Chapter 7

Immune Regulatory Network in Cervical Cancer Development: The Expanding Role of Innate Immunity Mechanisms

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Additional information is available at the end of the chapter

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Abstract

There is increasing evidence of a pivotal regulatory role of innate immune mechanisms in tumor-immune interplay. Among these diverse mechanisms, tumor-derived nucleic acids' sensing has recently emerged as one of the fundamental pathways linking innate and adaptive immunity, with DNA-sensor STING being the crucial member of this pathway. Another clear trend is understanding the striking diversity of innate and innate-like immune cell populations implicated in suppression or promotion of tumor growth. Papillomavirus-associated cervical cancer appears to represent a complex network of antiviral and antitumor innate immune mechanisms, whose regulation can be significantly influenced by developing neoplasia. In this chapter, we address new data on the problem of regulation of innate and acquired immunity in cervical cancer patients published in the past 2 years. To support the idea of multilevelness and diversity of changes in the innate arm of immunity, we also report our findings about (a) the expression of endogenous immune sensor STING in neoplastic tissue and peripheral blood lymphocytes, (b) altered frequencies of circulating natural killer and natural killer-like cell populations, as well as regulatory T lymphocytes from patients with precancerous or early cancerous lesions. Revisiting this problem may provide new insights into therapeutic options for cervical cancer.

Keywords: cervical neoplasia, innate immune system, antitumor immune response, innate-like lymphocytes, regulatory lymphocytes, immune suppression, DNA-sensing mechanisms

1. Introduction

Human papillomavirus (HPV)-associated cervical cancer is a type of oncopathology, one can consider as an example of a unique natural phenomenon of virus-related carcinogenesis, which realization is defined by dynamic interactions within a complex system "pathogen ('alien')-tumor ('altered-self')-host immunity." And while for the systems of "viral infection-immunity" and "tumor-immunity" interactions, the models well-describing molecular mechanisms supporting these interactions have been proposed, the situation when both pathological factors coexist seems to be much more complex. It is in these types of pathology that the dual (positive and negative) role of the immune system is most evident [1, 2], and it is for this reason that, obviously, despite a long history of studies, immunology of virus-related cancers still has a lot of blind-spots. The fact that clinical trials of immunotherapy methods to treat cervical cancer and other HPV-related cancers, which typically use unimodal approach, do not show the desired effect, particularly in advanced disease, underlines diverse multidirectional role of cellular, and molecular components of the immune system at different stages of disease development and points the need to study the combined multimodal approaches [3].

A large number of fundamental discoveries made recently in the area of oncoimmunology and immunology of infectious diseases have led to a substantial revision of the priorities in the studies of the antitumor immune response regulation mechanisms, including: (1) redefining the role for cellular components of the innate immune system, as well as the role for cells that represent a link between the innate and adaptive systems, in implementing an effective antitumor response; (2) understanding high phenotypic and functional heterogeneity (plasticity) of these components; (3) realization of the leading role of intrinsic (genetically encoded) mechanisms for stress-/damage-associated molecular pattern-dependent (neoantigen-independent) recognition and induction of immune response against transformed or virus-infected cells; (4) gaining insight into the expanding role of the immune checkpoint mechanisms (which normally have a protective, homeostatic function) tumors can adopt to resist antitumor immune response. It is clear that any attempts to activate (in clinical or experimental settings) specific T cell-mediated immunity, which is based on the T cell receptor (TCR) recognition of tumor-associated antigens (TAAs) presented by the major histocompatibility complex (MHC), to naught under the influence of immunosuppressive tumor micro- and macroenvironment. In this regard, current research has an explicit priority to study innate (genetically encoded) mechanisms of activation and suppression (i.e., immune regulation) of antitumor (and antiviral) response and cell subsets responsible for these mechanisms, as illustrated by thematic searching PubMed database for papers published in the last 2 years.

Among the innate mechanisms of immune recognition and immune regulation, the recognition of cell stress associated with the key hallmarks of carcinogenesis (such as uncontrolled cell mass accumulation, metabolic abnormalities, oxidative stress, and cell death program impairment) deserves special attention. These innate sensing mechanisms can be exploited not only in cells of the innate immune system itself, but also directly in neoplastic cells [4] and presumably even in adaptive immune cells of the system (see below). In general, they serve to detect mislocalized, normally non-immunogenic, molecules that can be regarded as damage-associated molecular patterns (DAMP), with the involvement of specific cell sensors that

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trigger downstream signaling to produce cytokines and other factors necessary for activation of effector functions of innate immune cells [4]. Among these signaling pathways, the innate response to extranuclear/cytosolic or extracellular DNA activated by various molecular DNA sensors (expressed virtually in all cell types) is an example of the most actively studied mechanisms, with the cGAS-STING molecular pair playing the main part. It is important to note that the mechanism of immune response to mislocalized/cytosolic DNA within the tumor site largely overlaps with the mechanism of recognition of viral infection (especially, in the case of DNA viruses such as HPV). However, even for such a common pathway of antitumor response induction, a dual (tumor-suppressing or tumor-promoting) role defined by the etiology or the stage of a disease has been reported.

More specialized populations of innate immune cells are also equipped with a large variety of receptors to detect mislocalized/ectopically expressed biomolecules in cancerous or virus-infected cells. Lymphoid cells (natural killer (NK) cells, NK-like T cells, and Tγδ lymphocytes), tumor-associated macrophages (TAMs, M1, and M2-polarized), tumor-associated neutrophils/myelocytes (TANs, N1, and N2-polarized), myeloid-derived suppressor cells (MDSCs), and other immature dendritic cells—all these cell populations (both tumor-infiltrating and circulating) are the main object of studies published recently. For most of them (including some innate-like T cell subpopulations), it has been established that they can significantly contribute to tumor progression, and at the same time, a crucial role in the elimination of malignant cells has been proved for innate-like lymphocytes (see below). The most difficult aspect of the functioning of these types of cells is their ability to produce the widest spectrum of cytokines that depends on the surrounding "context," thus defining their regulatory properties. In this sense, their activity should be considered in conjunction with the activity of regulatory/suppressor T and B cells (Treg, Breg) and different T helper subtypes, including pro-inflammatory Th17/Th22 cells, especially in light of the fact that the inflammation is appreciated as one of the most important tumor-promoting factors. Despite significant progress in the study of innate mechanisms of response to a developing tumor, which is implemented by the cell populations named above, many researchers point out that most of the information on this problem is obtained using laboratory mouse strains, which are certainly indispensable as experimental models, but this information cannot be simply extrapolated to the human body and thus requires a separate verification. This is especially important in case of virus-associated carcinogenesis because, due to high species-specificity of oncoviruses and their strong cell-type tropism, the range of *in vivo* models adequately reproducing the terms of the long-lasting, chronic infection, and gradual development of neoplasia in humans (which can take months and years), is limited [5].

HPV-associated cervical cancer as an object for studying the dynamics of "pathogen-tumor-immunity" interactions draws increasingly more attention due to the newly emerging findings demonstrating that during HPV-associated carcinogenesis, the immune system (and its innate components, in particular) acts as a double-edged sword and its role dramatically changes during the course of disease development [2]. Most HPV infections and low-grade lesions regress spontaneously in a short time; these cases are proposed to be considered as an "acute" infection [3], which is accompanied with the activation of inflammatory response superior in strength to a variety of mechanisms exploited by HPV to suppress inflammation and escape from immune recognition. However, in a number of cases, the infection turns into a persistent form, thereby

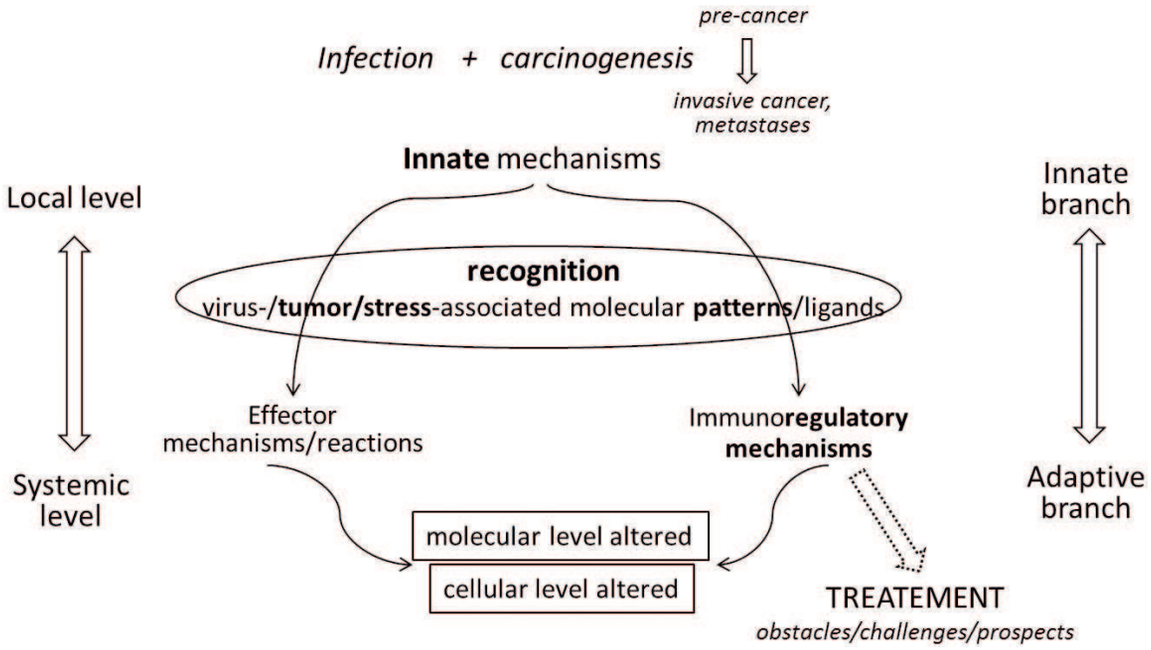

Figure 1. Scheme illustrating general relations between the key levels of immune response to cervical cancer that are addressed in the chapter.

increasing the risk of malignant transformation. In the later stages of carcinogenesis, in contrast to the stage of productive infection, HPV-transformed cells reprogram their environment in such a way that they gain the ability to recruit different populations of immune cells and to initiate chronic stromal inflammation, which contributes to further progression of precursor lesions into invasive cancer, facilitates tumor growth and metastatic spreading, and simultaneously promotes exhaustion of effector immune cells populations [2].

