

Interleukin 10 Pretreatment Protects Target Cells from Tumor- and Allo-specific Cytotoxic T Cells and Downregulates HLA Class I Expression

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Summary

Interleukin 10 (IL-10) is a cytokine with a variety of reported effects including inhibition of monocyte major histocompatibility complex (MHC) class II-dependent antigen presentation, type 1 helper T cell cytokine production, and inhibition of T cell proliferation. Herein we report the effect of IL-10 pretreatment on antigen presentation to tumor- and allo-specific CD8⁺ cytotoxic T lymphocytes (CTL). Prior incubation of human melanoma cells with recombinant IL-10 (rIL-10) for 48–72 h resulted in a dose-dependent, up to 100% inhibition, of autologous CTL-mediated, HLA-A2.1-restricted, tumor-specific lysis. Allo-specific CTL cytotoxicity against Epstein-Barr virus-transformed lymphoblastoid cell lines (LCL) was also inhibited, demonstrating a protective effect also on lymphoid cells. In contrast, IL-10 pretreatment of allogeneic LCL or K562 targets had either no effect or slightly enhanced cytotoxic activity mediated by freshly isolated or IL-2-activated natural killer cells. Flow cytometric analysis with monoclonal antibodies against HLA-A2, or nonpolymorphic determinants of MHC class I proteins, revealed a 20–50% reduction in cell-surface expression, whereas intercellular adhesion molecules 1, and 2, and lymphocyte function-associated antigen 3 levels were not affected. In addition, relative to untreated target cells, IL-10 pretreated tumor cells were unaltered in their capacity to affect CTL-mediated lysis by cold target inhibition, demonstrating that the effect of IL-10 is unrelated to the initial binding of CTL to their targets. These results are compatible with an effect of IL-10 on the MHC class I antigen presentation pathway, and suggest a novel mechanism of immune tolerance, based on escape from CTL-mediated tumor and allo-transplant rejection.

IL-10 was discovered because of its ability to suppress cytokine expression by type 1 helper T cells (Th1) and NK cells (1). More recently, it has been shown to have pleiotropic immunosuppressive effects. *In vitro*, IL-10 also blocks monocyte-dependent T cell proliferation (2), inhibits monocyte class II MHC expression (3), the upregulation of B7 on monocytes (4), and monocyte-associated production of nitric oxide and killing of parasites (5).

IL-10 is produced by a variety of cell types, including T cells, B cells, and macrophages (6, 7). Higher levels of human IL-10 were produced by Th2 than Th1 clones (8), and several human carcinoma lines, freshly isolated tumor biopsies, patient serum, and ascites fluid express or contain IL-10 (9–11). These observations suggest that the presence of IL-10 may be a common feature of several types of human tumors—a finding of potential importance regarding adverse effects on the host immune response.

In view of these findings, and since there is evidence sug-

gesting that tumors can be rejected by infiltrating CTL, we analyzed the effect that IL-10 pretreatment of tumors had on their sensitivity to MHC class I-restricted CTL. Our results show that pretreated melanoma and human B cell lines were impervious to otherwise lethal CTL—a phenomenon paralleled by a decrease in target cell surface MHC class I levels. In contrast, susceptibility to NK cell-mediated cytotoxicity was, at times, slightly enhanced.

Materials and Methods

Production of Melanoma Cell Lines and Tumor- and Allo-specific CTL Lines. Human metastatic melanoma specimens, resected at the Karolinska Hospital Department of Oncology, were processed by enzymatic digestion as described (12). The melanoma line 397 Mel, kindly provided by Dr. Paul Robbins (National Institutes of Health, Bethesda, MD) was used as an HLA-A2.1 negative control.

To produce a tumor line, adherent cells from the surgical spec-

imens were cultured in complete RPMI (5% FCS, 200 mM L-glutamine, 100 U penicillin/ml, and 100 µg/ml streptomycin). The tumor cells were analyzed by staining with the mAb anti-S-100/horseradish peroxidase (HRP) and anti-human-melanoma/HRP clone HMB45 (DAKO, Glostrup, Denmark). All tumors were typed, using the mAb HB 54 for the presence of HLA-A2.1, by FACScan® analysis (Becton Dickinson & Co., Mountain View, CA).

