn C. Antimicrobial peptides: an emerging category of therapeutic agents. *Front Cell Infect Microbiol* 2016; 6 : 194.

93. Frasca L, Lande R. Overlapping, additive and counter-regulatory effects of type II and I interferons on myeloid dendritic cell functions. *Sci World J* 2011; 11 : 2071-90.

94. Zha Z, Bucher F, Nejatfard A, *et al*. Interferon-γ is a master checkpoint regulator of cytokine-induced differentiation. *Proc Natl Acad Sci U S A* 2017; 114 : E6867-74.

95. von Buttlar H, Siegemund S, Büttner M, Alber G. Identification of Toll-like receptor 9 as parapoxvirus ovis-sensing receptor in plasmacytoid dendritic cells. *PLoS One* 2014; 9 : e106188.

96. Katashiba Y, Miyamoto R, Hyo A, *et al*. Interferon-α and interleukin-12 are induced, respectively, by double-stranded DNA and single-stranded RNA in human myeloid dendritic cells. *Immunology* 2011; 132 : 165-73.

97. Huang L-Y, Ishii KJ, Akira S, Aliberti J, Golding B. Th1-like cytokine induction by heat-killed Brucella abortus is dependent on triggering of TLR9. *J Immunol* 2005; 175 : 3964-70.

98. Zhang R, Xing M, Ji X, *et al*. Interferon-alpha and interleukin-6 in SLE serum induce the differentiation and maturation of dendritic cells derived from CD34+ hematopoietic precursor cells. *Cytokine* 2010; 50 : 195-203.

99. Schlesinger PH, Krogstad DJ, Herwaldt BL. Antimalarial agents: mechanisms of action. *Antimicrob Agents Chemother* 1988; 32 : 793-8.

100. Krogstad DJ, Schlesinger PH. The basis of antimalarial action: non-weak base effects of chloroquine on acid vesicle pH. *Am J Trop Med Hyg* 1987; 36 : 213-20.

101. Macfarlane DE, Manzel L. Antagonism of immunostimulatory CpG-oligodeoxynucleotides by quinacrine, chloroquine, and structurally related compounds. *J Immunol* 1998; 160 : 1122-31.

102. Pascolo S. Time to use a dose of chloroquine as an adjuvant to anti-cancer chemotherapies. *Eur J Pharmacol* 2016; 771 : 139-44.

103. Tuomela J, Sandholm J, Kauppila JH, Lehenkari P, Harris KW, Selander KS. Chloroquine has tumor-inhibitory and tumor-promoting effects in triple-negative breast cancer. *Oncol Lett* 2013; 6 : 1665-72.

104. Kuznik A, Bencina M, Svajger U, Jeras M, Rozman B, Jerala R. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J Immunol* 2011; 186 : 4794-804.

105. Rutz M, Metzger J, Gellert T, *et al*. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur J Immunol* 2004; 34 : 2541-50.

106. Park J, Kwon D, Choi C, Oh J-W, Benveniste EN. Chloroquine induces activation of nuclear factor-kappaB and subsequent expression of pro-inflammatory cytokines by human astroglial cells. *J Neurochem* 2003; 84 : 1266-74.

107. Park J, Choi K, Jeong E, Kwon D, Benveniste EN, Choi C. Reactive oxygen species mediate chloroquine-induced expression of chemokines by human astroglial cells. *Glia* 2004; 47 : 9-20.

108. Hart OM, Athie-Morales V, O'Connor GM, Gardiner CM. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. *J Immunol* 2005; 175 : 1636-42.

109. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* 2008; 89 : 1-47.

110. Lutz MB, Strobl H, Schuler G, Romani N. GM-CSF monocyte-derived cells and Langerhans cells as part of the dendritic cell family. *Front Immunol* 2017; 8 : 1388.