As a result of the fact that cervical cancer development is characterized by high genomic instability, the accumulated somatic mutations generate the enormous variety of neoantigens, which, together with the HPV-antigens, represent the potential targets for the T cell-mediated adaptive (TCR-restricted) response [6]. The range and immunogenicity (the ability to be presented to cytotoxic and helper T cells) of these antigens have been proved in high-throughput studies using integrated approaches to genome/transcriptome sequencing data analysis (see, for example, [7]). At the same time, the study by Qin et al. shows that increased mutation burden and neoantigen load correlates with HPV-dependent activation of master regulator genes that abrogate antitumor immune responses these neoantigens could cause by mobilizing immune regulatory, suppressive mechanisms. This again proves the rationale of studying the innate and innate-like lymphocytes, regulatory T/B lymphocytes, cells of myeloid lineage, as well as the mechanisms of antigen-independent innate immune response (including those involving DNA sensors) and the processes of immune regulation at different stages of cervical neoplasia development. In present chapter, the results of studies on these specific cell populations, mechanisms and processes published in last 2–3 years are described, with simultaneous discussion of our own experimental data on this problem, obtained from the patients with the diagnosis of pre- and microinvasive cervical cancer. Since a large number of constantly updated reviews are available on the issue of molecular strategies used by HPV to avoid immune response or other so-called cell restriction factors (see, for example, [8]), this question is not presented in the Chapter. In addition, we do

not discuss the preventive and therapeutic vaccines developed for cervical cancer, as one can find many specialized detailed articles devoted to this applied question (for example, [9–11]).

The issues which are accentuated in this Chapter are showed schematically in **Figure 1**. Those are: the relationships between local and systemic changes, cells of innate and adaptive arms of immunity, their regulatory and effector properties, their phenotypic and quantitative changes—at different stages of cervical cancer development. We give special attention to pre- and microinvasive cervical carcinoma when reporting our findings is due to the idea that these stages can be considered as tipping points in re-formatting of the host immune system.

2. Intrinsic molecular mechanisms bridging antiviral and antitumor immune responses in cervical cancer

2.1. The role of nucleic acid-sensing pattern-recognition receptors (PRRs) and related signaling pathways in controlling cervical cancer development: current concepts

To respond to ectopically localized nucleic acids of exogenous (infectious) or endogenous (tumor cell- or stressed cell-derived) origin, cells are "armed" with a set of nucleic acid-sensing pattern recognition receptors (PRRs). Members of this essential group of PRRs are expressed in cells of both immune (lymphoid/myeloid) and non-immune (for example, epithelial) origin and can recognize various forms of nucleic acids (single- and double-stranded DNA or RNA, DNA-RNA-heteroduplexes, CpG-islets, as well as specific chemical modifications or structures, typical for viral DNA/RNA, and messenger cyclic nucleotides) in different cellular compartments (cytosol, endosomes/phagosomes, and even in the nucleus). These include some representatives of Toll-like receptor family (TLRs: 3, 7, 8, 9), Absent in Melanoma 2 family, (AIM2, IFI16), RIG-I-like receptors (RLRs: RIG-I and MDA5), and other members of the DExD/H helicase family, as well as a "signaling pair" of cyclic GMP-AMP synthase (cGAS)—Stimulator of Interferon Genes (STING). In spite of the fact that these receptors/sensors activate different signaling pathways, they all eventually lead to the activation of transcription factors such as Interferon Regulatory Factors (IRFs) or Nuclear Factor kappa B (NF-kB), which are responsible for the production of type I interferons (IFN-I) or proinflammatory cytokines, respectively [12].

Among the listed molecular sensors, the STING protein is recognized as a signaling hub (**Figure 2A**): it can receive and redistribute signals coming from different upstream molecular partners, although the most well studied and, perhaps, most important for mammalian cells, is the cGAS-STING signaling axis [13]. Binding of cGAS with cytosolic DNA results in the synthesis of secondary messenger—cyclic dinucleotide cGAMP—a natural STING ligand; following interaction with cGAMP, STING (an endoplasmic reticulum membrane-resident protein) initiates assembly of a multiprotein complex (i.e., signaling platform) and, through activation of IRF3 transcription factor, triggers expression of a large number of genes, including IFN-I genes and IFN-stimulated genes (ISG). Moreover, the new data from high-throughput transcriptome analysis showed that depending on the cell type, STING can alter the expression of not only the immune response-associated genes, but also many other genes that govern crucial cellular processes (proliferation, apoptosis, and stress response) [14–16]. The existence of alternative pathways that lead to STING

activation (possibly ligand/agonist-independent) is also assumed, although the mechanisms have not yet been sufficiently described [17]. The key role of STING in antiviral innate immune defense has been confirmed by numerous studies, and it is not surprising, therefore, that different groups of viruses have evolved a variety of strategies to avoid/inhibit STING-dependent response, and oncoviruses are no exception: for most of tumor-associated mammalian viruses, STING, and other components of the STING-dependent signaling pathway were found to be specifically targeted by viral oncoproteins (in our previous paper, we summarized known mechanisms that are used by the oncoviruses, in particular, HPV, to evade STING-mediated recognition [18]).

The involvement of STING in regulation of the relationship between the tumor and the immune system (both innate and adaptive branches) mediated through the recognition of tumor DNA has been experimentally corroborated, although there are still many unresolved contradictions regarding its precise role in carcinogenesis: in different mouse tumor models, stimulation of expression, and/or activity of STING resulted in either restriction of tumor growth or tumor progression. What reasons could underlie these contradictions? On one hand, the STING-induced production of type I IFNs and activation of inflammatory reactions are obviously indispensable for the proper functioning of antigen-presenting cells (APC) and for further induction of adaptive antitumor response (discussed in [13, 19, 20]). On the other hand, the increased activity of STING leads to chronic inflammation within the locus of neoplasia which is a driving force of immunosuppression and tumorigenesis. In addition, there is still no clear understanding of exactly which cells within a tumor are responsible for STING-dependent recognition of tumor DNA. A previously proposed model, according to which it is phagocytizing cells (primarily dendritic cells and macrophages) that can engulf tumor DNA from dead/apoptotic tumor cells and activate the STING-signaling pathway, causes many doubts as it is not clear how endosomal/phagosomal DNA can reach cytosolic cGAS. Another model has been recently proposed, whereby the primary recognition of tumor DNA and synthesis of cGAMP occurs in tumor cells themselves because of the "leakage" of nuclear DNA into the cytosol (as a result of genomic instability, DNA damage, increased proliferation rates); cGAMP can diffuse to neighboring cells, including immune ones—presumably during the formation of immunological synapse—which are more efficient IFN-I producers and thus are able to promote recruitment of dendritic cells (DCs) and effector T cells [21]. APCs are widely recognized as such efficient producers, but other types of cells, for instance, lymphoid cells, can also be the candidates, considering that the level of STING mRNA/protein expression in lymphocytes was shown to be significantly higher than in macrophages [14, 16]. This model assumes that the initial stages of carcinogenesis are accompanied by an increased expression/activity of cGAS-STING, but as the tumor progresses, a disruption of cGAS-STING signaling—as a way to counterattack anti-tumor immunity—can occur. However, in virus-associated cancers, including cervical cancer, where STING activity can potentially be modulated by virus-derived and tumor-derived DNA, there may be the opposite sequence of events: in the initial phase of the establishment of a chronic infection, viral oncoproteins inhibit cGAS-STING pathway in infected cells (**Figure 2B**) and then, after undergoing malignant transformation, tumor cells gain the ability to support up-regulated state of cGAS-STING signaling in order to generate inflammatory immunosupressive microenvironment. Immunohistochemical study of HPV-infected cervical epithelium and low-grade cervical lesions indeed showed reduced expression of STING in relation to normal epithelium [22], but what changes are characteristic of high grade lesions and cervical cancer are as yet unknown.

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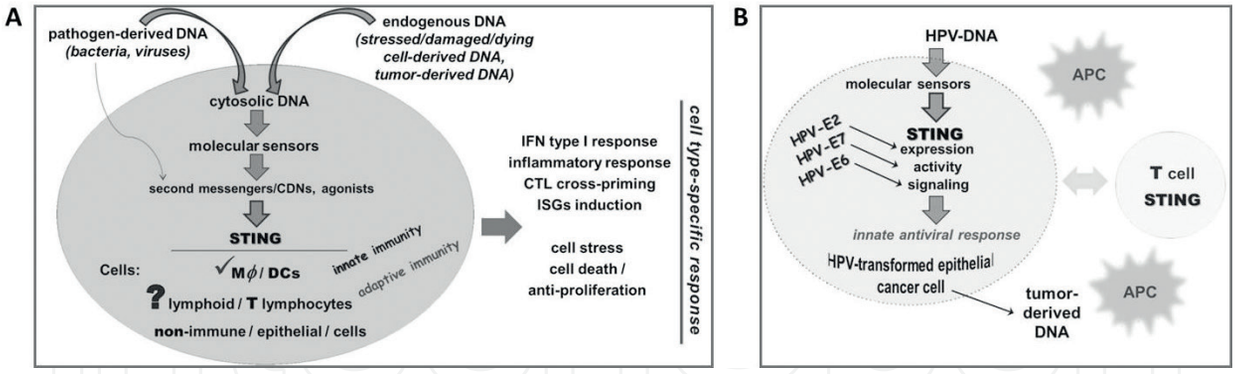

Figure 2. (A) Activating signals from various sources converge on STING to initiate cell type-specific innate response to cytosolic DNA. (B) In HPV-induced neoplastic lesions, STING can receive activating signals both from invading HPV-DNA and mislocalized self-DNA.

As mentioned before, STING-mediated signaling has been most thoroughly investigated in macrophages and dendritic cells while its role in other cell populations, specifically non-myeloid cells, is not fully understood. In this respect, recently published findings from *in vitro* and *in vivo* experiments (carried out using genetically engineered mice and STING ligands/agonists) demonstrating the functionality of canonical STING-dependent signaling in T cells [14–16] are of high importance. Surprisingly, besides activation of IFN-I response these experiments revealed T cell-specific ability of STING to modulate (inhibit) TCR-stimulated expansion and to induce cell death (through IRF3- and p53-dependent pathway), which is the fundamental difference from macrophages, in which stimulation of STING never leads to activation of death-associated genes [14–16]. The T cell-specific effect is extremely important for the prediction of therapeutic effect of STING agonists, which are currently undergoing extensive clinical trials as adjuvants in chemo-and immunotherapy of different types of cancer; however, in the case of cervical cancer the specificity of STING expression changes has not been investigated so far. At the same time, HPV-associated cervical cancer, in our opinion, can be used as a model object to study either cell type-specific or stage-specific involvement of STING in the innate/adaptive immune functioning at local and, most importantly, systemic level.