Tumor-specific CTL lines were obtained essentially as described (13). Single cell suspensions from metastatic lymph nodes were cultured in serum-free medium (AIM-V, Gibco, Paisley, UK), supplemented with 10 IU human rIL-2/ml (kindly supplied by Dr. Paul Simon, DuPont, Glenolden, PA). The cultures were stimulated every 10–14 d with irradiated tumor cells (one tumor cell to 20 T cells) by the appropriate autologous melanoma cell line. Cytotoxic activity against the K562 and HLA-A2.1⁺ lymphoblastoid cell lines (LCL) was used to assess specificity. CTL clones were obtained from a line established from a patient (KH) by standard limiting dilution. Allo-specific CTL lines were produced as described above for the production of tumor-specific lines, but PBMC of healthy HLA-A2.1⁻ donors and irradiated allogeneic HLA-A2.1⁺ PBMC were used as responder and stimulator cells, respectively.

Treatment of Tumor Cells with rIL-10. Melanoma or LCL were cultured in AIM-V serum-free medium containing 50–200 U human rIL-10/ml (sp act: 10⁷ U/mg; DNAX, Palo Alto, CA; a kind gift from Dr. A. Ogara) for 2–3 d, unless otherwise indicated. Control cultures in AIM-V medium alone were set up in parallel. The rIL-10-treated tumor cells were extensively washed during ⁵¹Cr labeling (which was performed before exposure to CTL effector cells). As a specificity control for the effect of rIL-10, medium containing rIL-10 (100 U/ml) was immunoadsorbed to beads (Dyna-beads; Dynal, Oslo, Norway) coated with the anti-human IL-10

mAb 19F1 (American Type Culture Collection [ATCC], Rockville, MD) (14), or with control beads without this mAb.

mAbs and FACS® Analysis. The antibodies W6/32 (antiononpolymorphic part of HLA class I), and HB 54 (anti-HLA-A2.1) were obtained from the ATCC. The mAb specific for CD28 (9.3) was kindly provided courtesy of Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). The HB 54 and the 9.3 mAbs, purified via protein-A, were used at a concentration of 5 µg/ml when added to cytotoxicity assays in blocking experiments. The antibodies LB-2 (intercellular adhesion molecule 1 [ICAM-1]) and BB-1 (B7) were purchased from Becton Dickinson & Co. Cells treated with mAbs were stained with a secondary FITC-coupled rabbit anti-mouse Ig (Dakopats, Glostrup, Denmark), and analyzed using a FACScan® flow cytometer (Becton Dickinson & Co.).

Cytotoxicity. A 6 h ⁵¹Cr-release assay was used to measure cytotoxic activity of CTL or NK cells against melanoma, LCL, or K562 cells. All samples were run in triplicate. Specific lysis was calculated according to the formula: percent specific lysis = 100 × [(experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm)].

Results

We studied the effect of rIL-10 pretreatment on the sensitivity of human melanoma cells to lysis by autologous tumor-specific CTL lines derived from tumor infiltrating lymphocytes (TIL). These CTL showed partial cross-reactivity against other HLA-A2.1⁺ melanoma targets, but did not kill autologous LCL cells (data not shown). In addition, cytolytic activity of these lines was blocked by anti-HLA-A2.1 mAb,

