Chapter 4

Understanding the Anti-Tumor Properties Mediated by the Synthetic Peptide GK-1

Jacquelynne Cervantes-Torres, Laura Montero, Noé Rodríguez-Rodríguez, Edda Sciutto, Gladis Fragoso and Diana Torres-García

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Abstract

Cancer exhibits adaptive features typical of complex systems, like resilience and robustness to environmental challenges through the emergent co-evolution of its components. These events promote carcinogenesis through dynamic interactions among numerous components and subsystems, including the immune system. During the past decade, our research group has provided substantial evidence that the peptide GK-1 has important immunomodulatory properties. In elderly mice, GK-1 acts as a potent adjuvant of the influenza vaccine through a mechanism that involves the activation of antigen-presenting cells (APCs) and an increased production of pro-inflammatory cytokines and chemokines (IFN-γ, TNFα, CCL2). To date, there is solid evidence supporting the antitumoral properties of GK-1 in murine cancer models. First, a lower occurrence and smaller size of spontaneous bronchiolar adenomas were found in elderly GK-1-treated mice compared to paired untreated mice. In two independent studies, GK-1 treatment reduced tumor growth and increased mouse survival in a murine model of melanoma and breast tumor. In the former model, a synergy between GK-1 and anti-PD-L1 treatment was observed, while in the latter, GK-1 alone controlled the metastatic burden. The effective activation of APCs induced by GK-1, restoring the antitumor-specific immunity, may underlie some of its antineoplastic effects.

Keywords: GK-1 peptide, melanoma, breast cancer, immunomodulator, antitumor

1. Introduction

1.1. Immunomodulation

Immunosurveillance comprises interactions between the immune system and cancer cells that take place even before the tumor formation [1, 2]. This process includes the recognition and control of transformed cells through antitumor immune responses, with three related outcomes: elimination, equilibrium, and escape [1–5]. In this regard, stimulating the innate immune system by immunogenic cells plays a role in the removal of incipient tumors, activating cells from the adaptive response like T and B cells, as well as promoting acute inflammation due to the concomitant production of immunostimulatory cytokines. Nevertheless, some transformed cells may not be eliminated. This escape phase of immunosurveillance is characterized by tumor growth promotion through a phenomenon called tumor-induced tolerance, which involves an increased expression of immunosuppressive components such as myeloid-derived suppressor cells (MDSC), regulatory T cells (Tregs), as well as T cell exhaustion and the production of immunosuppressive soluble factors [6, 7]. Indeed, some of these cells could be used as prognosis factors, since increased numbers of Treg and MDSC cells are related to a poorer outcome in cancer patients [8–13]; by contrast, a Th1 response is associated with a good prognosis in melanoma, breast, head, neck, colorectal, prostatic, and renal cancer [14–16].

The immune response can be modulated by compounds capable of enhancing (immunopotentiation) or diminishing (immunosuppression) the immune response, either in an antigen-specific or in a nonspecific manner; the latter implies that the immune system requires to be stimulated to restore the patient's immunocompetence. Immunomodulators are biological or nonbiological substances that can modify one or more components of the immunoregulatory network to achieve a specific antitumor immunity, such as inducing effector tumor-specific cytotoxic T lymphocytes (CTLs), activating macrophages and natural killer (NK) cells, and/or promoting the production of inflammation mediators [17–21].

Immunomodulators include adjuvants, vaccines, and immunoglobulins used to prevent or treat infectious diseases. They are characterized by their ability to activate cells of the innate immune system, mainly dendritic cells (DCs) and macrophages. Some examples of this type of agents are pathogen-associated molecular patterns (PAMPs) and molecules like squalene, aluminum salts, and peptides, which are often used as adjuvants in vaccines [21, 22].