2.2. Altered patterns of STING expression indicate its putative role in cervical cancer

Based on the above facts, a study of the expression profile of STING (at mRNA/protein level) in tissue samples as well as in the major populations of peripheral blood T lymphocytes obtained from patients with preinvasive and microinvasive cervical cancer compared to healthy women (control group) has been started by our research team. We also took into account that: (1) increased expression of markers of apoptosis can be observed in circulating T lymphocytes in patients with early (pre-clinical) stages of cervical cancer [23]; (2) patients with early-stage cancer or precursor lesions display a variety of systemic alterations in the immune system including altered phenotype/activity and frequencies of different T cell populations, as evidenced by the large number of data (including those described below); (3) HPV-DNA (and possibly tumor DNA) circulates in the body and thus can be detected in various tissues and lymphoid organs long before the first detectable signs of metastases [24], whereby it potentially exerts a systemic effect on the activity of the STING.

2.2.1. STING protein levels in different subsets of circulating lymphocytes from early-stage cervical cancer patients

Intracellular STING level was measured in circulating CD4 and CD8 T cells, as well as in CD4CD25 subset (**Figure 3**) by flow cytometry using anti-human STING monoclonal antibody (MAb; clone 723505). Since the majority of lymphocyte population were stained positively for STING (which is in compliance with previously reported data showing that STING is robustly expressed in lymphoid tissue, specifically in T cells [14]), making the percentage values less informative, the level of STING protein was expressed as relative Mean Fluorescence Intensity value (ΔMFI) normalized to MFI of isotype control (IgG) with correction for autofluorescence of corresponding T cell subsets (Fluorescence Minus One, or FMO, control) (**Figure 3**).

As we did not find published works reporting on the level of STING in peripheral blood lymphocytes analyzed by means of immunofluorescence techniques, we first compared different commercially available kits for intracellular protein staining. The results of intracellular STING evaluation in peripheral blood T cells appeared to be sensitive to the permeabilizing ability of a fixation/permeabilization buffer set used, specifically: when kits designed for staining of intracellular proteins (such as cytokines) were applied, the level of anti-STING MAb binding did not differ from isotype control (**Figure 4A**); whereas the use of a reagent kit intended for intracellular detection of antigens such as nuclear transcription factors resulted in significant anti-STING MAb binding compared to isotype control (**Figure 4B**). This might be due to specific localization of STING and availability of its epitopes: homodimeric STING resides in the ER membrane and upon activation may form aggregates and translocate to Golgi and perinuclear space [25] (according to the manufacturer, the immunogen aa215-379 for the clone 723505 of anti-STING MAb corresponds to the C-terminal cytoplasmic domain of human STING).

In early-stage cervical cancer patients (with carcinoma *in situ* or microinvasive carcinoma), the level of STING protein showed a decreasing trend in both CD4 and CD8 T subsets compared to

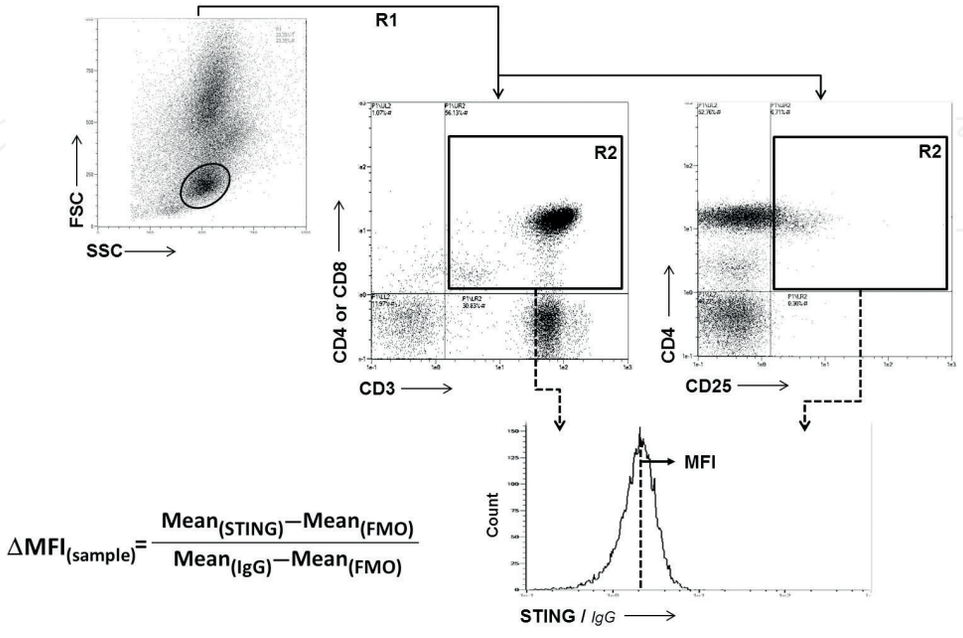

Figure 3. T cell gating and evaluation of STING protein level.

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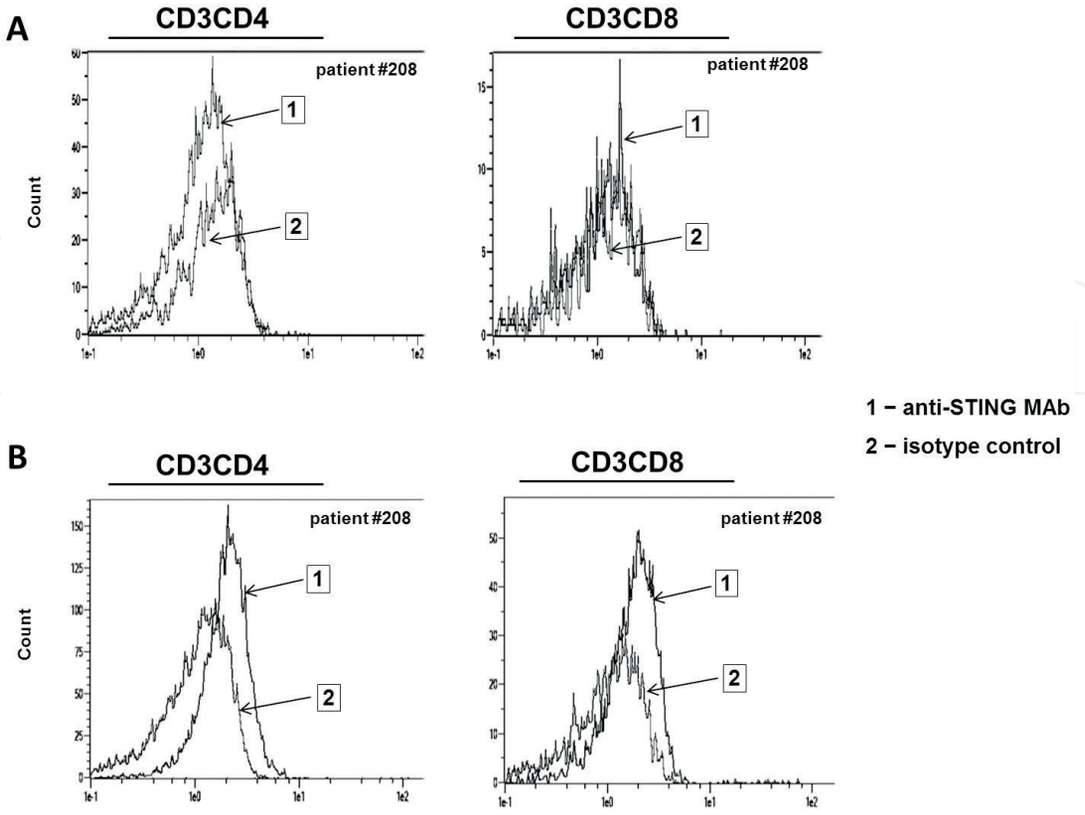

Figure 4. Representative examples of STING staining carried out using (A) a reagent buffer set for staining cytosolic proteins (e.g., cytokines), or (B) a reagent kit with stronger permeabilizing capacity for intracellular/nuclear protein staining.

healthy controls with this decrease being more pronounced in CD8 T lymphocytes (**Figure 5A**). No significant change was observed for CD4CD25 subpopulation. A notable increase in ΔMFI (CD4CD25)/ΔMFI (CD3CD8) ratio was revealed for circulating T cells from cancer patients (**Figure 5B**), implying that STING expression became more pronounced in CD4CD25 lymphocytes in relation to CD3CD8 subset. At the same time, the difference between STING levels in CD3CD4 and CD3CD8 cells from both controls and cancer patients was less significant; ΔMFI(CD3CD4)/ΔMFI (CD3CD8) ratios were close to 1 in all groups studied suggesting that the expression of STING is associated with both CD4 and CD8 T cell subsets. These results are, in a certain sense, in consistence with data reported previously by others for mouse models [14].

The percentage of STING-positive cells in total population of circulating lymphocytes from cervical cancer patients was on average lower than that in the control group, although this difference was not statistically significant ($p > 0.05$, U-test; **Figure 6**). When analyzing CD3 T cells, the same trend could be observed (while the total frequencies of T cells did not differ between patients and controls).