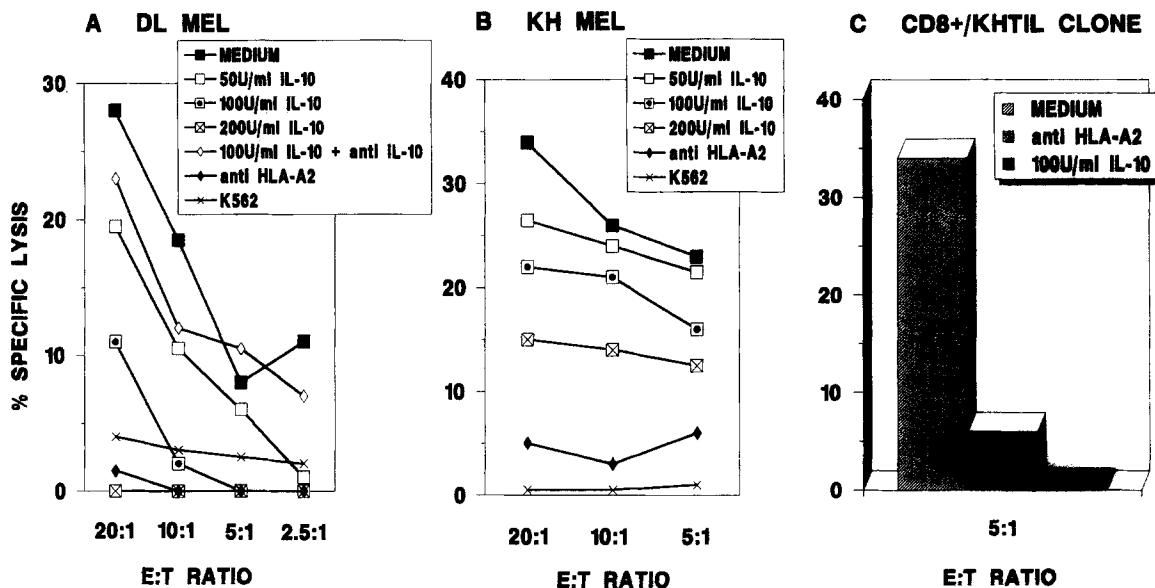


Figure 1. IL-10 pretreatment renders melanoma cells insensitive to lysis by tumor-specific CTLs. (A and B) Tumor-specific CTL lines, restricted by HLA-A2.1 as inferred from blocking by addition of the mAb HB 54, were generated from patient DL (A) and KH (B). CTL-mediated cytotoxicity against pretreated autologous melanoma lines (48 h in medium alone or in medium containing 50, 100, or 200 U/ml or rIL-10), was assessed by 6-h ⁵¹Cr-release assay. The effect of adsorbing IL-10 from medium containing 100 U/ml with beads coated with a mAb specific for IL-10 (19F1) before the incubation with melanoma targets is shown. The NK target K562 was included as a control. One representative experiment of three for each CTL target combination is shown. (C) A CD8⁺ CTL clone was isolated from KH and tested against autologous melanoma cells which were either untreated or pretreated with rIL-10 (100 U/ml for 48 h), or with an anti-HLA-A2.1 mAb (5 µg/ml) included during the assay. One representative experiment out of two is shown.

and they showed either no or only very marginal NK activity against the NK target K562 (Fig. 1, A and B). After rIL-10 pretreatment for 48 h, but not for 24 h or less (data not shown), a dose-dependent decrease in the susceptibility of DL and KH melanoma cells to lysis by autologous CTL was seen. Inhibition of cytotoxicity increased as a function of dose from 50 to 100%, with maximum inhibition at the highest tested dose of 200 U rIL-10/ml (Fig. 1, A and B). IL-10 preincubation did not effect the viability of the melanoma cells, as indicated by unaltered spontaneous release of ^{51}Cr and by trypan blue exclusion. Removal of the rIL-10 from the preincubation medium with an anti-IL-10-specific mAb abrogated a large part of the tumor-protective effect (Fig. 1 A). Although an HLA-A2.1-restricted CD8⁺ T cell clone, isolated from the KH line, was unable to lyse rIL-10-pretreated autologous melanoma cells, it was highly cytotoxic against nontreated tumor cells (Fig. 1 C). This obser-

vation excluded the possibility that the presence of a low number of CD4⁺ cells in the polyclonal CTL line was necessary for IL-10-mediated tumor cell protective effects, and confirmed that CD8⁺ cells were responsible for the observed cytotoxic activity.