1.2. Peptide-based therapies

Anticancer strategies based on peptides have several advantages over other chemotherapeutic approaches, like being nongenotoxic or possessing adjuvant properties; they also have a strong specificity, high affinity, good tissue penetration, and low toxicity with respect to small-molecule drugs and monoclonal antibodies [23–26]. Examples of anticancer peptides are (1) necrotic peptides (some of them are expressed in a wide diversity of species, including insects, fish, amphibians, and mammals, e.g., cecropins A and B found in mammals and various insects) [27]; (2) apoptotic peptides, cationic peptides known as host defense peptides (HDP) such as the bovine lactoferricin, magainin 2, hCAP109-135 (comprising the C-terminal

domain of human CAP18), and BMAP-28 from bovine myeloid cathelicidin [28–31]; (3) blocking peptides; (4) receptor-interacting peptides; (5) peptides that bind to cell-adhesion proteins; (6) protein kinase inhibitors; (7) protease inhibitors; (8) peptides with antiangiogenic properties; and (9) peptides with immunostimulatory activity [27].

With regard to receptor-interacting peptides, compounds like CpG, imiquimod, poly I:C (toll-like receptor (TLR) agonists), α-GalCer (glycolipid ligands), GM-CSF, IL-2, and IFNα/β have antitumoral activity, as well as adjuvant properties [32-34]. These compounds are capable of directly or indirectly enhancing APC functions and T effector activity. In this sense, some of the most employed immunotherapeutic agents in polytherapy induce the effector function of tumor microenvironment (TM)-associated T cells and macrophages [35–38]. For instance, CpG was a promising cancer immunotherapy adjuvant due to its capacity to induce a Th1 immune response and activate APCs through TLR9 signaling [35–38]; however, it failed to stimulate the immune response in clinical trials [39]. The identification of new adjuvants showing low toxicity and capable of stimulating a cellular Th1 response in humans would be a great advancement in the development of vaccines for infectious and noninfectious diseases such as cancer [40].

Unfortunately, several immunostimulators have failed to revert the immunosuppressive conditions in TM. For example, IL-2, IL-12, GM-CSF [41–44], and immunological adjuvants administered with highly immunogenic antigens like incomplete Freund's adjuvant, bacillus Calmette-Guerin [BCG], and MF59 have shown disappointing results [43, 44]. Moreover, these compounds have been associated with toxic effects [45–47].

2. Identification of the GK-1 peptide

Based on the nonspecific reactivity and immunopotentiator properties of GK-1, our group has been studying it as a promising adjuvant for cancer immunotherapy. This 18-amino acid peptide was first derived from the KETc7 protein, isolated from a *Taenia crassiceps* cysticercus cDNA library [48]; KETc7 is part of a broad family of proteins associated with membrane processes [49]. When searching for T cell epitopes *in silico*, GK-1 exhibited a strong association with MHC-I and, to a lesser extent, with MHC-II [49]. The immunomodulatory properties of GK-1 are associated with an efficient activation of cells involved in antigen presentation (such as DCs) by promoting the expression of the costimulatory molecules CD86 and MHC-II, as well as the secretion of soluble pro-inflammatory factors like IFN-γ, TNF-α, and CCL2 [50]. GK-1-treated DCs enhanced the proliferative response of antigen-specific CD4+ T cells both *in vivo* and *in vitro* [50]. GK-1 also induced the proliferation of CD8+ T cells and higher IFN-γ levels [51] even in the absence of adjuvant [52]. Considering that this peptide can promote APC function and enhance Th1 cell effector pathways, its capacity as an adjuvant of the influenza vaccine was evaluated. GK-1 increased the levels of specific IgG antibodies *in vivo,* before and after infection, in a murine model of influenza in elderly mice [53], favoring virus clearance after infection in both young and aged mice, which could be associated with an early infiltrate of mononuclear cells (lymphocytes and macrophages) to the lung parenchyma following the GK-1 peptide co-administration. Furthermore, lung histological examination showed better preserved alveolar spaces and less congested alveolar walls with respect to the vaccine-only animals [53].

