Hypoxia-Adenosinergic Immunosuppression: Tumor Protection by T Regulatory Cells and Cancerous Tissue Hypoxia

Michail V. Sitkovsky, Jorgen Kjaergaard, Dmitriy Lukashev, and Akio Ohta

Abstract   Cancerous tissue protection from tumor-recognizing CD8^{+} and CD4^{+} T cells (antitumor T cells) limits the therapeutic potential of immunotherapies. We propose that tumor protection is to a large extent due to (*a*) inhibition of antitumor T cells by hypoxia-driven accumulation of extracellular adenosine in local tumor microenvironment and due to (*b*) T regulatory cell-produced extracellular adenosine. The adenosine triggers the immunosuppressive signaling via intracellular cyclic AMP–elevating A2A adenosine receptors (A2AR) on antitumor T cells. In addition, the activated antitumor T cells in hypoxic tumor microenvironment could be inhibited by elevated levels of immunosuppressive hypoxia-inducible factor-1α. Complete rejection or tumor growth retardation was observed when A2AR has been genetically eliminated or antagonized with synthetic drug or with natural A2AR antagonist 1,3,7-trimethylxanthine (caffeine). The promising strategy may be in combining the anti-hypoxia-adenosinergic treatment that prevents inhibition of antitumor T cells by tumor-produced and T regulatory cell-produced adenosine with targeting of other negative regulators, such as CTL antigen-4 blockade. Observations of tumor rejection in mice and massive prospective epidemiologic studies support the feasibility of anti-hypoxia-adenosinergic combined immunotherapy.

Cancer immunotherapy by endogenous or adoptively transferred antitumor T cells is complementary to conventional treatments by surgery, radiotherapy, or chemotherapy (1–3). However, malignant cells can create a self-protective, immunosuppressive tumor microenvironment that prevents tumor destruction by antitumor T cells (4). Even when, for example, melanoma tumors do express antigens that can be recognized by T cells and despite the massive influx of tumor antigen-specific T cells to the tumor site, the incidence of complete tumor destruction is very low (5).

The long sought after explanation of the coexistence of tumors and antitumor immune cells in a cancer patient or in a mouse ("Hellstrom Paradox") has been a challenging problem to solve (6). Why do antitumor T cells fail to completely and reliably destroy tumors *in vivo* even when the ability to recognize tumors is not the limiting factor and there are large numbers of antitumor T cells present (5) or when very high numbers of highly lytic antitumor T cells are injected in a cancer patient or into tumor-bearing mice (7)? What is it in the tumor microenvironment *in vivo* that prevents tumor destruction by the tumor-specific and highly lytic *in vitro* antitumor CD8^{+} T cells?

Much of the failure could be due to such negative regulators of antitumor T cells like CTL antigen-4 (CTLA-4) and T regulatory (Treg) cells and/or by cytokines (8), but the tumor microenvironment itself can suppress antitumor T cells due to, for example, "nonimmune" molecules (metabolites with immunosuppressive properties; refs. 9, 10). The need to eliminate negative regulators of immune response is well recognized (8), and the pioneering use of CTLA-4 blockade (2, 3) or combined CTLA-4 blockade and Treg cells depletion (11) may have a synergistic effect in enabling the development of sufficient numbers of lethal and tumor-recognizing antitumor T cells. The path to prevent potential inflammatory pathologies due to autoimmunity in responding patients has been shown by the demonstration of clinically detectable antitumor immunity without toxicity in metastatic melanoma patients that have been periodically infused with anti-CTLA-4 antibodies after treatment with granulocyte-macrophage colony-stimulating factor–secreting autologous tumor cells (3).

Here, we discuss the available experimental evidence as well as hypothesize and propose (Fig. 1) that the tumor protection from antitumor T cells is also due to hypoxia-adenosinergic immunosuppression in the local tumor microenvironment.

