A Synthetic Peptide Homologous to Functional Domain of Human IL-10 Down-Regulates Expression of MHC Class I and Transporter Associated with Antigen Processing 1/2 in Human Melanoma Cells[1]

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Tumor cells treated with IL-10 were shown to have decreased, but peptide-inducible expression of MHC class I, decreased sensitivity to MHC class I-restricted CTL, and increased NK sensitivity. These findings could be explained, at least partially, by a down-regulation of TAP1/TAP2 expression. In this study, IT9302, a nanomeric peptide (AYMTMKIRN), homologous to the C-terminal of the human IL-10 sequence, was demonstrated to mimic these previously described IL-10 effects on MHC class I-related molecules and functions. We observed a dose-dependent down-regulation of MHC class I at the cell surface of melanoma cells after 24-h treatment with IT9302. The IL-10 homologue peptide also caused a dose-dependent inhibition of the IFN-γ-mediated surface induction of MHC class I in a melanoma cell line. We demonstrated, using Western blot and flow cytometry, that IT9302 inhibits the expression of TAP1 and TAP2 proteins, but not MHC class I H chain or low molecular protein molecules. Finally, peptide-treated melanoma cells were shown to be more sensitive to lysis by NK cells in a dose-dependent way. Taken together, these results demonstrate that a small synthetic peptide derived from IL-10 can mimic the Ag presentation-related effects mediated by this cytokine in human melanomas and increase tumor sensitivity to NK cells, which can be relevant in the designing of future strategies for cancer immune therapy. *The Journal of Immunology,* **2004, 173: 1731–1737.**

Interleukin-10 is a protein of 160 aa with a molecular size of 18.5 kDa, and it exists as a 37-kDa homodimer (1). IL-10 was discovered as a T cell growth and differentiation factor (2, 3) and has the ability to suppress cytokine production by monocytes (4), activated macrophages (5), and Th1 cells (6). IL-10 can also have pleiotropic immunosuppressive effects, including the capacity to block monocyte-dependent T cell proliferation (7), and can inhibit class II MHC expression on monocytes (8), dendritic cells (DC)[3] (9), and Langerhans cells (10). IL-10 can also inhibit the up-regulation of B7 on monocytes (11), monocyte-associated production of NO, and killing of parasites (12). Overexpression of IL-10 in IL-10 transgenic mice was shown to lead to decreased capacity to reject tumors (13).

Interest in the immune inhibitory effects of IL-10 in relation to antitumor responses was based on the recurrent presence of this cytokine in fresh tumor biopsies or ascites fluid derived from cancer patients (14, 15). Expression of IL-10 mRNA was found in various kinds of solid tumors, such as ovarian carcinomas (16), renal carcinomas (17), nonsmall lung cancer cells (18), and melanomas (19). The preferential expression of IL-10 in metastatic vs primarily lesions of melanomas has been established, indicating an increased metastatic potential of IL-10-secreting melanoma cells (20).

We have previously demonstrated that murine tumor cells treated with rIL-10 or transfected with the IL-10 gene showed a changed phenotype characterized by a down-regulation in MHC class I expression, resistance to lysis by tumor-specific CTL, and increased sensitivity to lysis by NK cells related to an inhibition in the function and expression of TAP molecules (21–23). In human melanoma, we demonstrated that human rIL-10 (rhIL-10)-pretreated melanoma cell lines became less sensitive to tumor-specific CTL and also showed a significant reduction of MHC class I expression (24). These observations have been confirmed in studies in which IL-10 was described to down-regulate HLA class I and class II and also ICAM-1 molecules in melanoma cell lines (25).

A synthetic nanomeric peptide IT9302 (Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn), with complete homology to an amino acid sequence located in the C-terminal portion of human IL-10 (residues 152–160), has been shown to be the functional domain of IL-10, sharing several of the functions of this cytokine (26). In this study, we have analyzed the effect of this IT9302 peptide on the expression of MHC class I-related Ag presentation proteins in melanoma cells. We demonstrated that the IL-10 homologous peptide down-regulates the MHC class I surface expression and also the IFN-γ-induced

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[3] Abbreviations used in this paper: DC, dendritic cell; LAK, lymphokine-activated killer; LMP, low molecular protein; MFI, mean fluorescence intensity; rhIL-10, human rIL-10.

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expression of MHC class I, TAP1, and TAP2 molecules, affecting NK sensitivity of treated tumor cells.

Materials and Methods

Cell lines and peptides

The FM55 and FM3D melanoma cell lines were obtained from the Danish Cancer Society through the courtesy of J. Zeuthen. The OCM1 is an ocular melanoma line kindly provided by M. Jager (University of Leiden, Leiden, The Netherlands). These cell lines were negative for IL-10 expression and positive for IL-10R expression, and were maintained in culture in RPMI 1640 medium supplemented with 5% FCS, 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies, Gaithersburg, MD). The nanomeric peptide IT9302 (AYMTMKIRN) and the control peptide IT6542 (SPGQGTQSE) corresponding to a nonfunctional IL-10 domain (26) were chemically synthesized using an automatic polypeptide synthesizer. The purity of the proteins was confirmed to exceed 95% by HPCL, and the m.w. was controlled by mass spectroscopy. In some experiments, an irrelevant nanomeric peptide derived from influenza matrix protein (GILGFVFTL) was used as a control.

Treatment of tumor cells with IT9302 and IFN-γ

Melanoma cells (6×10^5 cells in 3 ml) were cultured in AIM-V serum-free medium (Invitrogen Life Technologies, Gaithersburg, MD) in the presence of 1, 10, and 100 ng/ml peptide IT9302 or an irrelevant control peptide (100 ng/ml) during 24 h. To investigate the inhibition of IFN-γ-mediated induction of Ag presentation proteins, cells were pretreated during 2 h with different concentrations of IT9302 peptide or control peptide and then incubated in the presence of only medium, or medium containing 100 or 500 U/ml rIFN-γ IMUKIN (Boehringer Ingelheim, Ingelheim, Germany) for additional 22 h for FACS, or Western blot analysis. To specifically block the IT9302-mediated effect, medium containing 100 ng/ml IT9302 was preincubated with 10 μg/ml rabbit polyclonal anti-IT9302 Ab (26) or 10 μg/ml normal rabbit serum as control and then added to cell cultures.