The question of whether rIL-10 would also protect target cells of hematopoietic origin from class I-restricted CTL-mediated lysis was investigated by using EBV-transformed LCL as target cells. An allo-specific CTL line, which was partly inhibited by addition of a mAb specific for HLA-A2.1 but not with a mAb against CD28, was used for this purpose. Preincubation of LCL (KH and GA) with rIL-10 for 48 h (100 U/ml) resulted in decreased sensitivity to allo-specific CTL. The magnitude of this effect was similar to that seen with melanoma-specific cytotoxicity (data not shown).

We also investigated whether IL-10 preincubation would protect target cells from lysis by MHC-unrestricted NK cells,

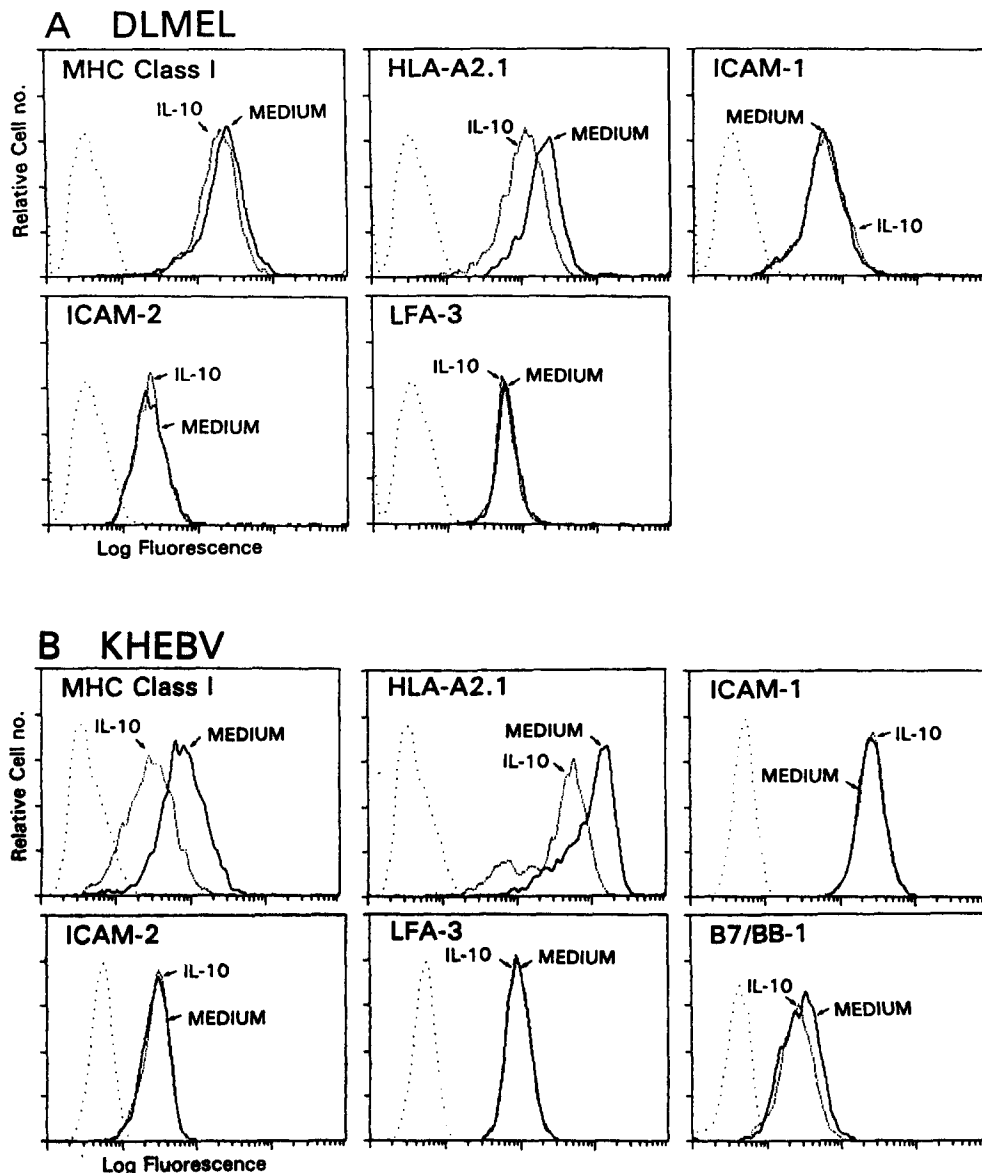


Figure 2. Surface expression of MHC class I is reduced by IL-10 pretreatment. DL melanoma cells (A) and KH LCL (B) were cultured in 100 U/ml of rIL-10 for 48 h, or in medium only, and analyzed by FACScan[®] after indirect staining with mAbs against a monomorphic determinant of HLA class I (W6/32), against HLA-A2.1 (HB 54), the adhesion molecules ICAM-1 (LB-2), ICAM-2 (CBR-IC2/2), LFA-3 (TS 2/9) or the B7 (BB-1) accessory molecules, as indicated.

and from IL-2-activated NK cells with LAK activity. Either no effect or sometimes a slight increase in the susceptibility to lysis by purified allogeneic NK cells or IL-2-activated killer cells was seen after preincubation of the KH LCL with 100 U rIL-10/ml for 48 h. This treatment failed to alter the susceptibility of K562 cells to either NK or IL-2 activated killer cell-mediated cytotoxicity (data not shown).