2.2.2. STING mRNA expression in peripheral blood mononuclear cells (PBMC) and neoplastic tissue samples

At the mRNA level, STING expression was analyzed in ficoll-isolated PBMC using semi-qPCR (RPLP0 and PGK1 genes were used as endogenous controls [26]): similar to flow cytometry results, in PBMC from patients with preinvasive/microinvasive cancer (stage 0-IA), STING-mRNA

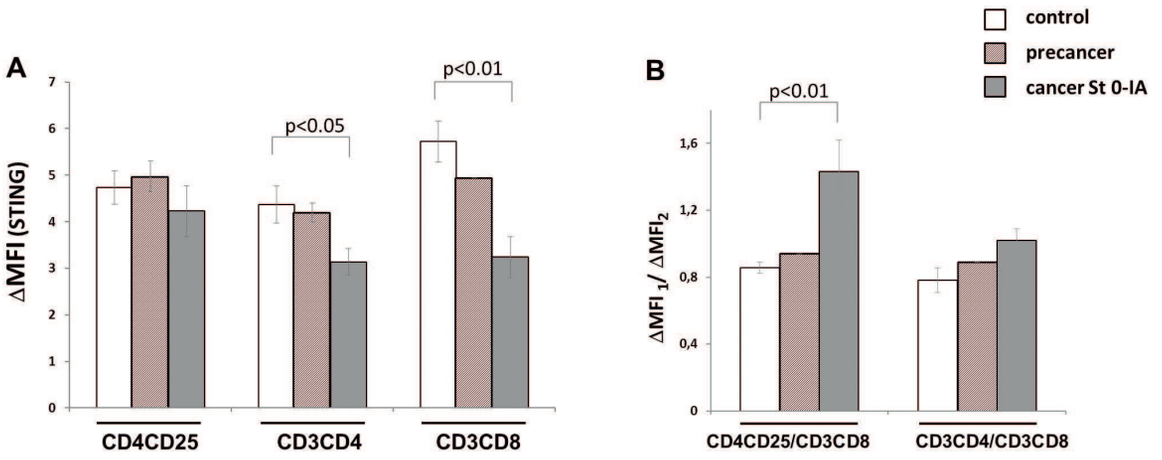

Figure 5. (A) The change of STING protein levels in major subsets of peripheral blood T cells from patients with precancerous cervical lesions or cancer (stage 0-IA, n = 20) relative to the control group of healthy donors (n = 15). (B) The ratio of the relative STING expression level in different T cell populations. Mean ± SEM values are displayed; p-value was assessed by U-test.

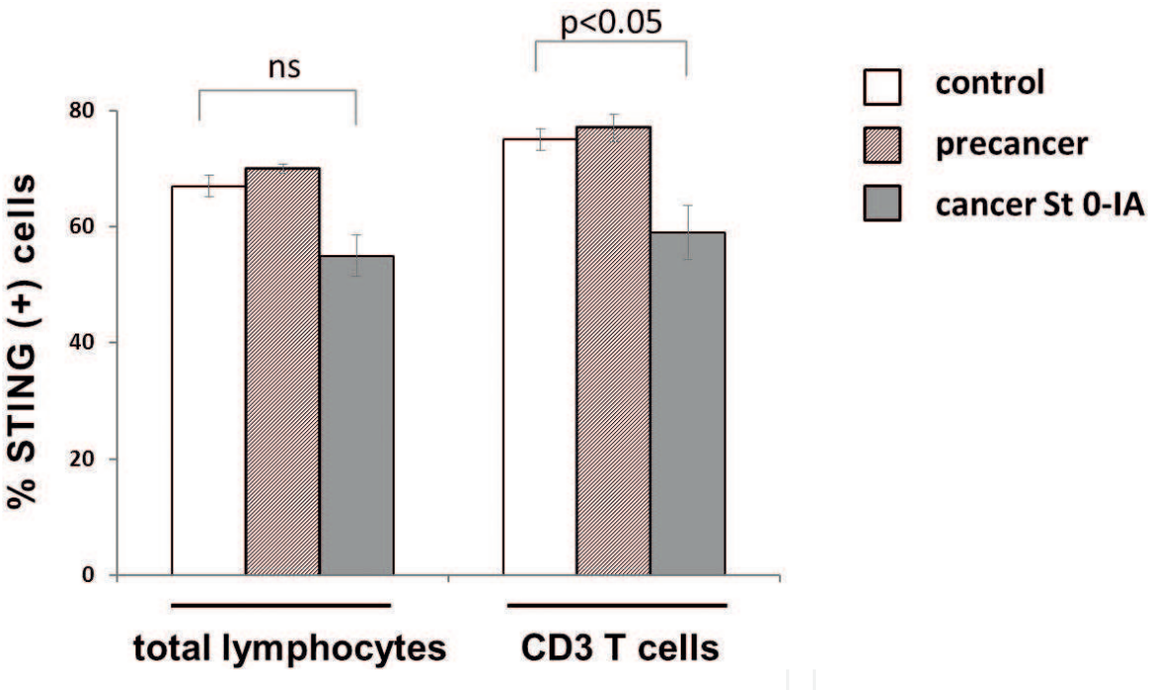

Figure 6. Percentage of peripheral blood lymphocytes stained positively for STING in patients with precancerous cervical lesions or cancer (stage 0-IA, n = 20) vs. healthy donors (control, n = 15). Mean ± SEM values are shown, ns—not significant.

level showed a slight decrease compared to the control group ($p > 0.05$, U-test; **Figure 7A**) suggesting the need for T cell (CD4/CD8) separation in further analysis. STING-mRNA expression was also assessed in samples of HPV-negative morphologically normal epithelium (control), HPV-positive precancerous lesions of the cervix, carcinoma in situ and microinvasive carcinoma (relative to four genes—EEF1A1, ACTB, GAPDH, and RPLP0—taken as endogenous controls due to their proved constitutive expression in cervical tissues [27]) (**Figure 7B**). In contrast to lymphocytes, a considerable (up to 50%) proportion of pathological samples

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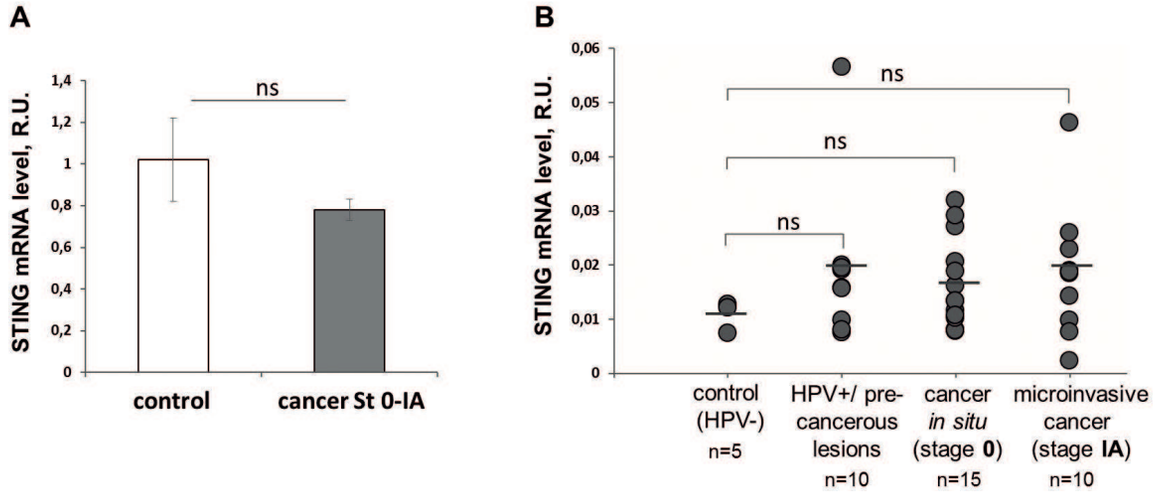

Figure 7. (A) The change of STING mRNA expression in PBMC isolated from cervical cancer patients compared to healthy women (controls); mean ± SEM values are shown. (B) The change of STING mRNA expression in cervical neoplastic lesions; group mean values are depicted as horizontal bars; ns—not significant.

in each patient group showed elevated STING expression as compared with normal non-infected epithelium (though the group mean values did not differ statistically). Up-regulation of STING at early stages of cervical carcinogenesis is consistent with some previous reports by other researchers and is, overall, in line with the conception of dichotomous role of STING-pathway in tumor development [28]. The data may also indicate STING's participation in as yet unexplored mechanisms promoting tumor development: for example, c-MYC proto-oncogene which overexpression is a hallmark of cervical cancer has been recently described as an essential transcription factor for the STING gene [29]).

Taking into account that cervical carcinogenesis can be associated with decreased proportion of STING-expressing T lymphocytes, as well as decreased level of STING protein in both T cell subsets (CD4 and CD8), one may assume the involvement of T cell STING in controlling papillomavirus infection and HPV-induced oncopathology. On the other hand, despite the lower level of STING observed in total CD3CD4 population of patients' peripheral blood lymphocytes, in CD25-positive subpopulation its expression was sustained at levels similar to the control: as CD25 is known to be a Treg marker, as well as a T-activation marker, this may be related to the processes of T cell activation/proliferation, interleukin (IL) 2-signaling, and T cell death. This assumption can be confirmed by recent findings [14, 15] demonstrating anti-proliferative or cell-death promoting activity of STING in TCR-stimulated T cells. Previously, we also showed up-regulation of apoptotic processes in circulating lymphocytes from early-stage cervical cancer patients [23], which was correlated with the expansion of CD25-positive cells (including FoxP3-expressing Treg) prompting further investigation of STING in T cells during virus-related carcinogenesis. Thus, the study of naturally occurred cervical neoplastic pathology that develops as a result of chronic viral infection suggests that STING being a key player in modulation of innate immune reactions may have an essential role in T cell functions. During the development of infection-related cancer, the importance of this specific role can be realized through redistribution of STING levels in different T cell subsets. Oppositely directed changes in STING expression observed in different compartments—blood T lymphocytes and neoplastic tissue—may illustrate the putative dual role of STING in virus-related

carcinogenesis, which, in turn, may represent an important point in prognosing therapeutic outcome of STING stimulation. While administration of STING agonists may occur beneficial, for example, for patients with T cell-derived cancer (or other lymphoproliferative disorders) due to promotion of apoptosis in malignant T cells [16], mobilization of STING activity in solid tumors may have an opposite effect due to increased apoptosis of T effectors.

Summarizing, it is worth noting that the abundance of STING in T cells may imply; on one hand, their engagement in the innate immune mechanisms (as was revealed by a study of Larkin and co-authors who observed induction of intact antiviral IFN-I response in mouse T cells upon stimulation with STING agonists [14]) or, on the other hand, the plausibility of noncanonical functions exerted by human STING in cells of the adaptive immune system, these issues to be further investigated in the norm and in various pathological states, including virus-induced cervical cancer. In conclusion, it is worth mentioning that such noncanonical activity of STING, specifically, ability to switch on the apoptotic pathway has been unraveled not only in T cells, but in murine B lymphocytes (normal and malignant) as well [30]. However, in another study, the expression of STING in human B cells could be detected only upon Epstein-Barr virus-mediated transformation, while normal B lymphocytes were unable to elicit IFN-I response upon treatment with STING agonists due to the absence of STING expression [31]. Regarding other types of lymphocytes, for instance, NK cells, there is limited or no information. According to our flow cytometry data, the level of STING protein in circulating natural killer cells from patients with cervical carcinoma in situ is notably lower than in CD3CD4 and CD3CD8 (ΔMFI for CD3negCD16pos population was 2.16±0.16), but nonetheless 35 ± 4% of NK cells appear to be STING-positive suggesting potential involvement of STING in NK cell functions.