mAbs and FACS analysis

Melanoma cells either untreated or stimulated for 24 h with IT9302 and with or without rIFN-γ were washed in PBS and incubated for 30 min on ice in RPMI 1640 and 5% FBS with mAb W6/32 (anti-MHC class I) (American Type Culture Collection (ATCC), Manassas, VA), mAb HB54 (anti-HLA-A2) (ATCC), or mouse IgG as negative control (BD Pharmingen, San Diego, CA). After two washings, the cells were incubated with a secondary anti-mouse IgG FITC (DakoCytomation, Hamburg, Germany) for 30 min. Cells were analyzed after four washings. For intracellular detection of TAP molecules, anti-TAP1 and anti-TAP2 mAbs (BD Pharmingen, San Diego, CA) were used after cell permeabilization with digitonin (Sigma-Aldrich, St. Louis, MO). Flow cytometry was performed using a FACScan flow cytometer and analyzed by the CellQuest analysis program (BD Biosciences, Heidelberg, Germany).

Western blot analysis

Cell pellets from harvested melanoma cell lines (1×10^6 cells) were suspended in 100 μl of lysis buffer (65 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME, 1% bromphenol blue) and maintained for 15 min at room temperature. Then samples were sonicated by 10 μm for 30 s, warmed at 95°C for 5 min, and then centrifuged. A total of 15 μl of each sample was electrophoresed through a 12% SDS-polyacrylamide gel. For immunoblots, proteins were electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked in PBS containing 5% milk (low fat). All additional immunostaining steps were performed in PBS with 3% milk and washed with PBS 0.05% Tween 20 (PBS-Tween 0.05%). Filters were incubated overnight at 4°C with the corresponding primary Ab: mouse mAb TO-1 anti-TAP1, mouse mAb SY-2 anti-TAP2 (both kindly provided by S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY)), or a rabbit polyclonal Ab against MHC class I H chain (also kindly provided by S. Ferrone). As an internal control, a commercial polyclonal Ab against β-actin was used (Sigma-Aldrich, St. Louis, MO). After washing with PBS/milk, the membranes were incubated with the respective secondary Ab (HRP-conjugated sheep anti-mouse Ig or HRP-conjugated sheep anti-rabbit Ig (Amersham Biosciences, Buckinghamshire, U.K.)) for 2 h at room temperature. Filters were then washed in PBS-0.05% Tween five times and developed with ECL system (Amersham Biosciences).

Cytotoxic assay

Lymphokine-activated killer (LAK) cells were obtained from normal donors' PBMC. After a gradient separation with Lymphoprep (Invitrogen Life Technologies), 25×10^6 PBMC were incubated during 5 days in 15 ml of RPMI 1640 and 10% FBS medium containing 500 U/ml IL-2 (kindly supplied by P. Simon, DuPont Merck Pharmaceutical, Wilmington, DE). A 6-h ^{51}Cr release assay was used to measure cytotoxic activity of LAK cells against peptide- and/or IFN-γ-treated melanoma cell lines or K562 cells as positive control. All samples were run in triplicate. Specific lysis was calculated according to the formula: percentage of specific lysis = $100 \times$ ((experimental cpm $-$ spontaneous cpm)/(maximum cpm – spontaneous cpm)).

Statistical analysis

The differences in TAP expression between triplicates were compared by Student's *t* test using a computer program, and were considered statistically significant at $p < 0.05$

Results

The peptide IT9302, homologous to the functional domain of human IL-10, down-regulates the surface MHC class I expression on melanoma cell lines

The nanomeric peptide IT9302 has been demonstrated to constitute the functional domain of human IL-10 (26). To investigate whether IT9302 could also mimic the capability of IL-10 to down-regulate MHC class I surface expression on melanoma cells, we treated the cutaneous melanoma line FM55 and the ocular melanoma line OCM1 with 0, 1, 10, and 100 ng/ml IT9302 during 24 h. The peptide concentrations were selected considering that 6125 ng of peptide corresponds in molarity to ~100 ng/ml human IL-10 (26). A dose-dependent inhibition of MHC class I could be observed in both treated melanomas, with a maximal inhibition of 60% for OCM1 (Fig. 1*A*) and 50% for FM55 (Fig. 1*B*) compared with the controls. These results were in line with previously observed effects using rhIL-10 (24). The effect of the IT9302 peptide and the control peptide on HLA class I expression was measured also on the HLA-A2$^+$ FM3D melanoma line. Of interest, while a strong effect on total MHC class I was noted (mean fluorescence intensity (MFI) of 92.5) following IT9302 incubation as compared with MFI of 152 with the control peptide and MFI of 162 for medium only for the FM3D line, using mAb HB54, the effect on HLA-A2 expression was only marginal (MFI of 35.6 following IT9302 incubation as compared with MFI of 47.8 with the control peptide and MFI of 45.5 with medium only) (Fig. 1*C*). The reduced effect of IT9302 on HLA-A2 as compared with the effect on total HLA class I could be explained by the known ability of HLA-A2 to present peptides in a TAP-independent way (27–29).

The peptide IT9302 inhibits the MHC class I induction mediated by IFN-γ

The IT9302 peptide was previously shown to inhibit the IFN-γ-induced MHC class II expression on human monocytes (26) in line with the known ability of IL-10 to antagonize the effect that IFN-γ has on Ag presentation (30, 31). In this study, we investigated whether the peptide homologue could antagonize the IFN-γ-mediated enhancement of MHC class I H chain expression. OCM1 melanoma cells were first treated with 0, 1, 10, and 100 U/ml IT9302 peptide, medium alone, or 100 U/ml control peptide for 2 h, and thereafter with 100 or 500 U/ml rIFN-γ for an additional 22 h. In this experiment, a 2- to 3-fold induction of MHC class I surface expression was observed after treatment with the two concentrations of IFN-γ. A dose-dependent inhibition of the basal level of MHC class I and, most importantly, of the IFN-γ-induced expression of MHC class I was observed when the cells were pretreated with increasing doses of IT9302 and thereafter IFN-γ (100 U/ml) (Fig. 2). The highest inhibition of MHC class I induction

