Identification of heme oxygenase-1–specific regulatory CD8+ T cells in cancer patients

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Treg deficiencies are associated with autoimmunity. Conversely, CD4+ and CD8+ Tregs accumulate in the tumor microenvironment and are associated with prevention of antitumor immunity and anticancer immunotherapy. Recently, CD4+ Tregs have been much studied, but little is known about CD8+ Tregs and the antigens they recognize. Here, we describe what we believe to be the first natural target for CD8+ Tregs. Naturally occurring HLA-A2–restricted CD8+ T cells specific for the antiinflammatory molecule heme oxygenase-1 (HO-1) were able to suppress cellular immune responses with outstanding efficacy. HO-1–specific CD8+ T cells were detected ex vivo and in situ among T cells from cancer patients. HO-1–specific T cells isolated from the peripheral blood of cancer patients inhibited cytokine release, proliferation, and cytotoxicity of other immune cells. Notably, the inhibitory effect of HO-1–specific T cells was far more pronounced than that of conventional CD4+CD25+CD127– Tregs. The inhibitory activity of HO-1–specific T cells seemed at least partly to be mediated by soluble factors. Our data link the cellular stress response to the regulation of adaptive immunity, expand the role of HO-1 in T cell–mediated immunoregulation, and establish a role for peptide-specific CD8+ T cells in regulating cellular immune responses. Identification of potent antigen-specific CD8+ Tregs may open new avenues for therapeutic interventions in both autoimmune diseases and cancer.

Introduction

The immune system responds vigorously to invading pathogens (nonself) while remaining unresponsive (tolerant) to the body's own components and circulating constituents (self). This tolerance to self components is the result of finely orchestrated events of thymic negative selection of potentially autoreactive T cells combined with mechanisms operative in the periphery, which act to control the activity of autoreactive T cells that escaped negative selection. Thus, autoreactive cells are present, and control of autoimmune reactions is based on both soluble and cellular mechanisms. In the case of the latter, a network of Tregs exists to downregulate immune responses in various inflammatory circumstances and ultimately assure peripheral T cell tolerance (reviewed in ref. 1). The best-characterized subset of these immune suppressive cells is naturally occurring Tregs, which is a unique subpopulation of CD4+ T cells that express CD25 (IL-2 receptor α chain) on the cell surface and the transcription factor forkhead box P3 (FoxP3) (2, 3). The importance of these FoxP3-positive Tregs in protection from severe autoimmune reactions is underscored by the fact that genetic defects in the *Foxp3* gene are associated with autoimmune disorders in mouse as well as humans (4, 5).Other molecules, including the glucocorticoid-induced TNFR family–related gene (*GITR*) (6), lymphocyte activation gene-3 (*LAG3*) (7), and CTL antigen-4 (CTLA-4) (4), have been used as markers for naturally occurring Tregs. More recently, low expression of CD127 (IL-7 receptor α chain) was described as a useful marker to define pure populations of these cells (8, 9). So-called CD8+ suppressor T cell–mediated regulation of immune responses was originally described in the early 1970s by Gershon et al. (10). These suppressor T cells are now commonly termed CD8+ Tregs, though the knowledge of CD8+ Tregs in general remains scarce compared with that of CD4+ Tregs. Several cell-surface molecules have been associated with CD8+ Tregs in different experimental systems. Many human and rodent CD8+ Tregs have been shown to express cell-surface markers characteristic of activated T cells, e.g., CD8+CD122+, CD8+CD25+, CCR7+CD45RO+CD8+, and CD8+CD45RC^{lo} Tregs (11–13). Expression of Foxp3 has also been shown to be present in CD8+ Tregs (12, 14). Like CD4+ Tregs, CD8+ Tregs contribute to immunoregulation. It is thought that naive CD8+CD25– cells can differentiate into CD8+ Tregs in the presence of antigen (15). The deficiency and/or altered function of Tregs is associated with autoimmunity (16). Hence, CD8+ Tregs have been associated with disease protection and recovery from EAE in rodents (17–19), and their dysfunction has also been implicated in the regulation of autoimmune diseases in humans, including inflammatory bowel disease and multiple sclerosis (20, 21). Furthermore, recent studies demonstrate that tumor cells can recruit these Tregs to inhibit antitumor immunity in the tumor microenvironment, thereby limiting the efficiency of immune surveillance and anticancer immunotherapy (11, 22–24). Tregs suppress the activity of effector T cells in different ways (25): expression of negative costimulatory molecules (26), induction of antiinflammatory biochemical pathways in effector T cells and APCs, direct or indirect killing of effector cells and APCs, the consumption of proinflammatory cytokines such as IL-2, or the production of immunoregulatory cytokines, such as IL-10, TGF-β, or IL-35 (2, 27–29).