3. GK-1 as an anticancer immunotherapy

In neoplasms, the host is often immunocompromised due to the presence of immunosuppressive cells and molecules in the TM, to prevent the removal of cancer cells [2]. This highlights the relevance of stimulating the host immune response against cancer antigens by administering immunoadjuvants along with chemotherapy, radiotherapy, or surgery [54]. In this regard, small peptides with a nonspecific immunostimulatory response like GK-1, long known to act as vaccine adjuvants, are potentially useful in cancer therapy. The antitumor effect of GK-1 has been studied in melanoma and breast cancer murine models.

3.1. GK-1 in a mouse melanoma model

Melanoma is the most malignant form of skin cancer, mainly affecting the Caucasian population [55, 56]. Until recently, systemic therapy for metastatic melanoma had been inefficient, with a 5-year survival rate for patients (<30%) [57, 58]. However, new therapies were recently approved to treat melanoma, such as pegylated-interferon-α2b (IFN-α2b) in the adjuvant setting; ipilimumab, an anti-CTLA4 monoclonal antibody, for metastatic disease; vemurafenib, an oral BRAF inhibitor indicated for patients with metastatic melanoma harboring BRAFV600 mutations, and more recently antibodies against PD-1 like pembrolizumab [59–61] and antibodies blocking PD-L1 pathways, as well as inhibitors of the mitogen-activated protein kinase (MAPK) pathway. Additionally, nonspecific immunomodulation by several cytokines (IL-2, IL-12, TNF-α, and IFN-γ) and TLR ligands [62–64] in addition to adoptive transfer approaches have been widely used [65]. For over a decade, DCs have also been used in immunotherapy against various types of cancer [66–68] as an alternative to chemotherapy, by vaccination with DCs loaded with tumor peptides (i.e., MAGE-AX [69–72] and/or with necrotic or apoptotic tumor cells to induce effector tumor-specific T cells [73, 74]). The efficacy of this immunotherapeutic approach was also evaluated against murine melanoma, using GK-1 as an immunostimulant.

GK-1 has been reported to increase the mean survival and significantly delays tumor growth in a melanoma model with B16-F10 cells, showing more necrotic areas along with the presence of numerous neutrophils (**Figure 1**). Neutrophilia inside pulmonary blood vessels was also observed, without evidence of macroscopic or microscopic metastasis. In a melanoma lung metastatic model, GK-1 decreased lymphocyte count, while increased the number of neutrophils and decreased the serum levels of IFN-γ; on the other hand, an increase in the levels of IFN-γ and IL-12 in the intratumor (lung metastases) environment, along with a decrease in IL-17, IL-4, IL-22, and IL-23 was also observed [75, 76]. The antitumor activities of IL-12 have been established in preclinical studies against various tumor cell lines; the increased concentration of the antitumor cytokine IL-12 found in primary tumors may enhance the damage to tumor cells, limiting the number of cancer cells detaching from the primary tumor [77–79]. Its antitumor activity is also mediated by the induction of IFN-γ [67, 78, 80], which upregulated the expression of MHC-I and -II by B16 cells *in vitro*, favoring a cytolytic response in MHC-I-restricted CTL (**Figure 1**) [81]. There is a consensus that the induction of a Th1 profile or the release of cytokines like IFN-γ and TNF-α by T cells is essential for an effective antitumor immune response in