3. Cellular component of innate immunity (natural killer lymphocytes, myeloid cell populations): its role in regulation of T cell-mediated antitumor/antiviral immunity

3.1. Regulatory functions of innate immune cells in relation to cervical cancer development: current knowledge

The regulatory role of myeloid cells (monocytes—dendritic cells and macrophages, and especially granulocytes) has been undervalued for a long time; however, recently emerged data have prompted reconsideration of significance of these cells, classically regarded as professional phagocytes or professional APC, in mediating regulatory/suppressor effects of tumor cells on T-effectors [32]. In addition, the systemic effect of local neoplastic lesions on deviations within these innate immune cell populations, which can become detectable even earlier than the distribution of tumor-infiltrating cell populations is changed, is becoming increasingly apparent [32]. In respect of these abnormalities, a number of fundamentally important data have been obtained for cervical cancer.

According to the model described by Smola et al., IL-6 secreted by HPV-transformed cells acts as a triggering factor that leads to multiple impairments in the key functions of myelomonocytic cells during the intraepithelial stage of cervical cancer development. Under the influence of IL-6 and chemokines, myelocytes are actively recruited into the site of neoplasia, where they can

differentiate into functionally impaired dendritic cells or M2-polarized macrophages to maintain pro-inflammatory environment. Despite they have mature phenotype, dendritic cells are not able to migrate to the lymph nodes to initiate adaptive response due to the lack of appropriate homing receptors; instead they accumulate within cervical cancer stroma and secrete protumorigenic and Th2-polarizing factors. Cervical cancer-infiltrating M2-macrophages not only fail to produce IFNs at levels required for T cell activation and proliferation, but also express ligands for the immune checkpoint molecules, for example PD-1L, thereby promoting cytotoxic T cell exhaustion [6, 32–34]. Interestingly, according to Swangphon et al., cervical cancer patients exhibit altered ratio of M1/M2-polarized (CD64+/CD163+) monocytes not only at the local level, but in systemic circulation as well; notably, circulating M1/M2 ratio was shown to be correlated with the number of stroma- or peritumoral area-infiltrating M2-macrophages (CD163+), and with severity of the disease [35]. Similarly, cervical cancer patients displayed increased numbers of circulating dendritic cells (CD11b+) expressing PD-1L [36]. Moreover, an increase in the number of tumor-promoting M2-macrophages/ monocytes has been found to occur not only locally, i.e., in the tumor site, or systemically, i.e., in circulation, but also in tumor-draining lymph nodes (TDLN) of cervical cancer patients implying that the number of PD-1L+ M2-macrophages and metastasis are interrelated; this association allows to suppose that metastasizing cancer cells have the ability to recruit CD14+ monocytes and drive their conversion into M2-macrophages further contributing to the expansion of highly suppressive Treg cells [34].

Progression of precursor lesions into cervical cancer is also accompanied by an increase in the number of infiltrating neutrophils (TANs) displaying suppressive phenotype. A negative correlation found between the amount of TANs and CD8 T cells in high-grade lesions (cervical intraepithelial neoplasia grade 3, CIN3) or cervical cancer samples suggests that TANs can potentially contribute to inhibition of T cell activity and thereby facilitate tumor growth [32]. This assumption was confirmed experimentally in *in vitro* cell system using co-cultures of SiHa-spheroids, *ex vivo*-stimulated T lymphocytes, and neutrophils, with the ratio of T cell/neutrophil numbers appeared to be the determining factor for the degree of suppression of T cell proliferation, their expression of activation markers, secretion of IFNγ, and cytotoxic activity against SiHa cells [32]. At the systemic level—in the peripheral blood of cervical cancer patients—higher frequency of immature low density neutrophils has been also revealed, with elevated serum levels of granulocyte colony stimulating factor (G-CSF) discovered not only in cervical cancer patients, but also in women with precursor lesions (CIN2-3). Furthermore, patients diagnosed with cervical cancer are characterized by a systemic increase in the frequency of the tolerogenic monocyte-derived dendritic cells (MoDCs), the differentiation of which is modulated by G-CSF: MoDCs that were differentiated from monocytes taken from patients with CIN3 or cervical cancer and showing higher serum level of G-CSF were able to significantly more intensively inhibit proliferation of T cells from healthy donors and to promote Treg differentiation in the *ex vivo* system [32]. The effect of cervical neoplastic lesions on the process of MoDCs differentiation (expression of maturation markers, the profile of secreted cytokines) has been also demonstrated in a study by Lopes et al. [37]. Altogether, these data once again prove that early neoplastic lesions can be accompanied by systemic deviations in innate immunity, which in turn can influence redistribution of innate and adaptive cell populations and their interactions with each other within the tumor locus. The entirety of systemic and local immune changes is also an important point to consider when developing antitumor therapies based on adoptive DC transfer, because it is obviously these changes that determine the absence of the desired therapeutic effect (such developments

aimed at overcoming the suppressive impact on DC are conducted using preclinical murine models of cervical cancer, see, for example, [38, 39]). In addition, a study performed by van Meir et al. showed that myeloid cells from cervical cancer patients can systematically respond to radiotherapy (RT): during the course of RT and 3–9 weeks after its completion (regardless the administration of cisplatin), increased frequencies of circulating CD3(-)CD19(-)HLA-DR(+) monocytes as well as CD3(-)CD19(-)HLA-DR(-) MDSCs were detected in parallel with the loss of T cell reactivity and stimulatory capacity of APC in *ex vivo* testing [40].

Unlike neutrophils and suppressor populations of myeloid cells, whose contribution to the progression of solid tumors has only recently come under intense investigation, the functions of natural killer cells have always been considered in the context of cancer immunosurveillance. However, in spite of the fact that for this group of innate lymphoid cells, a detailed spectrum of receptors allowing for recognition of transformed cells has been described and a vast diversity of mechanisms for their cytotoxic action has been established, attempts to use them in anticancer therapy occurred to be unsuccessful—the reasons for this situation are reviewed in [41], and among these reasons are the underappreciated regulatory properties of NK cells implementing via production of a wide range of cytokines, the specificity of which is largely determined by the surrounding molecular context. Nevertheless, recently there has been considerable revival of interest in NK cells brought about by the invention of chimeric antigen receptors (CAR) technology that made possible creation of engineered CAR-NKs with "improved" properties (e.g., increased migrating and proliferating ability, up-regulated expression of activating receptors) for their subsequent adoptive transfer into a cancer patient. Another promising concept seems to be the use of Cord-Blood NK cells that can retain a highly activated phenotype and whose expansion capacity substantially exceeds that of peripheral blood NK cells (successful implementation of this approach in the preclinical model system using cervical cancer cell lines has been recently reported in [42]).

Cervical cancer cells' ability to withstand NK cell-mediated response is clearly confirmed by the observation that the prevalence of NK cells in CD45(+)-infiltrating leukocytes is greatly reduced with the progression of intraepithelial neoplasia to invasive cancer [32]. In addition to the known mechanisms recruited by cervical cancer cells to escape from NK-mediated recognition (including down-regulation of activating NK-cell receptor ligands MICA/B, ULBPs, or aberrant expression of non-classical HLA-G [43]), inhibition of NK cell activity can be driven by intra-tumoral Tregs, as was confirmed in ex vivo experiments with Tregs and NK cells isolated from primary tumors of cervical cancer patients [44]. Whether these negative processes have any influence on circulating NK cells during the development of cervical cancer remains a poorly studied question.

Despite the high phenotypic heterogeneity of NK cells, they can be divided into two subsets depending on the level of expression of CD56 marker: CD56bright and CD56dim. These two populations differ not only phenotypically and functionally—they are differently represented in the systemic circulation and tissues [45]. CD56dim population comprises the vast majority (80–95%) of peripheral blood NK cells and is characterized by high expression of markers of mature phenotype (including CD16/FcγRIIIa required for activation of antibody-dependent cytotoxicity, perforin and granzyme B cytotoxic proteins); traditionally, this population is associated with anti-tumor response. Unlike CD56dim, CD56bright NK cells represent the minor population in peripheral blood, while in the secondary lymphoid organs and other tissues CD56bright cells account for the majority of peripheral NKs. In addition, they are characterized by the absence or low expression of CD16 (CD16dim/neg) and low cytotoxic activity, so their role in direct

killing of tumor cells is less clear; on the other hand, CD56bright NK cells are known for their high cytokine and chemokine production capacity (including IFNγ, TNFα, GM-CSF, IL-10, IL-13, CCL3, and CCL4), and immunomodulation of activity of other innate or adaptive immune cells is therefore believed to be a key feature of CD56bright NK cell subset.

Presently, increasing attention is being paid to CD56bright NK cells as new facts are emerging suggesting that there is no strict functional dichotomy between the so called regulatory CD56bright and cytolytic CD56dim subsets, and that CD56bright cells are capable of acquiring cytotoxicity upon appropriate stimulation with specific combinations of cytokines [45]. Indeed, it has been recently shown that priming of CD56bright NK cells with IL-15 is accompanied by a burst of cytotoxic activity against tumor cells; however, this has only been confirmed so far for hematological malignancies [46]. Upon treatment with different stimuli, CD56bright NK cells exhibit ability of suppressing proliferation of autologous CD4 T cells via both cytotoxic and immunoregulatory mechanisms, e.g., by secreting the immunosuppressive molecule adenosine (these mechanisms are reviewed in detail in [47]). For some types of solid cancers, in particular lung cancer and breast cancer, the proportion of CD56bright cells in a total amount of tumor-infiltrating NK cells was found to be significantly higher than in the corresponding normal tissues, however, they express low perforin and rather play an immunoregulatory role, but not cytotoxic [48].

3.2. Analysis of NK cell subpopulations in peripheral blood lymphocytes of early-stage cervical cancer patients

Taking into consideration, the proposed model that describes the ability of CD56bright NK cells to circulate among tissues, lymphoid organs, and peripheral blood [45, 48], it can be assumed that altered frequencies of these cells in the blood of cancer patients are highly relevant to immune regulation at the tumor locus. Quantitative assessment of circulating CD56bright NK cell population has been performed for head and neck cancer [49], prostate [50], and breast cancer [51, 52]); we also recently reported our findings concerning circulating NK subsets in women with CIN3 (including carcinoma in situ) and microinvasive carcinoma (stage IA1) of the cervix [23].