Figure 1 illustrates how the A2A adenosine receptor (A2AR)–mediated suppression of antitumor T cells (12, 13) is triggered after A2AR activation by (*a*) cancerous tissue-produced extracellular adenosine (14) and/or (*b*) Treg cell-produced extracellular adenosine (15, 16) and (*c*) due to suppression of antitumor T cells by hypoxia-inducible factor-1α (HIF-1α; refs. 17, 18). HIF-1α levels are elevated in T cells in tumor microenvironment due to stabilization by hypoxia (19) and due to activation of T cells by cytokines or T-cell receptor (TCR; ref. 20). The extracellular adenosine inhibits T cells
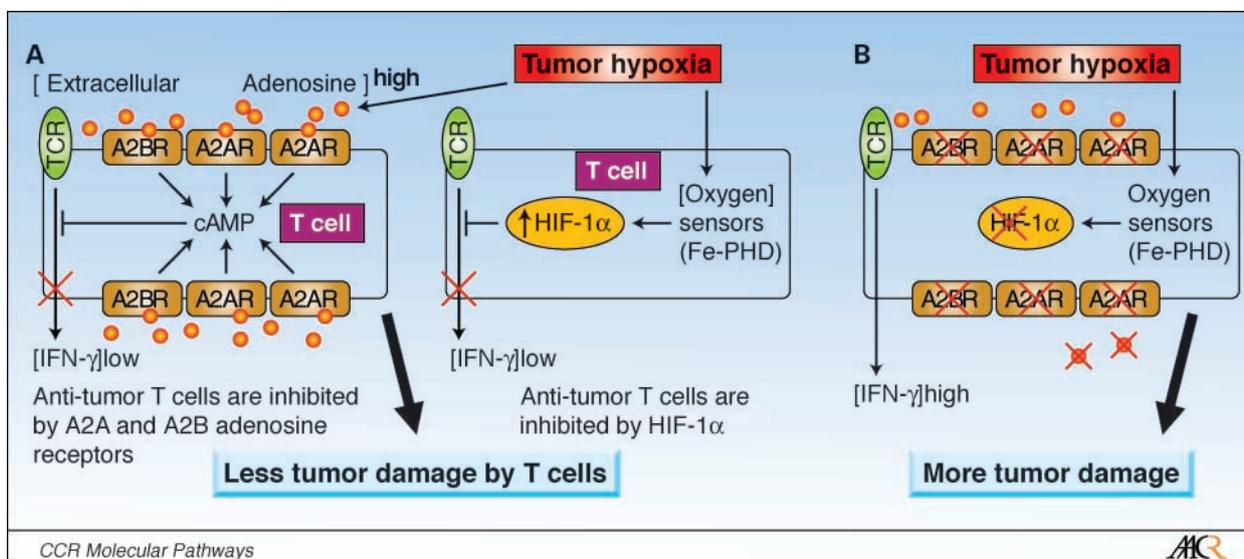
Fig. 1. Schema of the hypoxia-adenosinergic suppression of antitumor T-cell response (*A*) and suggested therapeutic approach to remove hypoxia/adenosine-mediated T-cell inhibition using antagonists of A2A and A2B adenosine receptors (*B*).

because A2AR triggers the accumulation of intracellular cyclic AMP (cAMP) that is a high-fidelity immunosuppressor (20).

It is proposed that in some, but not all, areas of cancerous tissues the tumor-infiltrating T cells are under the influence of low oxygen tension (defined here as <3% oxygen, hypoxia). In hypoxic tumor microenvironment, the hypoxia-driven accumulation of extracellular adenosine will result in signaling via cAMP-elevating A2AR and, maybe, via A2BR subtypes of A2 adenosine receptors (12–14) on all surrounding cells, including the antitumor T cells. Extracellular adenosine can be produced either due to diffusion or transport of intracellular adenosine by mechanisms yet to be clearly described or due to enzymatic hydrolysis of extracellular ATP.

The extracellular adenosine-triggered and A2AR/A2BR-mediated increases in intracellular cAMP result in immunosuppression due to inhibition of TCR-triggered signaling and effector functions of T cells. In addition, the T cells can be inhibited by HIF-1α. This important factor in the regulation of oxygen homeostasis is targeted for degradation by the oxygen-sensing, iron-containing prolyl hydroxylase in normoxic conditions but not in hypoxic conditions (21). Therefore, the antitumor T cells may have increased levels of the HIF-1α because they are located in hypoxic tumor microenvironment. The HIF-1α is also expected to be expressed at high levels in T cells because of TCR-mediated induction of HIF-1α (17, 18). Thus, the antitumor T cells may be inhibited by hypoxia-driven extracellular and intracellular immunosuppressive mechanisms, and the A2AR and A2BR and HIF-1α-mediated inhibition of TCR signaling in tumor microenvironment may result in inhibition of TCR-triggered effector functions of antitumor T cells (e.g., IFN-γ production; Fig. 1A). Therefore, therapeutic inactivation of A2AR and/or A2BR and/or HIF-1α may release antitumor T cells from inhibition in tumor microenvironment and facilitate the antitumor immune response (Fig. 1B).