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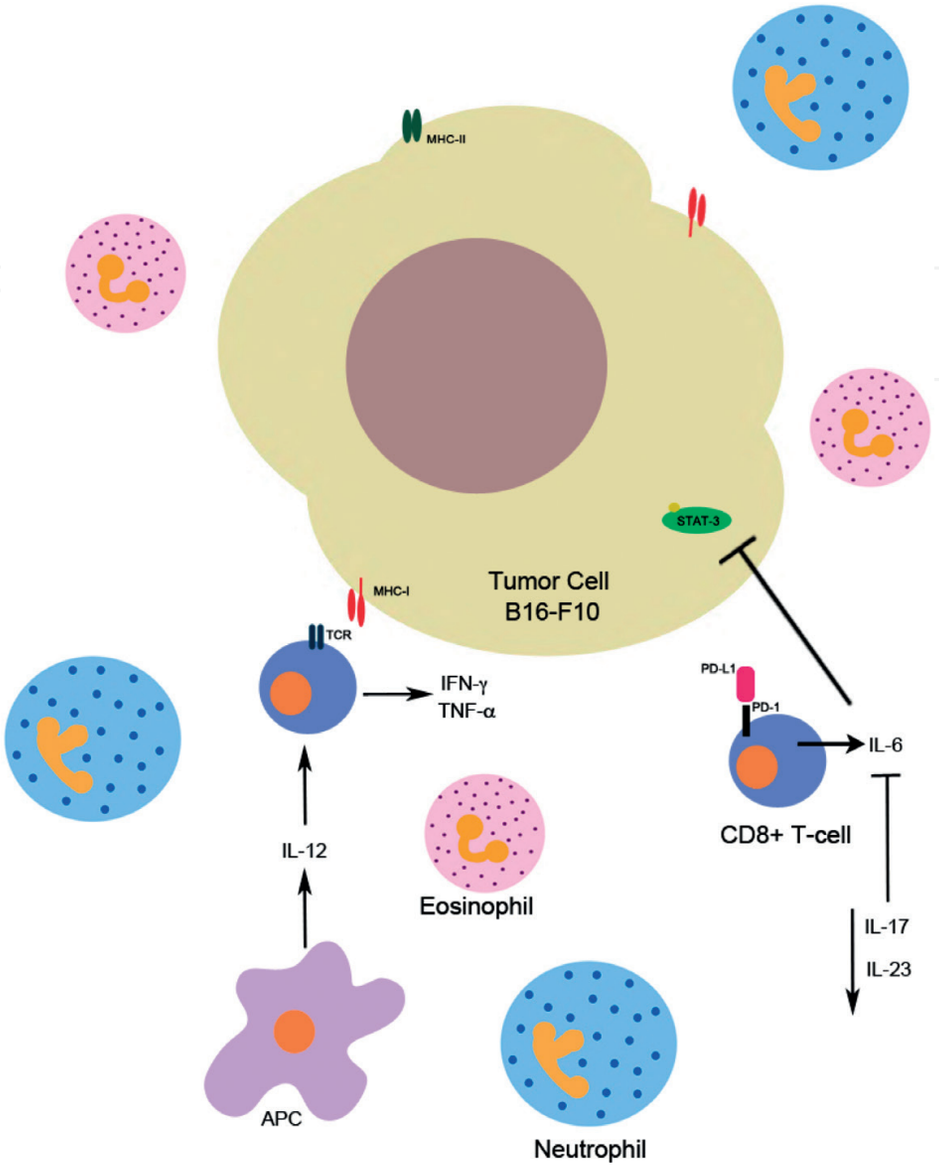

Figure 1. GK-1 in a preclinical mouse melanoma model. In a melanoma murine model with B16-F10 cells, GK-1 led to an increase in neutrophils with the increase of IFN-γ and IL-12 cytokines, along with a decrease in IL-17 and IL-23. On the other hand, the MAGE-AX/GK-1 treatment showed an increase in areas of cell death, characterized by eosinophilic regions and production of IFN-γ by CD8+ T-cells.

melanoma [82–84]. In fact, IFN-γ released from CTLs has been considered as a potent mediator of the antitumor response in bulky melanoma tumors [17, 85]. In contrast, IL-17 was proved to directly promote tumor growth and angiogenesis [86–88]. Indeed, it has been shown that IL-17 can promote tumor growth by a direct effect on IL-6 induction, which in turn activates STAT3 in both tumor and nontransformed cells in the TM [89]. Finally, IL-23 is an important molecular driver of Th17 cells in humans; IL-23 is increased in several tumors, and the expression of this cytokine antagonistically regulates local inflammatory responses in the TM, as well as the infiltration of epithelial lymphocytes [80]. Thus, the intratumoral subexpression of IL-17 and IL-23 in GK-1-treated mice could explain the reduced tumor progression (**Figure 1**).