Based on the intensity of CD16/CD56 staining, we could distinguish four main subsets of circulating NK cells within CD3-negative lymphocytes (gates P1–P4, **Figure 8**). As expected, we found no significant difference in the frequency of cells within CD16brightCD56dim gate (which encompasses the major pool of circulating cytotoxic NKs), as well as within CD16dim/negCD56dim and CD16brightCD56neg gates (comprising less abundant populations with poorly established functions) between patients and controls. As opposed to these subsets, a decrease in the frequency of CD16dim/negCD56bright NK cells and, accordingly, higher CD56dim/CD56bright ratio were observed in cervical cancer patients relative to the control group. We hypothesized this specific alteration reflects a systemic shift in the balance between effector and regulatory NK subsets that occur early in invasive cervical cancer development. One can also speculate this change, along with those described above for M2/M1, neutrophils and MoDC subsets, is part of a complex cervical cancer-related immunoregulatory network. As it is well known that activation of NK cells occurs locally, in our attempt to interpret the obtained data we therefore use the idea that circulating CD56bright NKregs are recruited to the lymphoid tissue (regional lymph nodes) and the primary tumor site, where they are thought to serve as precursors for cytotoxic/effector CD56dim NK cells [45]. This assumption encourages further investigation into the regulatory role of NK cells in cervical cancer progression.

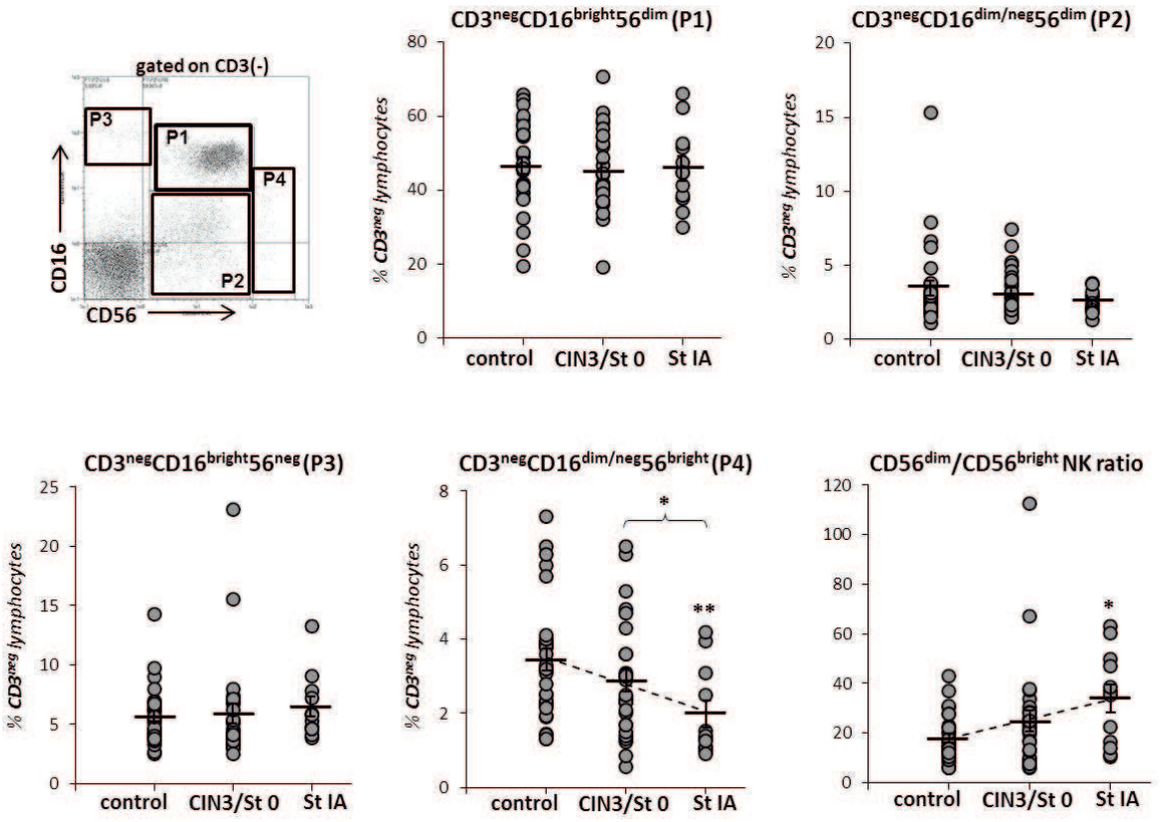

Figure 8. Percentage of peripheral blood CD56bright NK cells and CD56dim/CD56bright ratio within circulating NK cell population in patients (n = 30 for CIN3/stage 0, n = 15 for stage IA) vs. healthy controls (n = 30) as measured by flow cytometry. Lymphocytes were gated for CD3-negativity (a diagram on the left) and a population of interest was defined according to CD16/CD56 membrane expression levels (gates P1–P4). Here and below, individual values are shown as dots; bars correspond to the mean ± SEM values; statistically significant difference between the patient group and the control group are marked with asterisk: *$p < 0.05$, **$p < 0.01$ (U-test).

4. Innate-like T lymphocytes: pivotal players in the tumor: immunity interplay

4.1. The emerging role of NK-like T cells (NKT) and γδT lymphocytes in cervical cancer progression

Since recently, a heterogeneous group of innate-like T lymphocytes linking the two branches of immunity, innate and adaptive, is being increasingly acknowledged as a valuable source of novel opportunities for antitumor therapies development. This was facilitated by increasing realization of the pivotal role of innate-like T cells in tumor immune surveillance and their unique ability to recognize cancerous and virus-infected cells in a highly specific, though MHC-unrestricted, manner. Similar to innate immune cells, they are equipped with a rich set of germline-encoded receptors conferring them ability to undergo rapid activation upon interaction with ectopically expressed or stress-associated molecules on the surface of target cells. In addition, like conventional αβT cells, innate-like T lymphocytes (NKT and γδT) express T cell receptors (TCR), although their repertoire differs from αβT cells. The range of antigens the innate-like lymphocytes' TCRs are able to recognize is defined by their structural properties (for example, lipid antigens in the case of NKT cells

or phosphoantigens in the case of γδT cells) and therefore is thought to be restricted and universal; at the same time, these antigens are present within a plenty of natural ligands, which is doubtless advantageous from a therapeutic standpoint. Quick activation in response to antigenic exposure followed by intense production of a broad range of cytokines is another valuable characteristic of innate-like lymphocytes they have in common with typical innate lymphocytes; it is known, for instance, that even in the absence of stimulation NKT cells permanently stay in pre-activated state. That is why innate-like lymphocytes supposedly perform "guarding" functions by being the first to respond efficiently to pathological changes (infection, transformation) and stimulate further activation of dendritic cells and adaptive response. However, in spite of their apparent beneficial properties, there are several features that greatly impede potential manipulations of innate-like lymphocytes, among them are: high structural and functional population heterogeneity, low abundance, heterogeneous distribution of different subpopulations in tissue and blood compartments, ability to provoke chronic inflammation and to secrete not only Th1-cytokines, but Th2 as well. If structural heterogeneity of innate-like lymphocytes is defined by their receptor repertoire, their functional heterogeneity is believed to be driven by polarizing factors coming from the environment. Importantly, conclusions about existing functional subtypes of innate-like lymphocytes were made based mostly on the results of in vitro stimulation [53–55].

Like conventional T lymphocytes, NKT cells express αβTCR, but can undergo activation only on interaction with lipid antigens presented by CD1b (a nonpolymorphic MHC-I-like molecule). In spite of such a relatively narrow specificity, NKT cells however exhibit an important feature—ability for TCR-independent activation upon stimulation with proinflammatory cytokines IL-12, IL-18, IL-25, and IL-23. According to the structure and binding specificity of TCRs, two NKT subsets can be distinguished: NKT-I, or iNKT—invariant NKT cells (with α-galactosylceramide being a prototypic ligand), and NKT-II cells—variant NKT having less restricted specificity. NKT-II cells are thought to be the most prevalent NKT subset in humans (in contrast to, for example, mice, where NKT-I cells are known to be more abundant), although their identification and characterization is still a challenging task due to the lack of distinctive NKT-II markers or agonists specifically targeting their receptors. In general, following the results of *in vivo* modeling of various cancers, NKT-I cells have been associated with the protective antitumor response, while NKT-II have been implicated in immunosuppression/immunoregulation and tumor promotion. The mechanisms of antitumor activity of NKT-I cells consist in their ability for both direct tumor lysis and generation of copious amounts of IFNγ (along with other Th1 cytokines) required for recruitment and activation/full maturation of APC, CD8 cytotoxic T lymphocytes, and NK cells. Immunosuppressive effect of NKT-II cells is thought to be due to their ability to produce high levels of IL-4 and IL-13 that shift immune response towards Th2 type. Nevertheless, this functional dichotomy is at present actively debated, and there is growing conviction that it is not so firmly associated with NKT-I or -II subset; rather, it is determined by the context (for example, tissue location) or microenvironment where activation of NKT cells occurs [53]. (Due to limited space, in our characteristic of NKT cells and γδT cells, here and below we refer to several recently published comprehensive reviews that contain links to original papers).

In spite of the relatively low abundance of NKT cells, there is constantly growing body of evidence showing this cell population undergoes quantitative and phenotypic changes (both in peripheral blood and within the tumor locus) in patients with different types of cancer, however there is only scares information available for cervical cancer. It has been found that

HPV can escape from NKT cell-mediated CD1d-restricted recognition of infected keratinocytes and low-grade cervical neoplastic lesions via HPV-E5 dependent inhibition of CD1d expression (while normal keratinocytes express high levels of CD1d molecule) [56]. Despite this evasion mechanism, CIN2-3 lesions were shown to be associated with increased numbers of infiltrating iNKT, with these numbers being higher for HPV-positive lesions than for HPV-negative [57]. Elevated frequency of circulating NKT have been revealed in peripheral blood of women with CIN1 and HPV infection, compared to the control group or HPV-positive women without signs of neoplastic abnormalities [58]. It can be inferred from these findings that the population of NKT cells may undergo early changes upon persistent HPV infection and progressing neoplasia, although there is no data available for more advanced stages of the disease.