The important clues as to the mechanism of cancerous tissues protection via inhibition of antitumor T cells in tumor microenvironment have been provided by identification of

the anti-inflammatory immunosuppressive mechanism that protects normal tissues from excessive collateral damage during antipathogen immune response (9, 13, 22–24).

Local Tissue Hypoxia-Driven Protection of Normal Tissues

A2A and A2B extracellular adenosine receptors. There are four different adenosine receptors: A1, A2A, A2B, and A3 (25). The high-affinity A1 receptor and the low-affinity A3 receptor are Gi protein coupled. The cAMP-elevating Gs protein-coupled A2 receptors are subdivided into high-affinity A2AR and low-affinity A2BR. Adenosine receptors are known to be immunosuppressive. The CD8^{+} and CD4^{+} T cells, including antitumor CD8^{+} and human T cells, predominantly express A2AR and A2BR (12, 14, 26). The cAMP-elevating signaling through A2AR results in inhibition of TCR-triggered effector functions, including proliferation, expansion, and secretion by T cells of such important antitumor cytokines as IFN-γ and tumor necrosis factor-α (14, 22).

Tumor hypoxia and accumulation of extracellular adenosine. Many solid tumors are characterized by an insufficient oxygen supply and transient or chronic hypoxia in some microenvironments (27). Tumor hypoxia may contribute to the propagation of oncogenic signals in the tumor microenvironment as was shown in demonstration of the switch to the angiogenic phenotype, and tumor hypoxia is associated with poor prognosis (28). The gradient of extracellular adenosine from tumor center to periphery was shown within the melanoma tumor microenvironment (14). However, the current analytic methods do not allow determining the actual concentrations of extracellular adenosine in tumor microenvironment. Only relative levels of extracellular adenosine in the center of the tumor versus the edge of the tumor versus the bordering normal tissue could be reliably estimated.

Extracellular adenosine can be produced either due to diffusion or transport of intracellular adenosine by yet to be

clearly described mechanisms or due to enzymatic hydrolysis of extracellular ATP.

Hypoxia and intracellular sources of extracellular adenosine. The local tissue hypoxia that follows the damage to endothelial cells and microcirculation and the interruption of normal blood and oxygen supply (20, 29) is associated with (*a*) decrease in intracellular ATP, (*b*) increase in intracellular AMP, (*c*) inhibition of adenosine kinase (30), (*d*) accumulation of intracellular adenosine (30), and (*e*) subsequent transport or diffusion of intracellular adenosine and accumulation of adenosine in extracellular space. This mechanism is yet to be supported by studies of tumors.

Hypoxia and extracellular sources of extracellular adenosine. The generation of extracellular adenosine by hypoxia-triggered ectonucleoside triphosphate diphosphohydrolase CD39 and 5′-ectonucleotidase CD73 represents an important pathway of extracellular adenosine generation (16, 29, 31). The intravascular nucleotides released by inflammatory cells undergo phosphohydrolysis via CD39 ectoapyrase that converts ATP/ADP to AMP followed by CD73 5′-ectonucleotidase (to convert AMP to adenosine). Interestingly, the extracellular adenosine-generating CD39 is overexpressed in human melanomas (32). This mechanism is supported by genetic *in vivo* evidence (33).

T cell-inhibiting hypoxia and HIF in T cells. It was confirmed using hypoxic marker that T cells are not avoiding the hypoxic tissues (18). However, the hypoxia inhibits activities of T cells, including TCR-triggered IFN-γ production (34) and T cell signaling and proliferation (35). HIF-1α is the subject of great interest in cancer research (19) because it affects tumor growth and energy supply by promoting neovascularization and glycolysis as well as influencing cell invasion. Intracellular accumulation of HIF-1α and HIF-2α is associated with a negative prognosis in melanoma patients (36).