As the activity of both the tumor- and the allo-specific CTL lines was MHC class I restricted, we tested the possibility that the observed effect of IL-10 on CTL lysis would correlate with decreased cell surface expression of these proteins. Flow cytometric analysis, by indirect staining with mAb against HLA-A2.1 or mAb against nonpolymorphic determinants of MHC class I, of IL-10-pretreated melanoma and LCL revealed a 20–50% decrease of HLA-A2.1, and total MHC class I expression (Fig. 2). This decrease appeared to be more pronounced for HLA-A2.1 than total MHC class I expression (Figs. 2 and 3), and was dose dependent (Fig. 3 A). We noted that although pretreated melanoma cells (with 200 U IL-10/ml) were often totally protected from lysis by tumor-specific CTL, they retained 50% or more of their surface MHC class I (e.g., melanoma DL Fig. 1 A versus Fig. 3 A). It is notable that time-kinetic analysis showed that shorter time periods (<24 h) of exposure to rIL-10 was not protective (shown for the KH LCL in Fig. 3 B). In addition, after >5 d of culture, MHC class I levels returned to normal, perhaps because of consumption of the IL-10. No alterations in cell-surface expression of the adhesion molecules ICAM-1

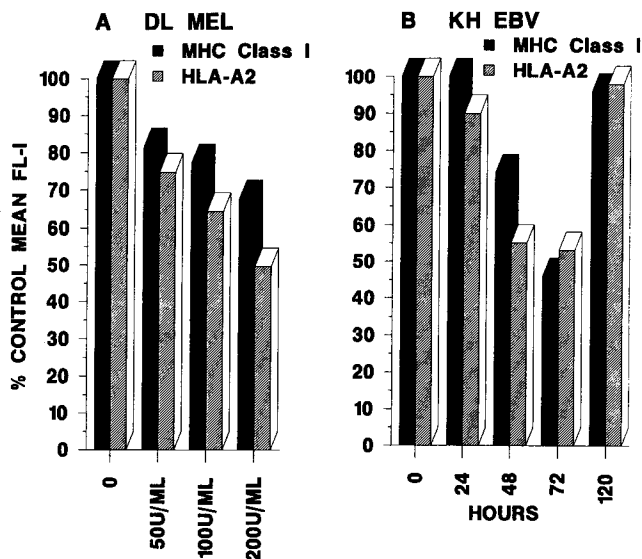


Figure 3. Dose dependency and time-kinetic analysis of the rIL-10 induced downregulation of MHC class I expression. (A) DL melanoma cells were cultured in medium containing the indicated doses of rIL-10 for 48 h, or in medium alone, and analyzed by FACSscan® after indirect staining with mAbs against the monomorphic determinant of HLA class I (W6/32) or against HLA-A2.1 (HB 54). The same melanoma cells used in this experiment were also used in cytotoxicity assays shown in Fig. 1 A. (B) An EBV-transformed B cell line from patient KH was cultured in rIL-10 (100 U/ml) for the indicated time intervals and tested for expression of MHC class I or HLA-A2.1.