γδT lymphocytes differ from both conventional T cells and NKT cells in their TCR chains composition and ability to recognize phosphoantigens, while many other features characteristic of NKT cells are shared by γδT as well, specifically: rapid activation, direct cytotoxicity against infected or transformed cells, reliance on natural killer receptors that enable fast (MHC-independent) response to stress-related ligands expressed on the surface of cancer cells, strong regulatory properties and ability to modulate activity of other immune cells via production of a wide range of cytokines. Further, similar to NKT cells, γδT also demonstrate functional heterogeneity (polarization) with regard to antitumor response, with this heterogeneity partially overlapping with the structural features of γδTCR, but nevertheless being mostly driven by differential environmental stimulation, as was mentioned for NKT cells. In humans, Vδ2 T cells were found to be the most frequent subpopulation of peripheral blood γδT cells (70%); Vδ1 T cells constitute the remaining 30% of γδT in circulation, although they represent the dominant γδT subset in epithelial and some other tissues. Antitumor activity of Vδ2 T cells is attributed to not only their ability to directly recognize (via congenital receptors) and kill tumor cells, but also their ability to effectively cross-present antigens to CD8 $\alpha\beta$T effectors and NKT cells, as well as facilitate DC maturation and co-stimulate cytolytic activity of NK cells. Pro-tumor role of Vδ1 T cells can be explained by their IL-17-producing ability; at the same time, however, these cells show unique specificity for B7-H6 molecule expressed exclusively on tumor cells, being able thereby to exert antitumor effect. Immunoregulatory (suppressive) function of γδT cells in antitumor immunity is thought to be mediated by IL-10 and TGF-β, or adenosine secreted by tumor-infiltrating γδT cells [54, 55].

The impact of γδT cells on pathogenesis of cervical cancer is largely unexplored. Gosmann and co-authors analyzed total population of CD45+IL-17+ cells infiltrating CIN2-3 lesions and found them to be represented by not only CD3CD4 T helpers (Th17), but also by γδT cells, although the percentage of γδT cells was significantly lower than that of Th17 [59]; this observation may indicate their putative involvement in the promotion of proinflammatory suppressive microenvironment as CIN progresses to invasive cancer. The cytotoxic activity of γδT cells isolated from PBMC against cervical cancer cell lines (HeLa, SiHa, and CaSki) pre-treated with bisphosphonate pamidronate was also confirmed in [60]. A vast amount of clinical data on the role of γδT cells in viral infections, as well as their correlation with cancer prognosis allows speculation on γδT cell involvement in pathogenesis of virus-associated cervical cancer. Whether this cell population experiences any changes at different stages of cervical cancer, and if so, in which tissue compartments or depending on which clinical-pathological parameters—remains an open question.

Taken together, innate and innate-like (NK, NKT, and γδT) lymphocytes proved to have non-redundant functions in antitumor immune response, which makes them attractive objects

for the development of adoptive cell transfer therapy combined with immune checkpoints blockade or neutralization of other immune-suppressive factors [55, 61]. At the same time, the results of preclinical studies and attempts to translate them to clinical settings explicitly point to our insufficient knowledge of the role of innate-like lymphocytes and the mechanisms, whereby they contribute to cancer progression [53, 55].

4.2. Analysis of CD3+CD56+ population and its CD3bright subset in PBMC from early-stage cervical cancer patients

CD56 is a natural killer prototypic marker, but, apart from NK cells, its expression is shared by T lymphocytes (NKT and γδT) and is commonly considered as a marker of an activated state, NK-like cytotoxicity and IFNγproduction [62]. Accordingly, CD3+CD56+ population comprises of both NKT and γδT lymphocytes, but within this population, a CD3bright subset can be observed [63]. Studies on phenotyping of CD3bright subpopulation have identified it as γδT lymphocytes [63, 64]. Furthermore, Paget and co-authors [64] established mouse CD3bright γδT cells were identical to Vδ1 sub-lineage (Vγ6/Vδ1+ TCR) and possessed high IL-17-producing capacity; the high CD3 expression (CD3bright phenotype) could hence be considered as a surrogate marker of γδT identity. In humans, the population of circulating CD3brightCD56+ cells has been analyzed in patients with chronic hepatitis B infection: it has been shown that, despite increased numbers, their activity and phenotype are substantially impaired, this impairment includes down-modulation of IFNγ and LAMP1 expression (i.e., markers of antiviral and killing activity) and, conversely, up-regulation of NKG2A [65].

Given, the described data on the ability of CD3bright T cells to respond to inflammation and chronic infection observed either in model animals or in humans, in the norm or under pathological conditions, we decided to examine whether changes of the frequency of this cell population could be detected in the circulation of patients at early stages of cervical cancer progression (**Figure 9**). Using clone UCHT1 of anti-human CD3 MAb, we were able to clearly identify subpopulation of CD3bright lymphocytes in peripheral blood of CUN3/cervical cancer patients and the controls. In spite of the relatively wide range of individual values in all studied groups, a trend towards a decreased number of circulating CD3brightCD56+ cells ($p > 0.05$) was observed for women with microinvasive carcinoma (gate P1, **Figure 9**); at the same time, no difference in the frequency of cells falling within gate P2 and the total frequency of CD3+CD56+ (including CD3+CD16+/-CD56+) cells was revealed (data not shown). Then, within the CD3bright population, we also analyzed the expression of CD16, a marker of antibody-mediated cytotoxicity, but did not find any significant difference between the controls and the patients groups (data not shown). According to Lambert et al., CD3bright T cells (i.e., γδT) do not express CD4, but express low levels of CD8 [63]. We compared the frequencies of CD3brightCD8low cells (gate P3) between the study groups, but again did not observe any difference. Therefore, in contrast to regulatory NK cells, the combination of CD3/CD16/CD56 markers is not sufficient to show if there are any significant changes occurring within the population of circulating innate-like T cells in early-stage cervical cancer. Although these results cannot be compared with the results reported by Pita-Lopez et al., who used the same combination of CD markers to analyze blood samples from women with low-grade lesions (CIN1), nevertheless, observations made for CD3brightCD56 cells, along with published data mentioned above underline the need for continuing investigation into innate-like lymphocytes at various stages of cervical cancer development and progression with the use of lineage-specific (e.g., anti-TCR) antibodies.

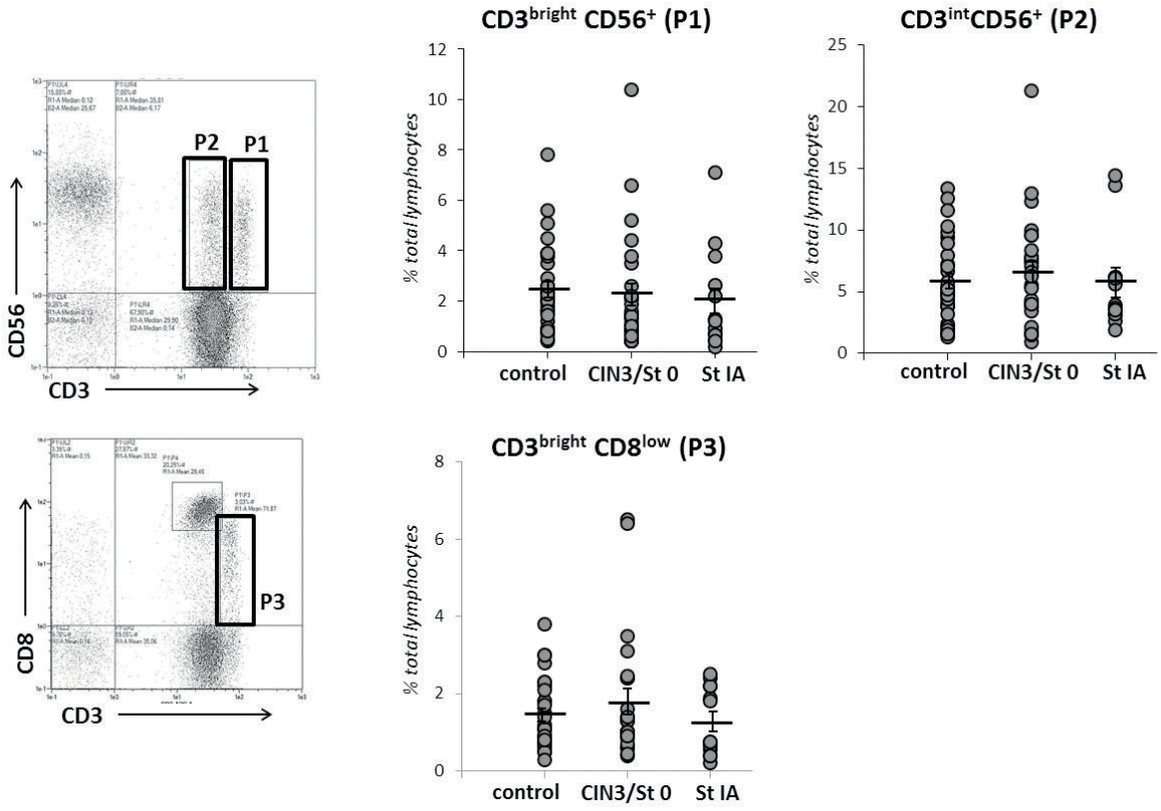

Figure 9. Percentage of peripheral blood T cells with NK-like phenotype in patients with CIN3 or microinvasive carcinoma (St IA) and healthy controls. Lymphocyte populations of interest were defined according to CD3/CD56 expression levels (gates P1-P3).

5. Regulatory T and B cells and immune checkpoint molecules in cervical cancer: at the crossroads of immune suppression mechanisms

Mobilization of intrinsic immune checkpoint mechanisms by a tumor to restrict antitumor immune response is one of the topical issues currently discussed; the phenomenon of effector cell exhaustion and the expression of checkpoint markers have been described for various innate/acquired immune cell populations, including innate-like cells. Regarding cervical cancer, one can observe an avalanche of new data emerged in recent 2 years on the expression of immune checkpoint markers, first of all PD-1/PD-1L, a hallmark of T cell exhaustion caused by chronic antigenic stimulation [66], as well as other members of B7 and CD28 protein families (e.g., B7-H3 [67] and B7-H4 [68]). For example, patients with CIN or cervical cancer show increased expression of PD-1 both in infiltrating lymphocytes and macrophages (TAMs) [69, 70], as well as in circulating CD4 and CD8 T cells [36], and, furthermore, in the sentinel lymph nodes [71]. Cervical neoplastic cells are considered as the primary source of PD-1 Ligand (PD-1L) [69, 70], with HPV16-E7 oncoprotein proved to be the driving force for elevated PD-L1 expression [72] and copy number gains of PD-L1 gene being one of the putative underlying reasons [73, 74]. Tumor-infiltrating and stromal M2 macrophages are another such source [34]. Finally, there is one more important source of PD-L1 among adaptive immune cells represented by regulatory T cells (Treg); a

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correlation between PD-L1 expression and FoxP3+Treg was reported by Ma et al. [75]. Moreover, CD4CD25 Tregs are able to upregulate PD-1 expression in patients with CIN/ cervical carcinoma [36], which, however, does not result in Treg exhaustion, but, conversely, favors upregulation of their immunosuppressive activity. Lastly, it has been reported that regional lymph nodes from stage IB1 cervical cancer patients, along with elevated PD-1, have increased expression of FoxP3 Treg-marker, which may shed light on the establishment of pre-metastatic niches [71], as well as on systemic expansion of suppressive mechanisms Tregs are engaged in.