The first observations of immunosuppressive and tissue-protecting effects of HIF-1α in studies of HIF-1α gene-deficient T and B cells (37) have been further confirmed and extended using T cell-targeted knockdown of HIF-1α gene in T cells (17). The HIF-1α-deleted T cells had increased TCR-triggered production of cytokines (e.g., IFN-γ). A similar conclusion about the negative role of HIF-1α in regulation of activated T cells was reached in *in vivo* studies of T cells in hypoxic inflamed areas (18) where the behavior of activated antitumor T cells in hypoxic cancerous tissues was modeled in studies of activated T cells in hypoxic-inflamed tissues during peritonitis. Although the confirmation of these data in tumor rejection assays is yet to be provided, these observations suggest that HIF-1α may collaborate with A2AR in inhibiting antitumor T cells (Fig. 1).

Cross-talk among HIF, extracellular adenosine, and A2 adenosine receptors. Recent demonstration of the inhibition of intracellular adenosine kinase by HIF-1α (38) suggests that hypoxia → HIF-1α → inhibited adenosine kinase does not rephosphorylate adenosine to AMP leading to elevated levels of intracellular adenosine, which then is subsequently transported or diffused into extracellular space by mechanisms yet to be fully understood. The signaling by hypoxia→ HIF-1α → inhibited adenosine kinase-generated extracellular adenosine may then up-regulate A2AR adenosine receptors (39). The adenosine A2 receptor can then mediate the normoxic induction of HIF-1α in macrophages (40), thereby completing the feed-forward loop. Finally, identification of a HIF-1α-dependent expression of extracellular adenosine-generating ectoenzyme CD73 further supports mutual dependence of HIF and A2 receptor-mediated immunosuppression (41).

Clinical-Translational Advances

Here, we describe preclinical studies that support the feasibility of a novel therapeutic approach to prevent inhibition of antitumor T cells in tumor microenvironment. This novel strategy requires the weakening or elimination of the tumor tissue hypoxia-driven immunosuppression in tumor microenvironment that is mediated by extracellular adenosine that inhibits antitumor T cells via their Gs protein-coupled cAMP-elevating A2AR (Fig. 1). This approach is supported by *in vivo* studies of tumor rejection in mice and by prospective epidemiologic studies.

Better rejection of established tumors has been accomplished by relieving the endogenously developed or adoptively transferred antitumor T cells from inhibition in tumor microenvironment by pharmacologic weakening of immunosuppressive hypoxia → adenosine → A2AR-mediated pathways *in vivo* (Fig. 1).

Genetic elimination of A2AR abrogates inhibition of antitumor T cells and improves tumor rejection by cytotoxic CD8+ T cells. The genetic elimination of A2AR resulted in complete tumor rejection or tumor growth retardation due to prevention of the adenosine-mediated inhibition of antitumor T cells in tumor microenvironment (14). Mice with deleted A2AR, but not the wild-type controls, successfully rejected CL8-1 melanoma and survived. Similar results were obtained with RMA T lymphoma. Importantly, the deficiency in A2AR did not prevent the establishment or the early growth of small inoculated tumors; rather, it improved the destruction of well-established tumors *after* the host has developed anti-CL8-1 or anti-RMA CD8+ T cells (14).

Pharmacologic antagonism of A2AR weakens inhibition of antitumor T cells and improves tumor rejection by cytotoxic CD8+ T cells. It was also shown that endogenously developed antitumor T cells or the adoptively transferred T cells are much more resistant to inhibition in tumor microenvironment if mice were treated with A2AR antagonists. Significantly delayed onset of rapid growth of CL8-1 melanoma was observed even if injections of A2AR antagonists ZM241385 or 1,3,7-trimethylxanthine (caffeine) commenced after tumor reached a large size (14).

The improved rejection of solid tumor or lung metastasis by adoptively transferred antitumor CD8+ T cells in mice treated with A2AR antagonist was reflected in growth retardation of s.c. grown solid LL-LCMV lung carcinoma by adoptively transferred TCR-transgenic CD8+ T cells and in better rejection of lung metastasis of CMS4 sarcoma by polyclonal antitumor CD8+ T cells (14). Similar effects have been observed with an A2AR antagonist, 1,3,7-trimethylxanthine, given i.p. or in drinking water. Importantly, the tested LL-LCMV tumor was engineered to express low numbers of the LCMV-gp33 epitope to better model tumors in humans (14). It is expected that longer-lived *in vivo* and A2AR-selective antagonists will have even stronger antitumor effects by preventing the inhibition of antitumor T cells. Such A2AR antagonists are being developed for the treatment of Parkinson's disease.