(LB-2), ICAM-2 (CBR-IC2/2), or LFA-3 (TS 2/3) on either melanoma or LCL was seen as a result of IL-10 preincubation (Fig. 2).

It was recently shown that IL-10 inhibited macrophage costimulatory activity by selectively inhibiting the upregulation of B7 expression (4). To initiate the investigation of alternative mechanisms for IL-10-mediated protective effects, we examined B7 levels on the KH LCL target after incubation with rIL-10 for 3 d. A marginal decrease in B7 levels was observed (Fig. 2 B). Anti-CD28 mAb did not block lysis (data not shown), indicating that lysis of the KH LCL by allo-specific CTL was not dependent on CD28-B7 interaction, and B7 was not expressed by our melanoma lines (data not shown). It therefore appeared unlikely that downregulation of B7 expression was responsible for decreased sensitivity to CTL-mediated lysis.

We also tested the effect of IL-10 pretreatment of tumor cells in cold target inhibition assays. No significant differences were found between IL-10 pretreated and untreated autologous DL melanoma cells in their ability to inhibit specific lysis, with both types of competitors giving a comparable degree of dose-dependent inhibition (Fig. 4). This “cold target competition” was, as expected, HLA-A2.1 restricted and antigen specific, since an HLA-A2.1⁺ melanoma and an HLA-A2.1⁺ LCL did not inhibit cytotoxicity, regardless of whether these had been pretreated with rIL-10. These results therefore argue for the possibility that IL-10-treated target cells effect postbinding events involved in CTL-mediated lysis, and further argue against IL-10-induced changes in nonspecific adhesion molecules as the explanation of the observed protection.

Discussion

IL-10 has been shown to suppress the induction of T cell responses by acting at several levels including inhibition of monocyte MHC class II expression, upregulation of accessory molecules, and blocking of monocyte-dependent T cell

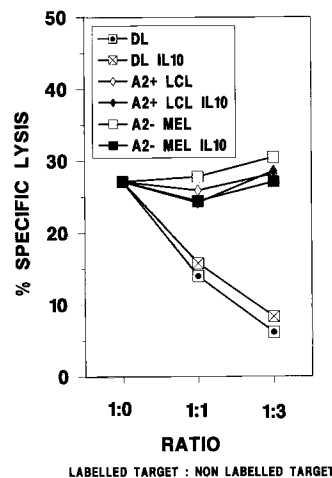


Figure 4. Unlabeled rIL-10-treated tumor cells are not superior inhibitors of melanoma-specific cytotoxicity. Unlabeled tumor cells were added to a constant number of ⁵¹Cr-labeled DL melanoma targets, and the CTL line from DL was used as effector cells at an E/T ratio of 30:1. As unlabeled “cold” competitors, the DL melanoma, the HLA A2.1⁺ LCL from patient KH, and the HLA A2.1⁻ melanoma (397 Mel), cultured with rIL-10 (100 U/ml) or with medium only for 3 d, were used at the ratio of labeled DL to cold competitor shown. When rIL-10-treated DL cells were labeled and used as targets, they showed a 45% decrease in sensitivity to lysis in the same experiment (data not shown).

proliferation (2–4). We found that this cytokine also renders target cells insensitive to MHC class I-restricted tumor- and allo-specific CTL lysis. This phenomenon was initially observed on melanoma targets, but the observation that EBV-transformed B cell lines are also protected by lysis by CTLs shows that IL-10 exerts this effect on cells of hematopoietic origin as well. This is in contrast to the inhibitory effect of IL-10 on MHC class II expression previously shown to be effective on monocytes but not on LCL (3).