Several studies have previously reported on increased frequency of circulating CD4 Tregs at initial stages of cervical cancer development [76–78]. Furthermore, we have recently confirmed systemic expansion of Tregs within not only CD4 cell subset, but within CD8 subset as well, at as early as preinvasive and microinvasive cancer (**Figure 10**); we have also revealed correlations between the number of circulating Tregs and the T cell expression of markers of apoptosis, whose induction is supposed to be one of the mechanisms mediating exhaustion of T effector pool during cervical cancer progression [23]. In parallel, the search of new mechanisms providing conditions for Treg expansion during the course of cervical cancer progression is continued: for example, it has been recently found that cervical cancer cells, as well as mesenchymal stromal cells isolated from cervical tumor tissue can upregulate CD73 ectonucleotidase to generate high amounts of adenosine, a potent inducer of Treg differentiation and recruitment [79, 80].

Apart from regulatory T cells, the potential involvement of regulatory B lymphocytes (Breg) in cervical cancer promotion should not be ignored. This can be supported by the results obtained

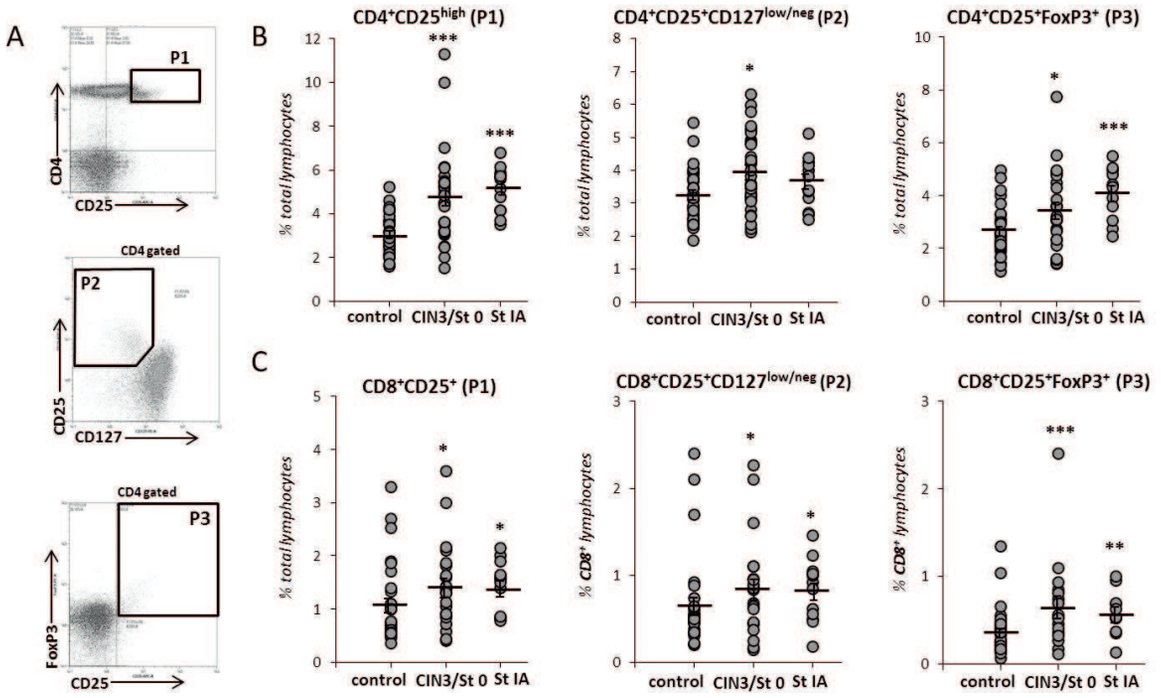

Figure 10. The frequencies of peripheral blood Treg lymphocytes in patients with CIN3 or microinvasive carcinoma (St IA) and healthy controls. (A) CD4 Tregs were gated according to the level of CD25, CD127, and FoxP3 expression; gating of CD8 Tregs was performed in a similar way. (B) The change in the frequency of circulating CD4 regulatory cells in patients compared to healthy donors. (C) The change in the frequency of circulating CD8 regulatory cells in patients compared to healthy donors: *p < 0.05, **p < 0.01, ***p < 0.001 (U-test).

by Tang et al. who used mouse model of HPV-related cancer to demonstrate that Bregs accumulate in tumor-draining lymph nodes, have altered phenotype (specifically, altered expression of cell surface markers, such as MHC II, PD-L1, and CD39), exhibit high regulatory potency, thus fostering tumor growth [81]. In humans, many types of solid tumors were found to be accompanied with increased numbers of both tumor-infiltrating and circulating Bregs capable of producing suppressor cytokines (e.g., IL-10) and immune checkpoint ligands, thus impairing T cell function (see reviews [82, 83]), suggesting this issue to be investigated for cervical cancer patients.

6. Conclusion

Further ways to develop approaches for the treatment of HPV-associated malignancies, including cervical cancer, belong to the area of combined therapies, where particular attention is to be paid to restoration the effectiveness of innate mechanisms of immune response, including those trigged by PRRs (e.g., STING). PRR agonists are expected to serve potent adjuvant function; another promising area is the use of agonists to stimulate NK, NKT, and γδT receptors. Despite substantial progress, there is clear understanding that stimulation of innate immune cells "per se" is senseless without concomitant inhibition of immunosuppressive factors (such as inhibitory molecules of immune checkpoint or other Treg-associated factors). Therefore, as illustrated by recent findings summarized in the chapter, there is obvious need for continuing comprehensive characterization of functional diversity of innate immune cells that organize cervical cancer immune regulatory network, exploration of noncanonical functions of innate immunity mediators, identification of precise resources of immune suppression and assessments of local and systemic changes in immune parameters.

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Conflict of interest

Authors have no conflict of interest to declare.

Abbreviations

APC Antigen-presenting cell

Breg Regulatory B cells

CAR Chimeric antigen receptor

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CD	Cluster of differentiation
cGAMP	Cyclic GMP-AMP
cGAS	cGAMP synthase
CIN	Cervical intraepithelial neoplasia
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
G-CSF	Granulocyte-colony stimulating factor
HPV	Human papillomavirus
IFN-I	Type I interferon
IL	Interleukin
IRF	Interferon regulatory factor
MAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cells
NK	Natural killer cells
NKT	Natural killer-like cells
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood monocyte cells
PRR	Pattern recognition receptor
SEM	Standard error of the mean
STING	Stimulator of Interferon Genes
TAM	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCR	T cell receptor
TDLN	Tumor-draining lymph nodes
TGFβ	Transforming growth factor beta

Th	Helper T cell
TLR	Toll-like receptor
Treg	Regulatory T cell

A. Appendices and nomenclature

A.1. Experimental procedures

Patients and specimens: Samples of peripheral blood and epithelial tissue were obtained from 55 patients who were diagnosed with CIN2-3 (including cancer *in situ*) or microinvasive carcinoma (FIGO stage IA1) and underwent surgery in Oncological Dispensary of the Republic of Karelia. CIN and cervical cancer diagnosis was based on comprehensive physical examination, extended colposcopy findings, cytology, and histopathology tests, in full compliance with the approved standards for the diagnosis and treatment of patients with gynecological malignancies. All women engaged in this this study were informed and gave voluntary written consent. The research was approved by the Committee on Medical Ethics of Petrozavodsk State University and the Ministry of Healthcare and Social Development of the Republic of Karelia, and was done in accordance with the Declaration of Helsinki and good clinical practice guidelines. All women from patient group were positive for oncogenic HPV types (with the prevalence of HPV16 > 80%). Thirty healthy non-pregnant women without cervical abnormalities and HPV-infection at the time of blood sampling served as normal controls. Venous blood was collected immediately before the surgery or any other treatment and immediately processed for multicolor flow cytometry. Tissue samples were submerged in RNA stabilizing reagent right after excision and stored at −80.

Flow cytometry: The following fluorophore-conjugated monoclonal antibodies were used: CD3-APC (Clone: UCHT1), CD4-FITC (Clone: MT310), CD8-FITC (Clone: DK25), CD16-FITC (Clone: DJ130c), CD56-RPE (Clone: C5.9) (Dako, Austria), CD25-APC (Clone: 4E3), CD45-VioBlue (Clone: 5B1), CD127-RPE (Clone: MB15-18C9), FoxP3-RPE (Clone: 3G3) (Miltenyi Biotec, Germany), STING/TMEM173-RPE (Clone: 723505, R&D Systems, USA). For blocking of non-specific antibody binding, FcR Blocking Reagent (Miltenyi Biotec) was used. For intracellular detection, cells were fixed and permeabilized using "FoxP3 Staining Buffer Set" (Miltenyi Biotec). Cells were acquired on a MACSQuant Analyzer flow cytometer (Miltenyi Biotec) and analyzed using MACSQuantify software.

Real-time PCR: Total RNA was extracted from tissue samples or ficoll-isolated PBMC with Trizol Reagent (Invitrogen). cDNA was synthesized from DNAse I-treated RNA (1 mg RNA per 1 reaction volume) using ProtoScript II (New England BioLabs, UK) or RevertAid First Strand cDNA Synthesis Kit (Fermentas, ThermoScientific, USA). Amplification was performed in StepOnePlus thermal cycler (Applied Biosystems, USA) using qPCRmix-HS-SYBR+HighROX reaction mix (Evrogen, Russia).

Statistical analysis: Data analysis was performed using R software. Mann-Whitney U-test was used to evaluate the differences between the patient and the control groups; the difference was considered to be statistically significant at $p < 0.05$.

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