Considering the capacity of GK-1 to enhance DC activation [50], BMDCs matured with TNF-α and stimulated with GK-1 and MAGE-AX were administrated to tumor-bearing mice in the melanoma model with B16-F10 cells; the treatment with MAGE-AX/GK-1 increased survival rates, while mice receiving GK-1 alone had a smaller increase in survival. Moreover, the combination MAGE-AX/GK-1 significantly delayed tumor growth and increased cell death areas, characterized by eosinophilic regions within melanomas. Similarly, both GK-1 alone and MAGE-AX/GK-1 increased the production of IFN-γ-producing CD8 cells, while GK-1 increased the percentage of IL-10 producing CD8+ T-cells [90]. The effect of MAGE-AX/GK-1 could be associated with higher levels of CD8+ lymphocytes in peritumoral lymph nodes, which have been correlated with the survival of patients suffering from melanoma and other cancer types [67, 91, 92].

Vera-Aguilera et al. [79] hypothesized that a combined GK1/anti-PD-L1 therapy could synergize and maximize the individual antitumor effect and extend survival. An increased survival was observed in mice treated with GK1/anti-PD-L1, as well as in mice treated with GK-1 or PD-L1 alone. Animals treated with GK1/anti-PD-L1 had smaller tumor masses. Additionally, GK1/anti-PD-L1 decreased the serum levels of IL-4, IL-5, IL-6, and IL-10. The mechanism by which the combined GK1/anti-PD-L1 treatment improved survival rates remains to be determined; however, the expression of PD-1 on T cells has been proved to be upregulated by IL-6 through the signal transducer and activator of transcription 3 (STAT3) [93], a point of convergence for several oncogenic signaling pathways leading to the expression of immunosuppressing molecules [94]. Similarly, the expression of PD-L1 and PD-L2 is also upregulated by numerous mechanisms, including the production of IL-4 and GM-CSF [93]. All these findings point to a possible synergistic mechanism associated with the reversion from an exhausted phenotype.

3.2. GK-1 in a breast cancer model

Considering the evidence described above, it is now clear that changes in the microenvironment could induce an antitumor response against the primary tumor and reduce the metastatic disease, which could allow us to control cancer progression. In this regard, immunomodulators like GK-1 can be used as anticancer therapies. In 2017, GK-1 was evaluated in a murine model of invasive breast adenocarcinoma, which spontaneously metastasizes to the lungs, liver, brain, and bone, similarly to breast cancer in humans [95–98]. GK-1 was associated with an increased survival in 4T1 tumor-bearing mice and a reduction in the primary tumor volume rate, which was accompanied by an increase of tumor cell death areas with morphologic features associated with necrosis (pyknosis, karyorrhexis, and karyolysis) and apoptosis (apoptotic bodies) at the primary tumors. These findings, along with an increase in IL-12 concentration in the primary tumor, denote deep changes in the TM induced by GK-1 [98], which could involve the infiltration of TCD8+, NK, and NKT cells in the primary tumor [77, 99, 100] (**Figure 2**). As described in the previous section, IL-12 has been associated with antitumor and antiangiogenic activities [100, 101], due to its capacity of inducing the infiltration of TCD8+ cells within tumor tissues [100]. In fact, it has been reported that a combined treatment with tamoxifen and IL-12 enhanced tumor inhibition due to an increase in apoptosis, and reduced tumor growth in a 4T1 cancer murine model [100].

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Those changes suppose a TM that could reduce the tumor growth rate, and the concomitant reduction of cancer cell egress by detachment from the primary tumor, which allows them to invade the stroma and break the basement membrane. These changes could explain the reduction of pulmonary metastasis associated with the GK-1 treatment [98]. Additionally, changes in lung microenvironment associated with the GK-1 treatment have been reported. In this sense, a reduction in the concentration of b-FGF, CCL-3, GM-CSF, CCL-2, TNF-α, and CXCL-9, along with an increased concentration of IL-6 has been found [98] (**Figure 2**). These changes could reduce metastasis development, possibly by inhibiting the proliferation of cells that are essential for the growth of secondary tumors, such as macrophage-associated metastasis (MAM) and MDSC [102, 103]. Considering these results, GK-1 could change the tumor microenvironment, inducing an active antitumoral immune response that could lead to a decrease in cancer burden.