The inhibition of CTL lysis induced by rIL-10 was associated with a moderate decrease in the expression of HLA class I, which to our knowledge, is the first example of a cytokine with this effect. A small but detectable decrease in MHC class I expression was reported after culture of normal mouse B cells with rIL-10 for 22 h (15), although this effect was not further analyzed by the authors. In our study, 2–3 d of *in vitro* culture with rIL-10 under serum-free conditions was required to detect a clear reduction in MHC class I levels, which might explain why others have not observed this effect. Alternatively, only melanoma and transformed B cells, as used in our study, but not normal B cells (15), are sensitive to this effect.

Our findings suggest that production of IL-10 by tumor and/or by tumor-infiltrating host cells might serve as a mechanism by which tumor progression occurs in the face of host CTL that are potentially lytic for tumor cells. Other immunosuppressive mechanisms acting locally within the tumor as well as in the peripheral blood of patients with human colorectal carcinomas include recently described alterations in signal-transducing ζ chains of CD3 and CD16 molecular complexes on T and NK cells (16). The possibility that locally secreted cytokines within tumors contribute to downregulation of lymphocyte functions is presently under investigation.

A variety of human solid and hematopoietic human tumor lines, including 38% of melanoma lines and 70% of colon carcinomas, were previously reported to produce IL-10 (9). Furthermore, IL-10 mRNA was detected in biopsies from ovarian carcinomas (10) and in freshly excised melanoma metastases (17). In addition, high levels of IL-10 were found in sera and ascites of patients with ovarian carcinomas (11). The ubiquitous expression of IL-10 in human tumors might therefore contribute to the often observed downregulation of MHC class I expression in tumors.

The finding that rIL-10-pretreated tumor cells were not superior cold target inhibitors than untreated cells tend to exclude the possibility that adhesion molecules involved in

initial effector/target binding are affected by rIL-10—a conclusion also supported by our FACScan[®] analysis of adhesion molecules. This experiment also argues against the possibility that IL-10 induces the secretion of rapidly acting factors that block CTL activity. Therefore, a mechanism involving changes within the antigen presentation machinery of the target cells appears likely. The unaltered or even increased susceptibility to NK lysis of pretreated targets is consistent with this proposal.

The total abrogation of CTL sensitivity in the presence of significant residual levels of class I antigens argues against a decrease in MHC class I expression on the tumor targets as the sole mechanism responsible for resistance to CTL lysis. One possibility would be that HLA molecules on the surface of IL-10-treated tumor cells may not contain the relevant T cell epitope in adequate amounts to trigger CTL lysis. Thus, investigations on the effect of rIL-10 on the TAP-1 and -2 molecules as well on the proteasome complex are presently in progress. Perhaps reduced levels of MHC class I antigens arise as an indirect consequence of an inhibition of the antigen processing machinery, leading to a deprived pool of peptides necessary for MHC class I assembly and cell surface expression. A posttranscriptional regulation of MHC class I expression has been postulated to explain the downregulation of these molecules in cervical carcinomas (18). The downregulation of peptide-transporter molecules TAP-1 and -2 accompanied by loss of class I MHC expression as recently reported *in situ* in cervical carcinomas and in small cell lung carcinoma cell lines is consistent with this proposal (19, 20).

As IL-10 did not affect and even slightly enhanced tumor sensitivity to lysis by NK cells, expression of IL-10 may have the opposite effect on NK-mediated tumor surveillance—inhibiting rather than promoting tumor growth. The previously observed finding of reduced growth of tumors transfected with an IL-10-expressing construct in T cell-deficient SCID and nude mice, known to have high NK activity, is compatible with this possibility (21). Further studies in animal models aimed at dissecting the effect of IL-10 on the different components of anti-tumor responses therefore seem worthwhile pursuing. Recently, high levels of IL-10 production *in vivo* were found to be associated with tolerance in SCID patients transplanted with HLA-mismatched hematopoietic stem cells (22). Our finding that IL-10 also protects target cells from allo-specific CTL might therefore have a wider implication in relation to tolerance to HLA-mismatched allografts.

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