Whether TCR-mediated signals are relevant for Treg function is still a matter of debate. Although the generation and maintenance of Tregs is believed to require the presence of the appropriate target antigens (30, 31), the identity of Treg-specific antigens

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: BC, breast cancer; FoxP3, forkhead box P3; HO-1, heme oxygenase-1; MM, malignant melanoma; PBL, peripheral blood lymphocyte; RCC, renal cell carcinoma; RT, room temperature; TAA, tumor-associated antigen.

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remains unknown. The thymic signals that confer lineage specificity have not been fully determined; however, it is known that at least some naturally occurring Tregs develop in the thymus (32), at least in the mouse. Thus, it can be hypothesized that explicit Treg-specific antigens do exist. In the present study, we tested the hypothesis that Treg-specific antigens are derived from proteins that are expressed in the late phase of inflammatory reactions and that contribute in a critical manner to inhibiting or terminating inflammation. Heme oxygenase-1 (HO-1) has such a vital immune protective effect (33). HO-1 is essential for the removal of heme, a potent prooxidant and proinflammatory agent. In addition, all 3 metabolites resulting from heme degradation by HO-1 (i.e., CO, ferrous iron, and biliverdin) have an immune protective effect (34). HO-1 plays an important role in modulating immune reactions implicated by various T cell subpopulations (reviewed in ref. 35). HO-1 deficiency in mice results in strongly increased generation of proinflammatory cytokines, e.g., IL-1β, IFN-γ, TNF, and IL-6 (36). HO-1–deficient mice develop progressive inflammatory diseases, such as splenomegaly and leukocytosis (34). Furthermore, HO-1 is extensively expressed in various tumor cells compared with surrounding healthy tissues and is further increased in response to chemo- or radiotherapy (37). The upregulation of HO-1 in cancer cells combined with its antiinflammatory effects prompted us to determine whether $CD8^+$ Tregs specifically recognizing HO-1 are present in cancer patients. To this end, HO-1–specific, HLA-A2–restricted, $CD8^+$ T cells were detected ex vivo and in situ in high frequencies and — most notably — these cells were able to suppress cell immune responses with outstanding efficacy.

Results

Ex vivo detection of HLA-A2/HO-1–restricted, $CD8^+$ T cells in peripheral blood lymphocytes from cancer patients. First, the amino acid sequence of the HO-1 protein was screened for the most probable HLA-A2 nonamer and decamer peptide epitopes and these peptides were assayed for actual binding as described (2). Hereby we identified a peptide HO212 (QLFEELQEL), which bound to HLA-A2 with the same high affinity as the HLA-A2 high-affinity–binding epitope HIV-1 $pol_{476–484}$ (ILKEPVHGV) (Figure 1A). Next, we evaluated the frequency of HLA-A2–restricted HO212-specific $CD8^+$ T cells by flow cytometry (FACS) in peripheral blood lymphocytes (PBLs) from 11 malignant melanoma (MM) patients, 13 renal cell carcinoma (RCC) patients, and 11 breast cancer (BC) patients using HLA-A2/HO212 pentamers directly after preparation, without any prestimulation step (Figure 1). Figure 1B illustrates an example of an HO212-specific T cell response in an RCC patient with an HLA-A2/HIV-1 $pol_{476–484}$, HLA-A2/HIV-1 $gag_{76–84}$, and HLA-A2 MART-$1_{26–35}$ (which were included for non-MM patients) pentamers serving as negative control stainings. The ex vivo presence of HO212-specific cells in all 3 nonrelated cancer types was significant ($P < 0.0001$) compared with that in healthy individuals. HLA-A2/HO212–pentamers$^+$ cells constituted up to 0.2% of $CD8^+$ T cells (Figure 1C).