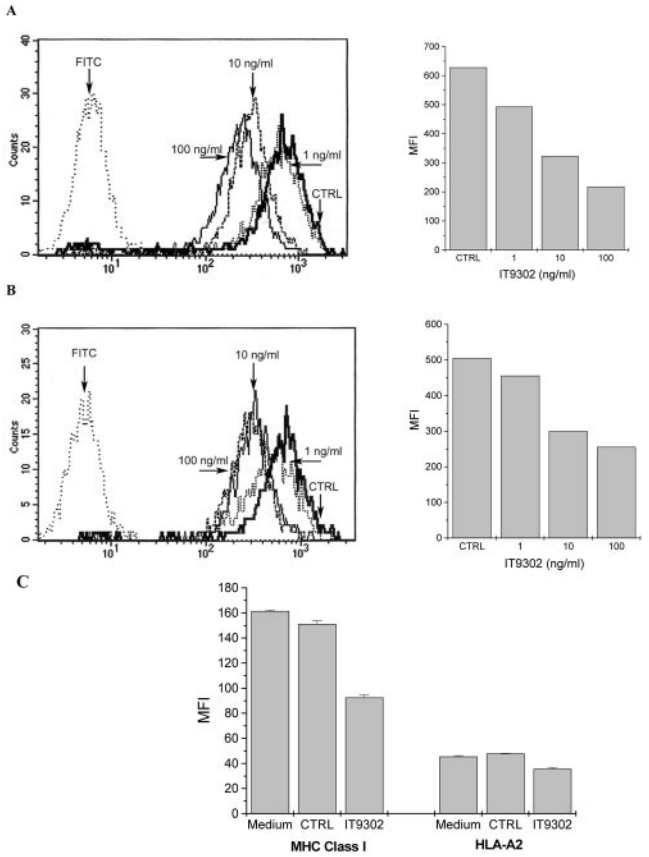

FIGURE 1. The peptide IT9302 down-regulates the surface MHC class I expression on melanoma cell lines. Melanoma cell lines OCM1 (*A*), FM55 (*B*), and FM3D (*C*) (HLA-A2$^+$) were treated with different concentrations of peptide IT9302 or with 100 ng/ml control peptide IT6542, and after 24-h incubation at 37°C analyzed by flow cytometry for MHC class I expression with the mAb W6.32 or with mAb HB45 (anti HLA-A2), as described in *Materials and Methods*. The experiment was performed three times with similar results.

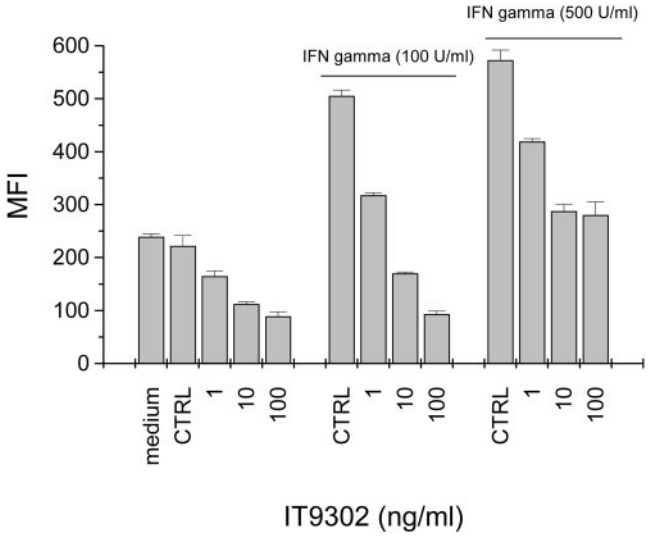

FIGURE 2. IT9302 inhibits IFN-γ-induced MHC class I expression. The melanoma cell line OCM1 was pretreated in duplicate cultures with different concentrations of peptide IT9302, with the control peptide GILGFVFTL, or with medium only. After 2 h of incubation, two different concentrations (100 and 500 U/ml) of rIFN-γ were added to the medium, and the cells were incubated for an additional 22 h and then analyzed for MHC class I expression with the mAb W6.32, as described in *Materials and Methods*. The experiment was performed three times with similar results.

was observed in cells pretreated with 100 ng/ml IT9302 (Fig. 2). The weaker inhibition of MHC class I by IT9302 peptide observed when the cells were treated with a high dose of IFN-γ (500 U/ml) indicated that MHC class I expression is delicately regulated by a balance between both cytokines (Fig. 2).

The peptide IT9302 inhibits IFN-γ-mediated induction of TAP1 and TAP2, but not MHC H chain or low molecular protein 2 (LMP2) in melanoma cell lines

We and others have previously shown that IL-10 could inhibit the function and expression of TAP1/TAP2 molecules (22, 23, 32). Many tumors, including melanomas, are known to express low levels of TAP molecules, which can be induced by IFN-γ (33–36). We could confirm that this was also the case for the FM55 melanoma line, as measured by RT-PCR (data not shown), and for OCM1 line measured by Western blot (Fig. 3).

To clarify whether IT9302 could also down-regulate TAP molecules, we induced their expression by treatment with IFN-γ. Using Western blot analysis, we could observe that the constitutive expression of TAP1, TAP2, and LMP2 in the melanoma line OCM1 was very low, at the limit of detection (Fig. 3). In contrast, OCM1 cells treated with 100 U/ml rIFN-γ showed a strong expression of TAP1, TAP2, and LMP2 proteins (Fig. 3). IT9302 markedly inhibited the IFN-γ-induced TAP1 and TAP2 expression in a dose-dependent manner, with a strong inhibition noted already at a concentration of 10 ng/ml peptide. The IFN-γ-induced expression of the LMP2 protein (Fig. 3) or MHC H chain (data not shown) was, however, not affected by the IT9302 peptide. The TAP1 and TAP2 were selectively down-regulated by IT9302, as the expression of the housekeeping protein β-actin used as control in this experiment was unaffected. Also, in the FM55 melanoma line, IT9302 and rhIL-10 did inhibit TAP1 and TAP2 expression (data not shown). Using intracellular staining and analysis by flow cytometry, it was observed that the level of expression of TAP1 (Fig. 4*A*) and TAP2 (Fig. 4*B*) could be almost completely recovered in the presence of a polyclonal Ab directed against peptide IT9302, but not using a normal rabbit serum as control (data not shown), both in the absence