Molecular and cellular mechanisms of antitumor effects of anti-hypoxia-adenosinergic treatment. Tumor growth retardation in

mice that mounted antitumor CD8^{+} T cell response and that were treated with A2AR antagonist could be explained best by antagonist-mediated tumor "starvation." The analysis of tumors in A2A receptor antagonist-treated mice revealed strong inhibition of neovascularization. This may be due to an increased IFN-γ production (42) by "deinhibited" CD8^{+} T cells in the tumor microenvironment (14). This explanation does not exclude the contribution of Fas ligand– and perforin-mediated T-cell-mediated tumor lysis. Thus, the antitumor CD8^{+} T cells may not only directly kill tumor cells via cell-mediated cytotoxicity mechanisms but also prevent tumor cell survival by limiting new blood vessel formation and thereby limiting the supply of nutrients and oxygen to cancerous tissues.

Treg Cell Connection

FoxP3^{+}CD25^{+}CD4^{+} Treg cells that are important in immunological self-tolerance by suppressing immune responses (43) are harmful when they oppose antitumor T cells by inducing tumor-specific local immune tolerance (3, 43). Inhibition of tumor-infiltrating Treg cells is considered highly desirable to improve the immunotherapy of cancer (2, 44–46). The correlation between CTLA-4 blockade-induced tumor necrosis and the ratio of intratumoral CD8^{+} effector T cells to FoxP3^{+} Treg cells in post-treatment biopsies (3) has important clinical implications and emphasizes the clinical significance of weakening Treg cells in cancer immunotherapy.

The "bonus" of antiadenosinergic treatment may be in the ability of A2AR antagonist to weaken the immunosuppression not only by the tissue-produced extracellular adenosine but also by Treg cell-produced adenosine (46). This is because Treg cells are now recognized to be producers of extracellular adenosine on their own (15, 16), thereby contributing to the A2AR-mediated immunosuppression caused by inflamed or cancerous tissue-produced extracellular adenosine (9, 14). Treg cells express high levels of the extracellular adenosine-generating ectoenzyme CD73, 5′-ectonucleotidase (15) that can generate extracellular adenosine from extracellular 5′-AMP after it was generated by CD39 ecto-ATPase/ADPase. The CD39 is coordinately coexpressed with CD73 on Treg cells (16). Although the accumulation of extracellular adenosine in tumor microenvironment has been demonstrated (14), it is yet to be conclusively proven that Treg cells do generate extracellular adenosine and suppress effector T cells *in vivo* by activating immunosuppressive A2AR on effector T cells. This will further implicate the A2AR on T cells (12, 14, 47) in immunosuppression by Treg-produced adenosine (15, 16).

Thus, the antiadenosinergic treatment that eliminates or weakens the local tissue hypoxia-driven immunosuppression discussed here will also inhibit at least part of Treg cell-mediated immunosuppression and will be complementary to other ongoing efforts to dissociate the development of antitumor T cells from development of Treg cells or to prevent the effects of Treg cells (3, 46).

Limitations. The success of the proposed strategy is dependent on the existence of ongoing antitumor T-cell response. In the absence of antitumor T cells, the elimination of A2AR is not going to have an effect on tumor rejection, except in situations when it can interfere with A2A or A2B adenosine receptor-mediated and tumor tissue hypoxia-driven neovascularization (48). Indeed, the weakly immunogenic B16 melanoma did not elicit anti-B16 T cells; therefore, there were no differences in growth of B16 in wild-type and A2AR-deficient mice (14). The CL8-1 melanoma is derived from weakly immunogenic B16, but it is more immunogenic because it is transfected with H-2K[b]. In contrast to B16, the CL8-1 melanoma cells have been completely rejected in parallel assays in A2AR-deficient but not in wild-type mice. Similarly, no antitumor effects of A2AR antagonist have been observed in the absence of antitumor T cells (14). Thus, the proposed strategy must be applied only when there are antitumor T cells.