Phenotype of HO-1–reactive T cells. We next analyzed the surface markers of the HO-1–specific cells. Ex vivo stainings of HO-1–reactive T cells revealed a rather naive or central memory phenotype. Hence, first PBLs from 5 patients were stained with HLA-A2/HO212 pentamers as well as anti-CD28, anti-CD127, and anti-CD62L. As exemplified in Figure 2A, most of the pentamer$^+$ cells were $CD28^+CD127^+$, whereas in all but 1 patient, most of the specific T cells were negative for CD62L. We further examined the expression of CCR7, CD25, and CD27 as well as CD45RA in 2 patients (Figure 2B). The pentamer-positive cells expressed CCR7 as well as CD27, whereas they were CD25 negative. The CD45RA staining revealed that most of the pentamer-positive cells were CD45RA positive.

The 1 CD62L-positive patient was confirmed by PCR (Figure 2C). Hence, we isolated HO-1–specific T cells by FACS from the patient and confirmed the expression of CD28 and CD127 as well as CD62L. Sorted T cell phenotypes that were negative or positive for the respective phenotypes were used as controls.

Finally, we examined the FoxP3 expression in HO-1–specific T cells isolated from 4 different patients by PCR. The HO-1–specific T cells did not express FoxP3 in any of the patients, as shown in Figure 2C.

Detection of HO-1–reactive T cells in situ. FITC-conjugated HLA-A2/HO212 dextramers were subsequently used to stain acetone-fixed, frozen tissue sections of tumor samples (i.e., MM) and biopsies of inflammatory skin diseases as described previously (17, 18). HLA-A2/HO212–reactive cells were visualized using a confocal laser microscope. The sections were costained with a Cy5-conjugated anti-CD8 antibody. Representative scans are depicted in Figure 3. HLA-A2/HO212–reactive, $CD8^+$ T cells were present in 3 out of 8 tumors. Moreover, in 6 of these patients, the tumor-draining lymph node (i.e., the sentinel lymph node) was also available to test for HLA-A2/HO212 reactivity. To this end, for all patients for whom HLA-A2/HO212–reactive cells could be detected in the primary tumor, the respective sentinel lymph node also harbored HO-1–reactive $CD8^+$ T cells (Figure 3, C and D).

HO212 suppresses IFN-γ release. Since many of the examined cancer patients harbored circulating ex vivo–detectable HO212-specific T cells and we could visualize such cells among the inflammatory infiltrate in tumor lesions of a substantial number of patients in situ, we further examined these cells for their functional activity. To this end, circulating T cells from HLA-A2–positive cancer patients were tested for HO212 peptide–specific IFN-γ production by means of the ELISPOT assay. Despite the significant frequency of HLA-A2/HO212–pentamer$^+$ T cells, we could not detect any IFN-γ production in direct ELISPOT ex vivo (data not shown). Thus, we subsequently performed an indirect ELISPOT, i.e., PBLs were stimulated once with HO212 peptide in vitro before examination by ELISPOT. This procedure was chosen to extend the sensitivity of the ELISPOT as described (38, 39). Again, HO212-specific T cells did not release IFN-γ in response to HO212 peptide in any of the cancer patients (data not shown). Shortening of the in vitro culture time from 7 to 4 days after the addition of IL-2 combined with a higher concentration of IL-2 increased the nonspecific background IFN-γ release (due to in vitro activation) in some cultures. Interestingly, in 8 out of 23 cultures, such nonspecific background IFN-γ release was almost completely inhibited when HO212 peptide was present. This is depicted for 2 patients in Figure 4A, which illustrates the difference in IFN-γ release in cultures with the HO212 peptide compared with cultures with the control peptide from HIV. Prompted by this observation, we determined whether the presence of the HO212 peptide would inhibit antigen-specific T cells. For this purpose, PBL T cells from 5 HLA-A2–positive cancer patients were stimulated once in vitro with either a well-established immunogenic HLA-A2–restricted tumor-associated antigen (TAA), i.e., MART-$1_{27–35}$ (40), Bcl-$X_{L173–182}$ (41), or $Survivin_{96–104}$ (38), alone or in combination with HO212 peptide. ELISPOT analysis revealed that the presence of the HLA-A2–restricted HO-1 peptide HO212