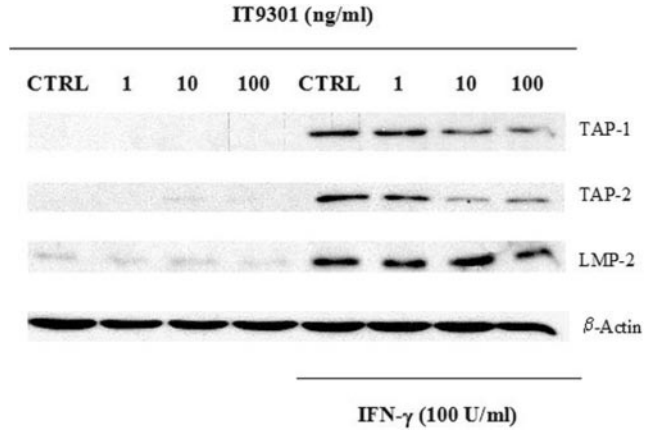

FIGURE 3. IT9302 inhibits IFN-γ-mediated induction of TAP1 and TAP2, but not LMP2, in melanoma cell lines. Melanoma cell line OCM1 was pretreated with different concentrations of peptide IT9302 or with the GILGFVFTL control peptide, and 2 h later treated with 100 U/ml rIFN-γ or with only medium. After additional 22-h incubation, cells were analyzed by Western blot using TAP1, TAP2, and LMP2-specific Abs, as described in *Materials and Methods*. β-actin-specific mAb was used as internal control.

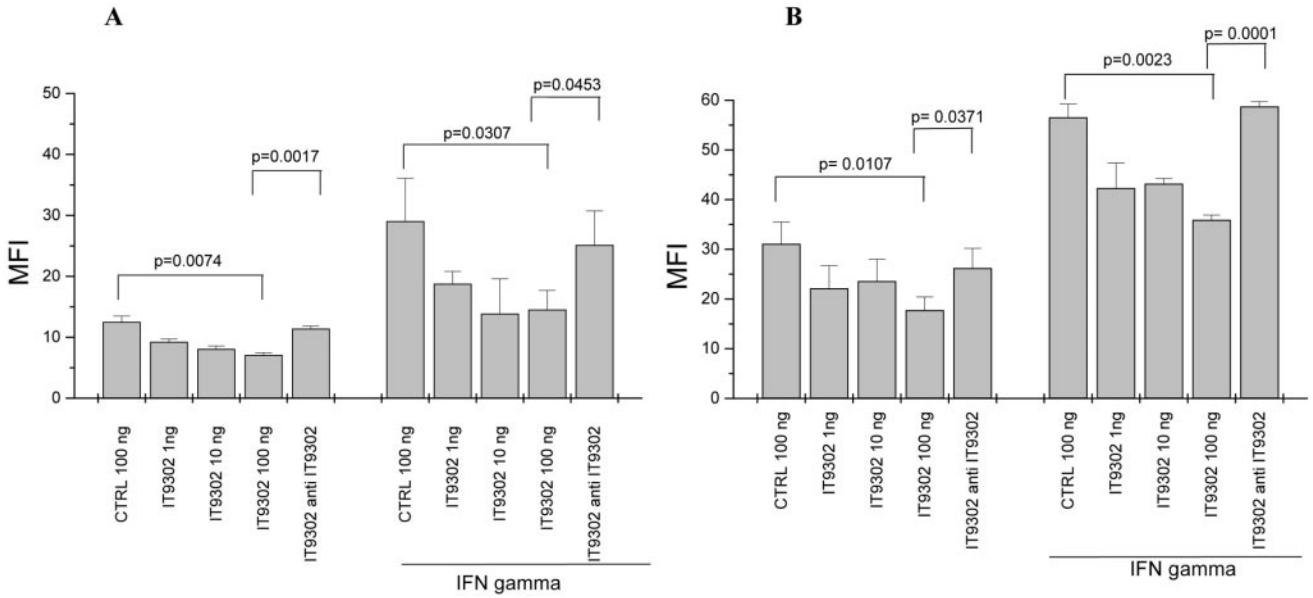

FIGURE 4. A polyclonal Ab specific to IT9302 can neutralize peptide inhibition of TAP1 and TAP2. Melanoma cell line OCM1 was pretreated in triplicate cultures with different concentrations of peptide IT9302 or with the control peptide IT6542, and 2 h later treated with 100 U/ml rIFN-γ or with only medium. To block the peptide effect, medium containing 100 ng/ml IT9302 peptide was preincubated with 10 μg/ml rabbit anti IT9302 polyclonal Ab. After 24-h incubation, cells were analyzed by flow cytometry using anti-TAP1 (*A*) and anti-TAP2 (*B*) mAbs, as described in *Materials and Methods*.

and presence of IFN-γ, confirming the direct effect of this peptide on the expression of TAP1/2 molecules.

The peptide IT9302 induces a higher sensitivity to LAK-mediated cytotoxicity in melanoma cells

We have previously demonstrated that IL-10 could convert murine tumor cells into an NK-sensitive phenotype (21–23). To investigate whether the IT9302 peptide also could enhance NK sensitivity of human melanoma cells, we performed cytotoxicity assays with LAK cells as effector cells. The melanoma cell line OCM1 pretreated with different concentrations of the IT9302 peptide was found to be more sensitive to LAK cells than melanoma cells treated with a control peptide. The effect of the IT9302 peptide was dose dependent, and the NK sensitivity of the OCM1 line treated with 100 ng/ml IT9302 approached that of the standard NK target cell K562 (Fig. 5*A*). A comparable effect was observed using the melanoma line FM55 (Fig. 5*B*). These results showed that the changes observed in surface expression of MHC class I translated into enhanced sensitivity of tumor cells to lysis mediated by LAK cells.

Discussion

It has been demonstrated recently that small molecules such as oligopeptides or other low m.w. nonpeptide structures can act as functional agonists for intact proteins, including some cytokines (26, 37–39). Those small molecules can interact with cell membrane-specific receptors and mediate intracellular signaling with similar consequences as those induced by the whole protein. They thus offer new and promising avenues to therapeutic interventions based on structurally defined small molecules, which may have several advantages as compared with the corresponding whole protein. In this study, we have analyzed the effect of the synthetic peptide IT9302 on the expression of MHC class I-related Ag presentation proteins in melanoma cells expressing IL-10R. Our observations demonstrate that the IL-10 homologous peptide down-regulates the MHC class I surface expression and also the IFN-γ-induced expression of TAP1 and TAP2 molecules, affecting NK sensitivity of the tumors. These results therefore are the first to show that a peptide homologue of IL-10 markedly can affect MHC class I-related molecules and function in human tumors.