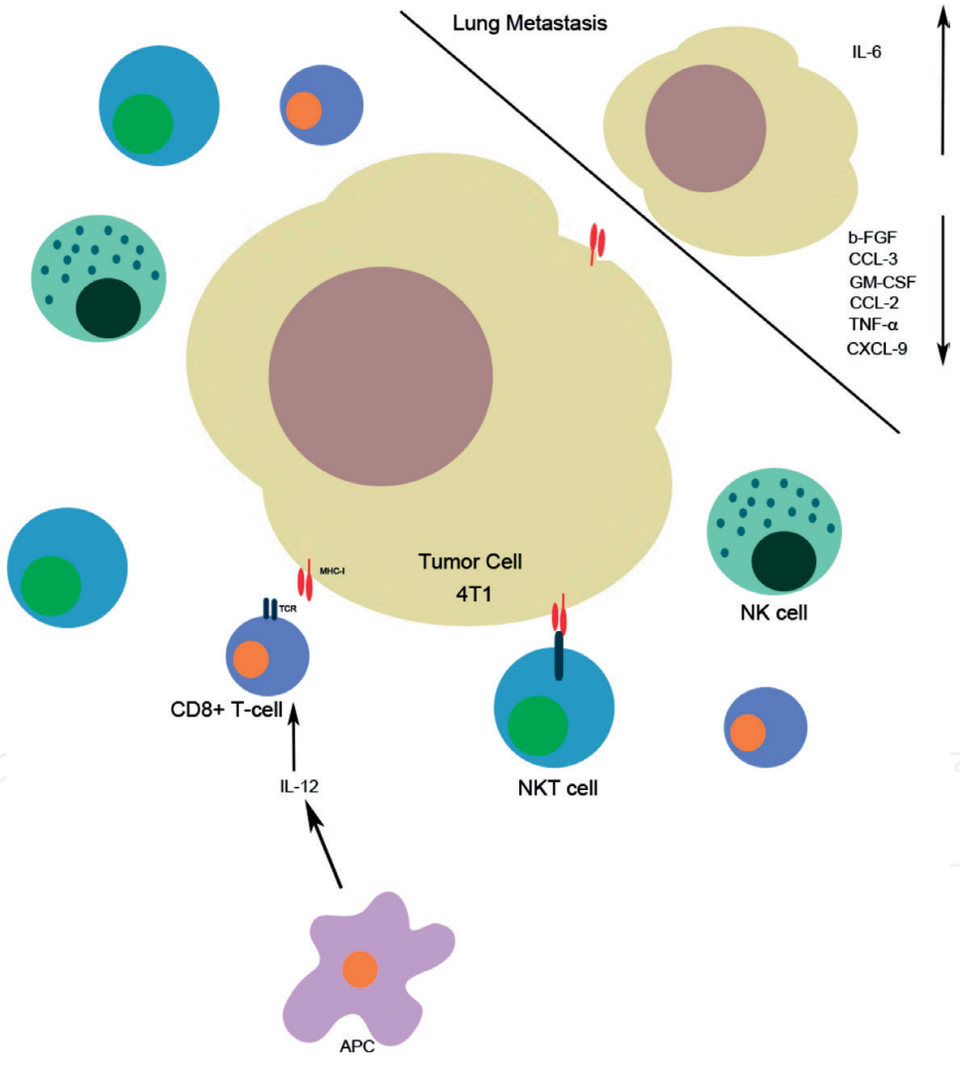

Figure 2. GK-1 in a breast cancer model. GK-1 was associated with an increased IL-12 concentration in the primary tumor, which could involve the infiltration of CD8+ T-cells, NK, and NKT cells. IL-12 is a cytokine produced principally by APC, such as monocytes, macrophages, and dendritic cells. This cytokine can induce specific CD8+ T-cells that are primed against tumor antigens and could serve as a tumor-specific CTL. Additionally, in the lungs, the GK-1-treatment induces a reduction in the concentration of b-FGF, CCL-3, GM-CSF, CCL-2, TNF-α, and CXCL-9, along with an increased concentration of IL-6, which correlates with a minor lung-metastatic burden.