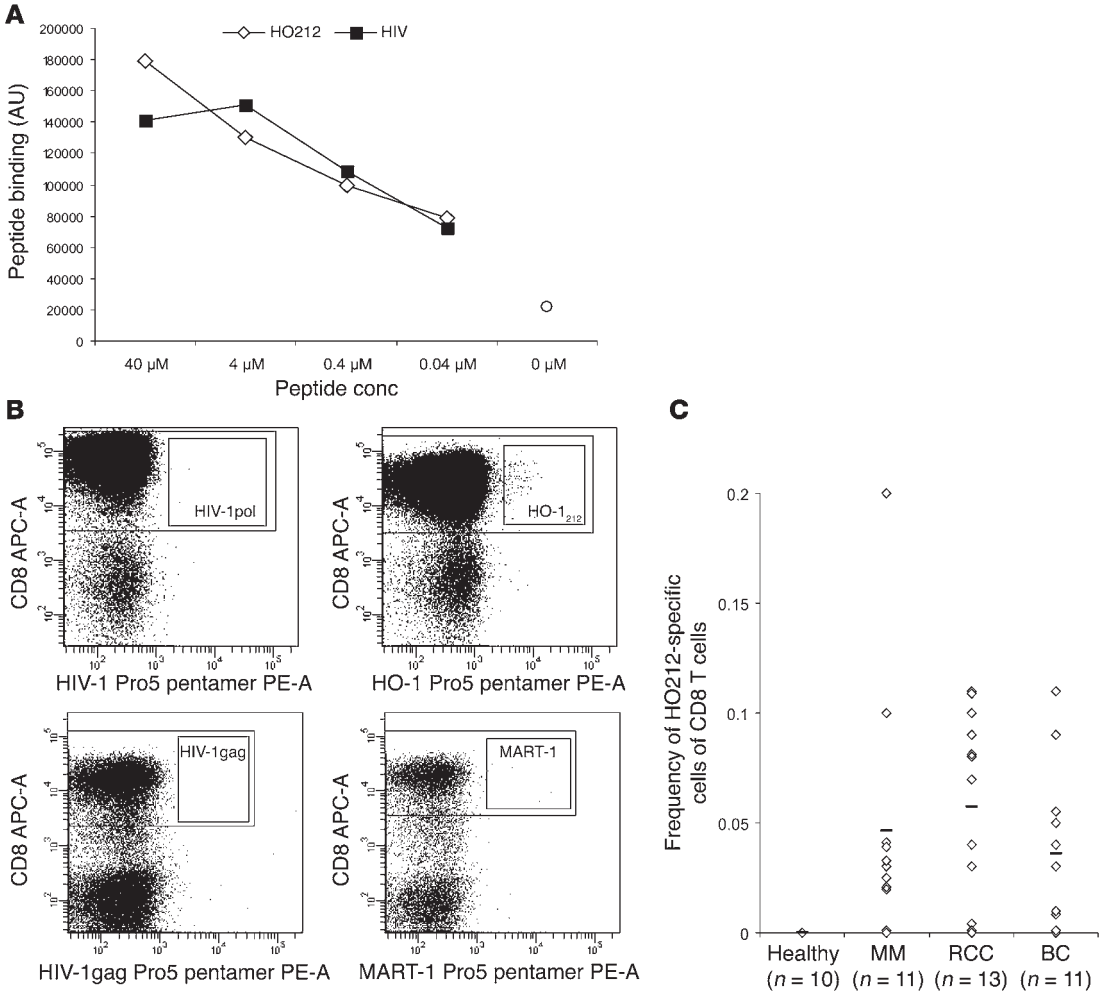

Figure 1

Detection of HO-1–reactive T cells ex vivo. (**A**) Peptide binding to HLA-A2 as examined by the assembly assay. The binding of HO212 (QLFEELQEL) was compared with that of the known HLA-A2–restricted epitope HIV-1 $pol_{476–484}$ (ILKEPVHGV). (**B**) An example of HO212-specific PBMCs from an RCC patient visualized by flow cytometry staining using HLA-A2/HO212–PE pentamer and CD8-allophycocyanin (APC-A). As negative controls, PBMCs from the same patient were stained with CD8-allophycocyanin as well as the following pentamer complexes: HLA-A2/HIV-1 $pol_{476–484}$–PE, HLA-A2/HIV-1 $gag_{76–84}$–PE, and HLA-A2 MART-$1_{26–35}$–PE (non-MM patients). (**C**) PBMCs from healthy donors or from patients with BC, MM, or RCC were stained with the pentamer complex HLA-A2/HO212 and analyzed by flow cytometry ex vivo. Point represent patients, and horizontal bars represent median frequency of specific cells.

during in vitro stimulation almost completely abolished the otherwise robust specific IFN-γ release in response to MART-1, BCL-X_L, or survivin ($P = 0.01$) (Figure 4B).

HO212-reactive T cells inhibit the effector function of tumor specific T cell cultures and clones. Due to the relative high frequency of the HO-1–specific cells, it was possible to isolate HO-1–specific T cells from PBLs from cancer patients using HLA-A2/HO212 pentamers and examine the inhibitory function ex vivo. We used such isolated HO212-reactive T cells in coculture experiments. To this