The synthetic nanomeric peptide IT9302, which we have used in this study, has a complete homology to an amino acid sequence located in the C-terminal portion of human IL-10 (residues 152–160), which has been shown to be the functional domain of IL-10 (26). The functions of IL-10 that this peptide previously was demonstrated to mimic are: inhibition of IL-1β-induced IL-8 production by monocytes, inhibition of spontaneous IL-8 expression by in vitro cultured monocytes, down-regulation of TNF-α production by $CD8^+$ lymphocytes, induction of IL-4 production by $CD4^+$ lymphocytes, and inhibition of IFN-γ induction of MHC class II on monocytes (26). Our results therefore demonstrate that the same domain on IL-10, namely the C-terminal portion, also determines the effect of this molecule on MHC class I expression. In previous studies, we have demonstrated that IL-10 has an inhibitory effect on Ag class I presentation, diminishing the expression of surface MHC class I in murine tumors (21–23) and human melanoma cells (24). We, in this study, demonstrated a similar inhibitory effect mediated by the IL-10 agonist peptide IT9302. Two melanoma cell lines, FM55 and OCM1, were shown to decrease surface MHC class I expression in a dose-dependent manner when they were treated with IT9302.

Several of the inhibitory effects mediated by IL-10 are opposite to those reported for IFNs, which were shown to up-regulate the expression of several members of the MHC class I Ag presentation pathway (30, 31). This was associated with increased sensitivity for CTL, but protection from NK cell cytotoxicity (40). Therefore, IL-10 is able to block not only the induction of IFN-γ by Th1 cells (4), but also to have an opposite effect from IFN-γ on Ag presentation (31). The net effect on Ag presentation of having both IFN-γ and IL-10 expressed simultaneously, as sometimes seems to be the case in, e.g., ovarian carcinomas (16), was analyzed using our melanoma model. First, we studied whether IT9302 could inhibit the MHC class I induction mediated by IFN-γ. Our results showed that the pretreatment of melanoma cells with the IT9302 peptide

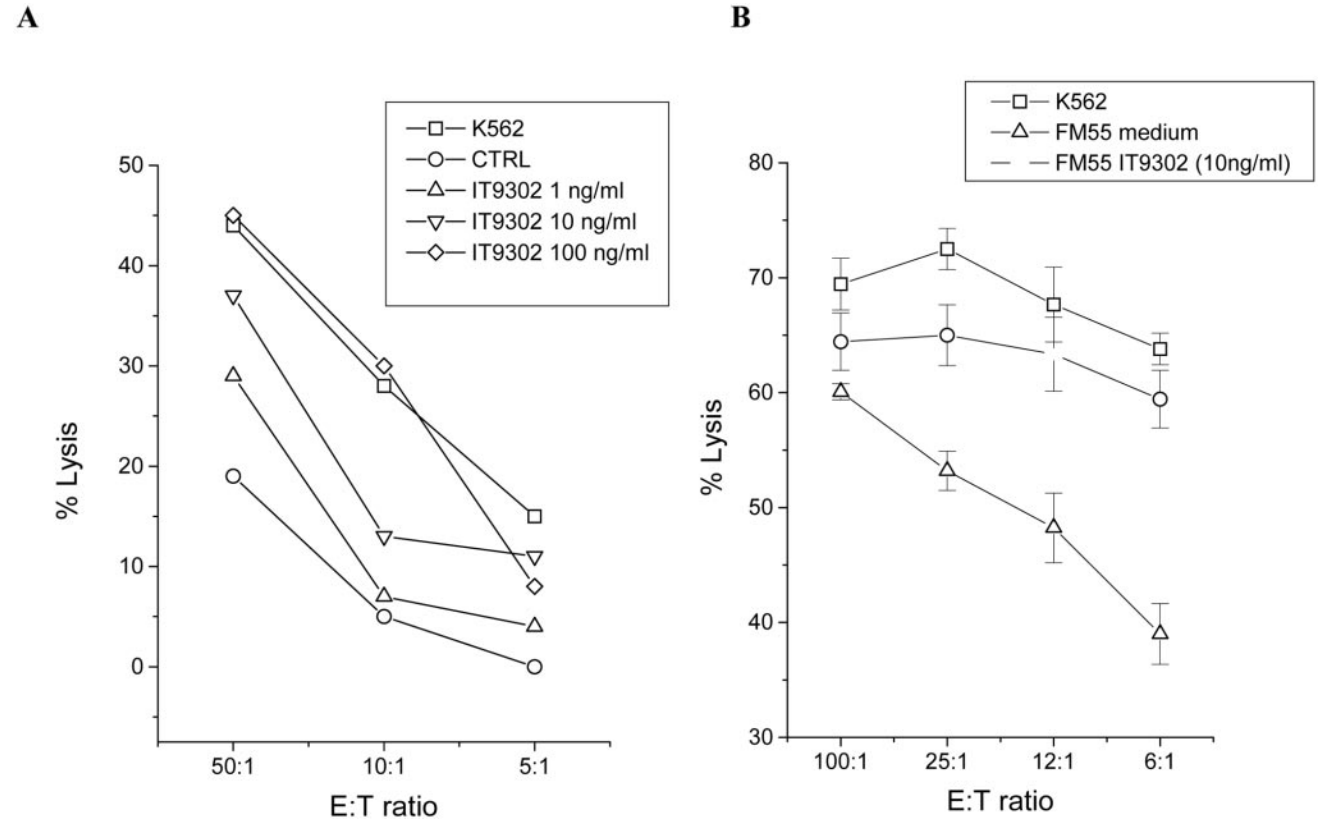

FIGURE 5. The peptide IT9302 induces a higher sensitivity to LAK-mediated cytotoxicity in melanoma cell lines. *A*, The melanoma cell line OCM1 was treated with 1, 10, and 100 ng/ml peptide IT9302, or with the control peptide IT6542 (100 ng/ml). *B*, The FM55 melanoma cell line was treated with medium alone or containing 10 ng/ml IT9302 and then analyzed in a ^{51}Cr release assay with LAK cells as effector cells, as described in *Materials and Methods*. The NK-sensitive cell line K562 was used as positive control.

could inhibit both the constitutive and the IFN-γ-induced surface MHC class I expression in a dose-dependent manner. IT9302 could more efficiently inhibit MHC class I expression induced by lower concentrations of IFN-γ (10–100 U/ml) than MHC class I induced by the higher concentration (500 U/ml), indicating that the MHC class I regulation responds to a balance between both cytokines. IL-10 is known to inhibit IFN-γ-induced class II Ag expression on mononuclear cells (8). This function appears to be located to the segment of IL-10, which is represented by the IT9302 peptide, and our results therefore demonstrate that also the ability of IL-10 to block induction of MHC class I is confined to the same region of IL-10.