4. Discussion

The ability of the GK-1 peptide to increase survival, significantly to delay tumor growth, and to reduce metastasis is discussed in this review. Considering that the immune system plays a crucial role in the outcome of cancer, orchestrating the response that may lead either to the control or dissemination of tumors [8, 78, 104], understanding the mechanisms that underlie the efficient response to the peptide is imperative.

It has been reported that the production of pro-inflammatory cytokines both by tumor and surrounding cells, along with the production of growth factors and chemokines, can promote the development of neoplasia by facilitating carcinogenesis programs, inducing a sustained cellular proliferative rate, inhibiting apoptosis and stimulating angiogenesis [105, 106]. As described above, GK-1 therapy contributed to decrease the levels of IL-4, IL-10, b-FGF, and GM-CSF; these chemoattractants, along with hypoxia, promote macrophage shift from a M1 to a M2 phenotype. M2-like tumor-associated macrophages (TAM) stimulate immunosuppression and increase blood vessel density, favoring angiogenesis. In a breast cancer model, lower CCL2 and CCL3 levels in the lungs of mice treated with GK-1 could be decreasing the migration of inflammatory monocytes such as MAM and MDSC, which promote metastasis [8, 13, 102, 107]. These changes in the microenvironment seem to contribute to control tumor burden and metastasis.

On the other hand, M1-like macrophages can contribute to tumor regression by recruiting cytotoxic CD8+ T (CTL) and NK cells [108–110]. In this regard, IL-12 induction by APCs could be contributing to the increase in the proliferation of CD8+ and CD4+ lymphocytes and the induction of a Th1 response, as previously reported [51, 52, 111]. Several studies have suggested a correlation of higher density levels of cytotoxic (CTL) and memory T lymphocytes (CD3+ CD45RO+) infiltrated in the primary tumor with increased survival rates of patients with different types of neoplasms [91, 112–116].

According to recent findings, the GK-1 peptide can induce a M1 phenotype and promote the efficient activation of DCs, which could be leading to the maintenance of an effector response against tumor growth, capable of counteracting the immunosuppressive response due to T cell exhaustion or DC dysfunction.

5. Conclusions

Considering the possible mechanisms of action of GK-1 and the information available, we propose that this peptide can decrease tumor growth and metastasis by changing the tumor microenvironment. GK-1 appears to reactivate the immune system affected by the tumor-associated suppressive microenvironment, thereby allowing immune cells to become activated. Although more studies focusing on the anticancer effect of GK-1 are required, this research gives new evidence on the possible clinical uses of GK-1 beyond its well-established adjuvant effect.

These results have also provided us with the rationale to evaluate the effectiveness of the GK-1 immunotherapy to revert the exhaustion of peripheral T-cells in several types of cancer.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Abbreviations

APC	antigen-presenting cell
CCL-22	chemokine ligand-22
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
HDP	host defense peptides
LAG-3	lymphocyte-activation gene 3
MDSC	myeloid-derived suppressor cells
NK	natural killer cells
PAMPs	pathogen-associated molecular patterns
PD-1	programmed cell death 1
PD-L1	programmed death-ligand 1
Treg	regulatory T cell
STAT3	signal transducer and activator of transcription 3
TM	tumor microenvironment
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor 2

Author details

Jacquelynne Cervantes-Torres†, Laura Montero†, Noé Rodríguez-Rodríguez, Edda Sciutto, Gladis Fragoso and Diana Torres-García*

*Address all correspondence to: dianatg@comunidad.unam.mx

Biomedical Research Institute, National Autonomous University of Mexico, Ciudad de México, Mexico

† First joined authors

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