end, 100 HO-1–specific T cells were isolated by FACS from PBLs from a cancer patient and added to 4×10^5 autologous $CD8^+$ cells isolated from PBLs with the MART-$1_{27–35}$ epitope. IL-2 was added and the cells cultured for 7 days before analysis in ELISPOT. The IFN-γ secretion upon peptide-specific stimulation was compared with $CD8^+$ cells cultured under the same conditions but in the absence of HO212-reactive T cells. The ELISPOT assay clearly demonstrated that HO212-reactive T cells efficiently inhibited MART-1–specific IFN-γ release (Figure 5A). Secretion of various cytokines, e.g., IFN-γ, is frequently used as a surrogate marker for activation. Thus, although it has been shown that IFN-γ ELISPOT reactivity in most cases correlates with the capacity to exhibit cytotoxic function, the formal proof for this notion can only be obtained directly. Hence, to extend our observations, we determined whether HO-1–specific T cells could have a direct effect on the functional capacity of peptide-specific T cells in conventional 51Cr-release assays. In this regard, 189 isolated HO212-reactive T cells were added to 3×10^5 cells from a specific T cell bulk culture generated from the same patient that harbored cells that recognized a TAA, i.e., RhoC (42); after a 5-day incubation, the RhoC-specific T cells were analyzed in a conventional 51Cr-release assay using peptide-pulsed T2 cells as target cells. When compared with the lysis of the parental culture (without the addition of HO-1–reactive T cells), the HO212-