It has been described that several tumors, including melanomas, commonly have a defective TAP expression (35, 36, 41). In confirmation of this, the OCM1 and FM55 melanomas tested in this study expressed very low levels of these proteins in a constitutive form (Figs. 3 and 4, *A* and *B*). Only after treatment with IFN-γ was it possible to efficiently detect the expression of TAP1 and TAP2 by Western blot. Both proteins were substantially reduced when the tumor cells were pretreated with IT9302, while MHC class I H chain protein and LMP2, also involved in MHC class I Ag presentation, were not affected. The reduced effect of IT9302 on HLA-A2 (<20%), as compared with the effect on total HLA class I (~60%) (Fig. 1*C*), could be explained by the known ability of HLA-A2 to present peptides in a TAP-independent way (27–29). The close relation between TAP inhibition and low surface expression of MHC class I has also been clearly established in various studies (33–36), including our previous works (21–23).

The effect of IL-10 on MHC class I Ag presentation has been observed also in normal, nonmalignant cell types, including B cells and DC (32, 42), and in tumors and infected cells (43–45). Thus, cellular and viral IL-10 were shown to affect Ag presentation and MHC class I expression in EBV-infected human B lymphocytes through their ability to reduce TAP1 expression (32). These authors speculate that IL-10 could therefore affect TAP1 levels in vivo, contributing to the persistence of EBV-infected cells in the human host. Furthermore, autocrine IL-10 secretion was shown to inhibit MHC class I in DC, affecting their Ag presentation capability (42). Therefore, it would be important to investigate whether the observed effects of IT9302 can be generalized also to B cells and DC, which would have a direct implication on the ability of this peptide to modulate Ag presentation in vivo.

As cells with defective TAP1/2 function, due to mutations (34, 35) or to IL-10 expression (22, 23, 32), are more susceptible to NK cell-mediated cytotoxicity, we investigated whether the pretreatment of melanoma with IT9302 could also induce a similar effect. Our results showed that IT9302 peptide pretreatment made the melanoma lines OCM1 and FM55 more sensitive to NK-mediated lysis (Fig. 5). NK cells may therefore more readily reject IL-10-expressing tumors. Several lines of evidence indicate that the dose and the mode of administration of IL-10 may affect the in vivo tumor rejection (46). High doses of IL-10 may have a stimulatory effect on the immune response, activating T cells, NK cells, and also macrophages and monocytes. An IL-10 transfectant of the murine mammary tumor cell line (TSA) producing high levels of IL-10, is more rapidly rejected than the control line, an effect explained as an activation of CTL and NK cells (47). Similar effects have been observed in several strains of mice and with a variety of IL-10-producing transfected tumors, such as the murine melanoma

CL8–1 (48), the murine melanoma B16 (49, 50), and the mammary carcinoma 410-4 (51). The role of NK cells in these systems is supported by experiments showing that Chinese hamster ovary cells transfected with murine IL-10 (52), and the murine lymphoma cell line (RMA) transfected with the IL-10 gene (our unpublished results) are more readily rejected in T cell-deficient SCID mice. Taken together, these observations demonstrate that IL-10 has profound effects on the NK cell-mediated tumor rejection in vivo, possibly mediated via the effect of IL-10 on TAP1/2 function, as described in this work. Recently, it has been demonstrated that the IT9302 peptide in vivo stimulates the production of proinflammatory cytokines in the serum of rabbits after experimentally induced acute pancreatitis (53), indicating that the peptide can exert its effect across the species barrier. In view of these results, it would be of particular importance to investigate the effects on host antitumor responses following in vivo administration of the IT9302 peptide to mice with NK-sensitive experimental tumors. This may lead to new therapeutic strategies based on coadministration of the IT9302 peptide and NK-activating cytokines to cancer patients.