Supporting Epidemiologic Studies

Prospective epidemiologic studies of the effects of caffeinated, but not decaffeinated, coffee on the incidence of cancer are unique among similar studies of all other ingredients of food because coffee is consumed primarily because it contains the most widely used psychoactive drug, caffeine. Well-controlled biochemical, pharmacologic, and neurobiological studies with genetic *in vivo* controls firmly established that psychoactive effects of caffeine are due to its properties as a selective A2AR antagonist. The consumption of coffee at moderate to heavy levels creates biologically active levels of caffeine that are sufficiently high to antagonize A2AR but are too low to enable the effect of caffeine on its other potential molecular targets. Thus, studies of coffee consumption are studies of consumption of a "drug" that antagonizes A2AR receptor both on brain cells and on antitumor T cells. Therefore, the observed antitumor effects of coffee are likely to be the effects of A2AR antagonist caffeine (10, 49).

In support of preclinical studies in mice that show better antitumor effects of T cells in mice consuming A2AR antagonist are epidemiologic prospective studies that established an inverse correlation between coffee consumption and incidence of immunogenic tumors in humans. Studies of 21,238 Norwegian women and 21,735 men revealed that drinking coffee is associated with significantly decreased risk of cutaneous malignant melanoma in the buccal cavity and pharynx in women (50). Another similarly large study of 25,049 Norwegian women and 25,708 men also reported that drinking coffee is associated with a significantly decreased risk of cutaneous malignant melanoma only in women (51).

It is in agreement with the overall idea of this review that the strongest anticancer effects of coffee have been observed only with immunogenic melanoma, only in women and not in men. First, it is not even expected that A2AR antagonist caffeine will lower the incidence of nonimmunogenic tumors because the A2AR antagonist is capable of antitumor effects only by facilitating an ongoing antitumor T-cell response (10). Second, the protecting effects of coffee on the incidence of melanoma in women but not in men (50, 51) may be explained by higher levels of immunosuppression in men due to testosterone production. To counteract this, it was suggested to administer prostate cancer immunotherapy after androgen ablation (52). Therefore, the lack of the antimelanoma effects of caffeine in men may be explained by the immunosuppressive effects of testosterone that cancel the immunoenhancing effects of A2AR antagonist caffeine.

Possible Side Effects and Exclusion Criteria

Autoimmunity considerations. It was noticed that rejection of melanoma in some A2AR-deficient mice was accompanied by melanocyte-directed autoimmunity and hair loss (14) that was regrown at days 70 to 90 post-tumor inoculation in tumor-rejecting and surviving mice. Similar signs of spontaneously resolving autoimmunity have been observed in vaccinated melanoma-rejecting mice (53) and in melanoma patients undergoing immunotherapy with melanoma antigen-specific T cells (54). The autoimmunity was also observed in studies of chimeric mice, which had HIF-1α deleted in T and B cells (36).

It is not expected that autoimmunity might affect the overall utility of the proposed approach because no autoimmunity was observed after the most clinically relevant pharmacologic treatment using A2AR antagonist. Even with A2AR-deficient mice, which represent the extreme case of elimination of adenosinergic protection, the self-resolving autoimmunity (vitiligo) was observed only in some, but not in all, tumor-rejecting survivors. However, the autoimmunity may be a bigger problem if the A2AR antagonist is combined with, for example, CTLA-4 blockade and/or Treg cell depletion (2, 3, 8, 11).

The autoimmunity may be avoided by careful analysis of patients. A recent report described an example of dissociated inflammatory pathologies due to autoimmunity from clinically detectable antitumor immunity in metastatic melanoma patients infused with anti-CTLA-4 antibodies after treatment with granulocyte-macrophage colony-stimulating factor–secreting autologous tumor cells (3).

The antagonists of A2AR should not be given to cancer immunotherapy patients during episodes of acute inflammation because such treatment may not only prevent the undesirable inhibition of antitumor T cells (10) but also will eliminate the protection of normal tissues from collateral damage by other activated immune cells (9). Thus, the "tradeoff" of tumor destruction versus normal tissue damage should be made with an understanding that the "price" of complete tumor destruction will be to manage the short-term autoimmunity in cancer patients treated with antiadenosinergic drugs and to avoid targeting the hypoxia-adenosinergic pathway, including avoiding the application of supplemental oxygen (55) during episodes of acute inflammation of lung or liver (56).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Michail V. Sitkovsky, Jorgen Kjaergaard, Dmitriy Lukashev, et al.

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