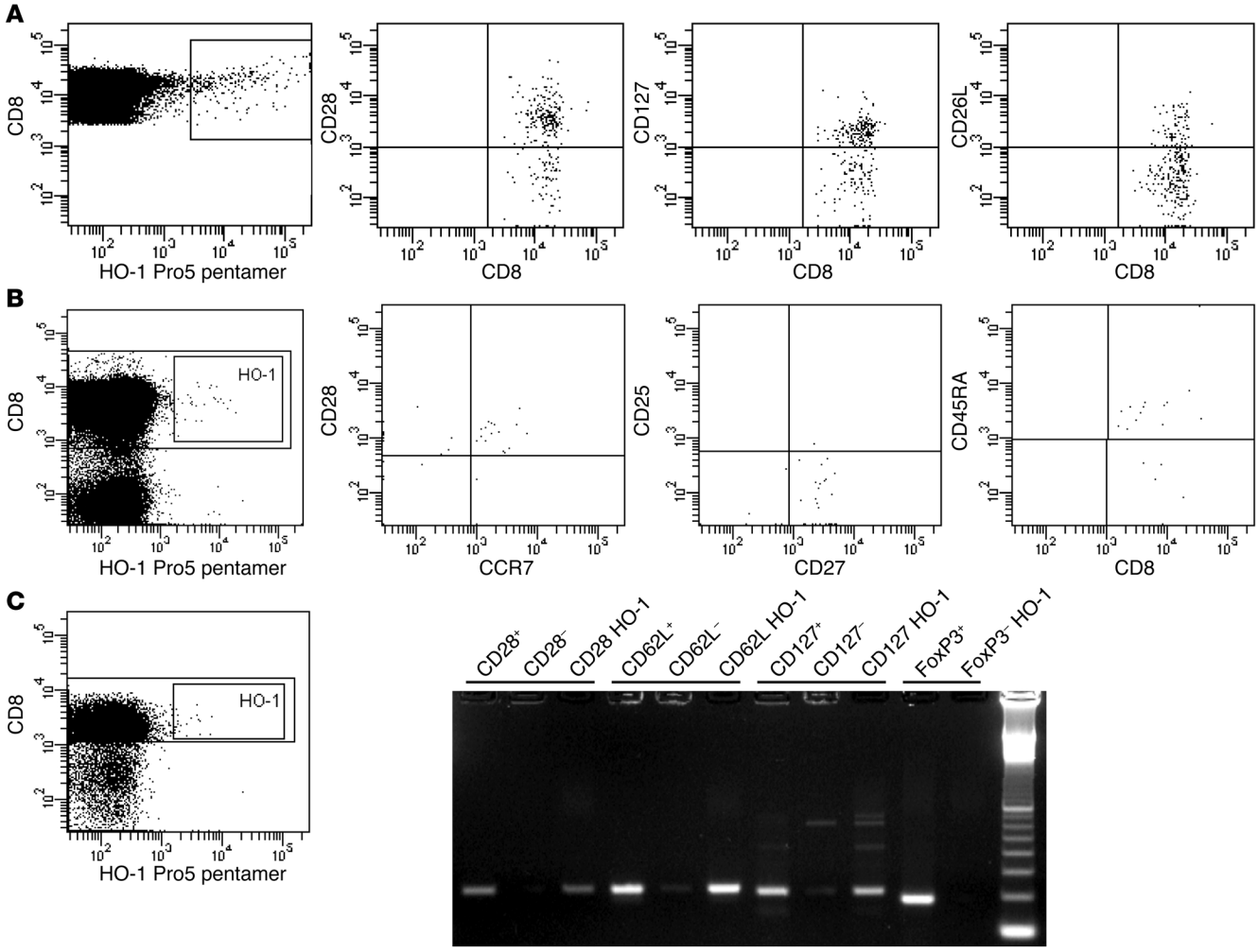

Figure 2

Phenotypes of HO-1–reactive T cells. (**A**) An example of surface expression markers of HO212 pentamer–positive cells ex vivo. The diagrams show CD8, CD28, CD127, and CD62L expression of HLA-A2/HO212–positive cells in PBLs from a cancer patient. (**B**) Diagrams show CD28, CCR7, CD25, CD27, CD8, and CD45RA expression of HLA-A2/HO212–positive cells in PBLs from a cancer patient. (**C**) RT-PCR analyses of HO-sorted cells for expression of CD28, CD62L, CD127, and Foxp3. Sorted T cell phenotypes served as positive (sorted $CD28^+$, $CD62L^+$, and $CD127^+$ cells analyzed in the first lane in each triplet) and negative controls ($CD28^-$, $CD62L^-$, and $CD127^-$ cells analyzed in the second lane in each triplet), and T cells sorted according to their phenotype served as tetramer-sorted HO-1–positive T cells. RT-PCR for expression of Foxp3 is given by analyses of PBMCs and tetramer-sorted HO-1–positive T cells. 100-bp ladder (Invitrogen) is shown to the far right. Aliquots of 6 μl volume were electrophoresed in 2% NuSieve agarose gel and stained with EtBr.