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References

1. Vieira, P., R. de Waal-Malefyt, M. N. Dang, K. E. Johnson, R. Kastelein, D. F. Fiorentino, J. E. De Vries, M. G. Roncarolo, T. R. Mosmann, and K. W. Moore. 1991. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. *Proc. Natl. Acad. Sci. USA 88:1172*.
2. MacNeil, I. A., T. Suda, K. W. Moore, T. R. Mosmann, and A. Zlotnik. 1990. IL-10, a novel growth cofactor for mature and immature T cells. *J. Immunol. 145:4167*.
3. Chen, W. F., and A. Zlotnik. 1991. IL-10: a novel cytotoxic T cell differentiation factor. *J. Immunol. 147:528*.
4. De Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med. 174:1209*.
5. Fiorentino, D. F., A. Zlotnik, P. Viera, T. R. Mosmann, M. Howard, K. W. Moore, and A. O′Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol. 147:3815*.
6. Fiorentino, D. F., A. Zlotnic, P. Viera, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol. 146:3444*.
7. Taga, K., H. Mostowski, and G. Tosato. 1993. Human interleukin-10 can directly inhibit T cell growth. *Blood 81:2964*.
8. De Waal-Malefyt, R., J. Haanen, H. Spits, M. G. Roncarolo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J. E. de Vries. 1991. IL-10 and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II MHC expression. *J. Exp. Med. 174:915*.
9. Caux, C., C. Massacrier, B. Vanbervliet, C. Barthelemy, Y. J. Liu, and J. Banchereau. 1994. Interleukin 10 inhibits T cell alloreaction induced by human dendritic cells. *Int. Immunol. 6:1177*.
10. Peguet-Navarro, J., C. Moulon, C. Caux, C. Dalbiez-Gauthier, J. Banchereau, and D. Schmitt. 1994. Interleukin-10 inhibits the primary allogeneic T cell response to human epidermal Langerhans cells. *Eur. J. Immunol. 24:884*.
11. Ding, L., P. S. Linsley, L.-Y. Huang, R. R. Germain, and E. M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J. Immunol. 151:1224*.
12. Gazzinelli, R. T., I. P. Oswald, S. L. James, and A. Sher. 1992. IL-10 inhibits parasite killing and nitric oxide production by IFN-γ-activated macrophages. *J. Immunol. 148:1792*.
13. Hagenbaugh, A., S. Sharma, S. M. Dubinett, S. H. Y. Wei, R. Aranda, H. Cheroutre, D. J. Fowell, S. Binder, B. Tsao, R. M. Locksley, et al. 1997. Altered immune responses in interleukin 10 transgenic mice. *J. Exp. Med. 185:2101*.
14. Gastle, G. A., J. S. Abrams, D. M. Nanus, R. Osterkamp, J. Silver, F. Liu, M. Chen, A. P. Albino, and N. H. J. Bander. 1993. Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. *Int. J. Cancer 55:96*.
15. Gotlieb, W. H., J. S. Abrams, J. M. Watson, T. J. Velu, J. S. Berek, and O. Martínez-Maza. 1992. Presence of interleukin 10 (IL-10) in the ascites of patients with ovarian and other intra-abdominal cancers. *Cytokine 4:385*.
16. Pisa, P., E. Halapi, E. Pisa, E. K. Gerdin, C. Hising, A. Bucht, B. Gerdin, and R. Kiessling. 1992. Selective expression of interleukin 10, interferon-γ and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. *Proc. Natl. Acad. Sci. USA 89:7708*.
17. Nakagomi, H., P. Pisa, E. K. Pisa, Y. Yamamoto, E. Halapi, K. Backlin, C. Juhlin, and R. Kiessling. 1995. Lack of interleukin-2 (IL-2) expression and selective expression of IL-10 mRNA in human renal cell carcinoma. *Int. J. Cancer 63:366*.
18. Huang, M., J. Wang, P. Lee, S. Sharma, J. T. Mao, H. Meissner, K. Uyemura, R. Modlin, J. Wollman, and S. M. Dubinett. 1995. Human non-small lung cancer cells express a type 2 cytokine pattern. *Cancer Res. 55:3847*.
19. Kruger-Krasagakes, S., K. Krasagakis, C. Garbe, E. Schmitt, C. Huls, T. Blankenstein, and T. Diamantstein. 1994. Expression of interleukin 10 in human melanoma. *Br. J. Cancer 70:1182*.
20. Dummer, W., B. C. Bastian, N. Ernst, C. Schanzle, A. Schwaaf, and E. B. Brocker. 1996. Interleukin-10 production in malignant melanoma: preferential detection of IL-10-secreting tumor cells in metastatic lesions. *Int. J. Cancer 66:607*.
21. Salazar-Onfray, F., M. Petersson, L. Franksson, M. Matsuda, T. Blankenstein, K. Kärre, and R. Kiessling. 1995. IL-10 converts mouse lymphoma cells to a CTL-resistant, NK sensitive phenotype with low but peptide-inducible MHC class I expression. *J. Immunol. 154:6291*.
22. Salazar-Onfray, F., J. Charo, M. Petersson, S. Freland, G. Noffz, Z. Qin, T. Blankenstein, H.-G. Ljunggren, and R. Kiessling. 1997. Down-regulation of the expression and function of TAP in murine tumor cell lines expressing interleukin-10. *J. Immunol. 159:3195*.
23. Petersson, M., J. Charo, F. Salazar-Onfray, G. Noffz, Z. Qin, G. Klein, T. Blankenstein, and R. Kiessling. 1998. Constitutive IL-10 production accounts for the high NK sensitivity, low MHC class I expression and poor TAP1/2 function in the prototype NK target YAC-1. *J. Immunol. 161:2099*.
24. Matsuda, M., F. Salazar, M. Petersson, G. Masucci, J. Hansson, P. Pisa, Q.-J. Zhang, M. G. Masucci, and R. Kiessling. 1994. Interleukin-10 pre-treatment protects target cells from tumor- and allo-specific cytotoxic T-cells and down-regulates HLA class I expression. *J. Exp. Med. 180:2371*.
25. Yue, F. Y., R. Dummer, R. Geertsen, G. Hofbauer, E. Laine, S. Manolio, and G. Burg. 1997. Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules. *Int. J. Cancer 71:630*.
26. Gesser, B., H. Leffers, T. Jinquan, C. Vestergaad, N. Kirsten, S. Sindet-Pedersen, S. Lindkaer, K. Thestrup-Pedersen, and C. Gronhoj. 1997. Identification of functional domains on human interleukin 10. *Proc. Natl. Acad. Sci. USA 94:14620*.
27. Lauvau, G., B. Gubler, H. Cohen, S. Daniel, S. Caillat-Zucman, and P. M. van Endert. 1999. Tapasin enhances assembly of transporters associated with antigen processing-dependent and -independent peptides with HLA-A2 and HLA-B27 expressed in insect cells. *J. Biol. Chem. 274:31349*.
28. Wolfel, C., I. Drexler, A. Van Pel, T. Thres, N. Leister, W. Herr, G. Sutter, C. Huber, and T. Wolfel. 2000. Transporter (TAP)- and proteasome-independent presentation of a melanoma-associated tyrosinase epitope. *Int. J. Cancer 88:432*.
29. Matsui, M., M. Ikeda, and T. Akatsuka. 2001. High expression of HLA-A2 on an oral squamous cell carcinoma with down-regulated transporter for antigen presentation. *Biochem. Biophys. Res. Commun. 280:1008*.
30. Restifo, N. P., P. J. Spiess, S. E. Karp, J. J. Mulé, and S. A. Rosenberg. 1992. A nonimmunogenic sarcoma transduced with the cDNA for interferon γ elicits $CD8^+$ T cells against the wild type tumor: correlation with antigen presentation capability. *J. Exp. Med. 175:1423*.
31. Hiltbold, E. M., and H. K. Ziegler. 1996. Interferon-γ and interleukin-10 have cross-regulatory roles in modulating the class I and class II MHC-mediated presentation of epitopes of *Listeria monocytogenes* by infected macrophages. *J. Interferon Cytokine Res. 16:547*.
32. Zeidler, R., G. Eissner, P. Meissner, S. Uebel, R. Tampe, S. Lazis, and W. Hammerschmidt. 1997. Down-regulation of TAP1 in B lymphocytes by cellular and Epstein-Barr virus-encoded interleukin-10. *Blood 90:2390*.
33. Cromme, F. V., J. Airey, M. T. Heemels, H. L. Ploegh, and J. M. Walboomers. 1994. Loss of transporter protein, encoded by the TAP-2 gene, is highly correlated with loss of HLA expression in cervical carcinomas. *J. Exp. Med. 179:335*.
34. Seliger, B., T. Dunn, A. Schwenzer, J. Casper, C. Huber, and H. J. Schmoll. 1997. Analysis of the MHC class I antigen presentation machinery in human embryonal carcinomas: evidence for deficiencies in TAP, LMP and MHC class I expression and their up-regulation by IFN-γ. *Scand. J. Immunol. 46:625*.
35. Kaklamanis, L., A. Townsend, I. A. Doussis-Anagnostopoulou, N. Mortensen, A. L. Harris, and K. C. Gatter. 1994. Loss of major histocompatibility complex-encoded transporter associated with antigen presentation (TAP) in colorectal cancer. *Am. J. Pathol. 145:505*.
36. Chen, H. L., D. Gabrilovich, R. Tampe, K. R. Girgis, S. Nadaf, and D. P. Carbone. 1996. A functionally defective allele of TAP1 results in loss of MHC class I antigen presentation in a human lung cancer. *Nat. Genet. 13:210*.
37. Hu, J. Y., Y. Le, W. Gong, N. M. Dunlop, J. L. Gao, P. M. Murphy, and J. M. Wang. 2001. Synthetic peptide MMK-1 is a highly specific chemotactic agonist for leukocyte FPRL1. *J. Leukocyte Biol. 70:155*.
38. Christophe, T., A. Karlsson, C. Dugave, M. J. Rabiet, F. Boulay, and C. Dahlgren. 2001. The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH2 specifically activates neutrophils through FPRL1/lipoxin A4 receptors and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J. Biol. Chem. 276:21585*.
39. Osman, M. O., N. O. Jacobsen, J. U. Kristensen, B. Deleuran, B. Gesser, C. G. Larsen, and S. L. Jensen. 1998. IT 9302, a synthetic interleukin-10 agonist, diminishes acute lung injury in rabbits with acute necrotizing pancreatitis. *Surgery 124:584*.

40. Welsh, R. M., K. Karre, M. Hansson, L. A. Kunkel, and R. W. Kiessling. 1981. Interferon-mediated protection of normal and tumor target cells against lysis by mouse natural killer cells. *J. Immunol. 126:219*.
41. Seliger, B., A. Höhne, A. Kuth, H. Bernhard, T. Meyer, R. Tampe, F. Momburg, and C. Huber. 1996. Analysis of the major histocompatibility complex class I antigen presentation machinery in normal and malignant renal cells: evidence for deficiencies associated with transformation and progression. *Cancer Res. 56:1756*.
42. Corinti, S., C. Albanesi, A. la Sala, S. Pastore, and G. Girolomoni. 2001. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J. Immunol. 166:4312*.
43. Kundu, N., and A. M. Fulton. 1997. Interleukin-10 inhibits tumor metastasis, down-regulates MHC class I, and enhances NK lysis. *Cell. Immunol. 180:55*.
44. Caspar-Bauguil, S., B. Puissant, D. Nazzal, J. C. Lefevre, M. Thomsen, R. Salvayre, and H. Benoist. 2000. *Chlamydia pneumoniae* induces interleukin-10 production that down-regulates major histocompatibility complex class I expression. *J. Infect. Dis. 182:1394*.
45. Spencer, J. V., K. M. Lockridge, P. A. Barry, G. Lin, M. Tsang, M. E. Penfold, and T. J. Schall. 2002. Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. *J. Virol. 76:1285*.
46. Garcia-Hernandez, M. L., R. Hernandez-Pando, P. Gariglio, and J. Berumen. 2002. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumor and vascular cell proliferation. *Immunology 105:231*.
47. Giovarelli, M., P. Musiani, A. Modesti, P. Dellabona, G. Casorati, A. Allione, M. Consalvo, F. Cavallo, F. di Piero, C. De Giovanni, et al. 1995. Local release of IL-10 by transfected mouse mammary adenocarcinoma cells does not suppress but enhances antitumor reaction and elicits a strong cytotoxic lymphocyte and antibody-dependent immune memory. *J. Immunol. 155:3112*.
48. Suzuki, T., H. Tahara, S. Narula, K. W. Moore, P. D. Robbins, and M. T. Lotze. 1995. Viral interleukin-10 (IL-10), the human herpes virus 4 cellular IL-10 homologue, induces local anergy to allogeneic and syngeneic tumors. *J. Exp. Med. 182:477*.
49. Gerard, C. M., C. Bruyns, A. Delvaux, N. Baudson, J. L. Dargent, M. Goldman, and T. Velu. 1996. Loss of tumorigenicity and increased immunogenicity induced by interleukin-10 gene transfer in B16 melanoma cells. *Hum. Gene Ther. 7:23*.
50. Zheng, L. M., D. M. Ojcius, F. Garaud, C. Roth, E. Maxwell, Z. Li, H. Rong, J. Chen, X. Y. Wang, J. J. Catino, and I. King. 1996. Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism. *J. Exp. Med. 184:579*.
51. Kundu, N., T. L. Beaty, M. J. Jackson, and A. M. Fulton. 1996. Antimetastatic and antitumor activities of interleukin 10 in a murine model of breast cancer. *J. Natl. Cancer Inst. 88:536*.
52. Richter, G., S. Kruger-Krasagakes, G. Hein, C. Huls, E. Schmitt, T. Diamantstein, and T. Blankenstein. 1993. Interleukin 10 transfected into Chinese hamster ovary cells prevents tumor growth and macrophage infiltration. *Cancer Res. 53:4134*.
53. Osman, M. O., B. Gesser, J. T. Mortensen, K. Matsushima, S. L. Jensen, and C. G. Larsen. 2002. Profiles of pro-inflammatory cytokines in the serum of rabbits after experimentally induced acute pancreatitis. *Cytokine 17:53*.