reactive T cells very efficiently inhibited the specific killing of the RhoC-specific T cell line (Figure 5B). Finally, we used the even more robust functional system of established antigen-specific T cell clones to test the inhibitory capacity of HO212-reactive T cells. To this end, the addition of 241 HO212-reactive T cells to 1.5×10^4 cells from a RhoC-specific T cell clone resulted in complete inhibition of killing of the target cells (Figure 5C). Likewise, the addition of HO-1–specific T cells to other TAA-specific T cell clones, i.e., cultures of survivin-specific T cell clones or MART-1–specific T cell clones, inhibited the functional capacity of these clones (data not shown). To determine whether the HO-1–specific T cells had a directly protecting effect on target cells, 433 isolated HO212-reactive T cells were added to 10^5 FM3 MM cells and cultured overnight. The FM3 cells were subsequently used as target cells in a conventional 51Cr-release assay and compared with FM3 cells cultured with 433 $CD8^+$ T cells isolated from the same patient. An autologous MART-1–specific T cell clone lysed almost 25% of the FM3 cells; however, culture with HO-1–specific cells decreased lyses to less than 15% (Figure 5D).

HO212-reactive $CD8^+$ T cells inhibit the proliferation of T cells with higher efficacy than conventional Tregs. The functionality of naturally occurring $CD4^+CD25^{hi}FOXP3^+$ Tregs can be tested via their ability to inhibit T cell proliferation. Thus, in the next series of experiments, we directly compared the inhibitory potential of HO212-reactive T cells with that of naturally occurring Tregs in proliferation assays. It has been demonstrated that $CD4^+CD25^{hi}FOXP3^+$ Tregs can be sorted for functional assays by means of a combination of antibodies directed against CD4, CD25, and CD127 (8), since CD127 expression is inversely correlated with FoxP3 expression and suppressive function of human $CD4^+$ Tregs. We adapted this strategy

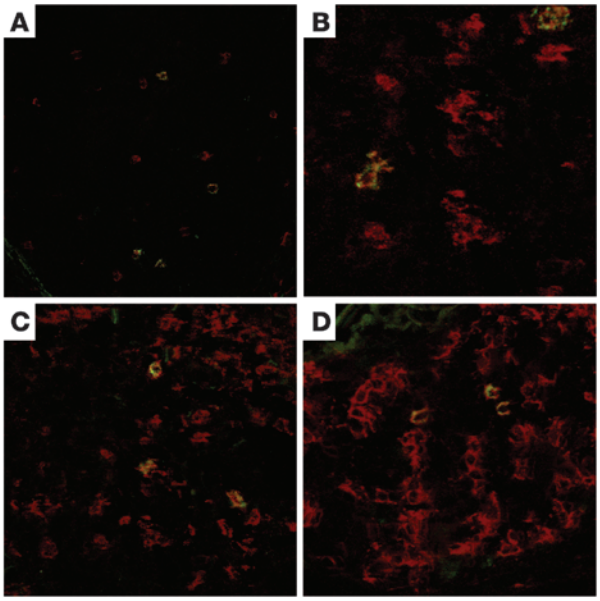

Figure 3
Detection of HO-1–reactive T cells in situ. Frozen tissue sections from primary MM (**A** and **B**) or the respective sentinel lymph nodes (**C** and **D**) were subjected to FITC-conjugated HLA-A2/HO212 dextramers and a Cy5-conjugated anti-CD8 antibody. HLA-A2/HO212–reactive cells appear green, anti-CD8 antibody–positive cells red, and double-positive cells yellow. Original magnification: ×10 (**A**); ×40 (**B**); ×20 (**C** and **D**).

and sorted $CD4^+CD25^{hi}CD127^-$ cells to compare their regulatory potential directly with that of HO212-reactive T cells isolated from the same patients. The suitability of these settings to isolate naturally occurring Tregs was demonstrated by the fact that around 90% of the cells within the $CD4^+CD25^{hi}CD127^-$ gate also expressed FOXP3 (data not shown).

The $CD4^+CD25^{hi}CD127^-$ T cells inhibited the proliferation of activated $CD4^+$ and $CD8^+$ T cells at a regulatory to responder cell ratio of 1:2 by 33% and 49%, respectively (Figure 6). Increasing the regulatory to responder ratio to 1:1 resulted in inhibition of $CD8^+$ T cell proliferation by 75%. Decreasing the ratio to 1:4 almost abolished the inhibitory effect; proliferation of $CD8^+$ cells was only inhibited by 10%. As depicted in Figure 5, addition of the same number of control cells ($CD4^+CD25^-CD127^+$ T cells or CMV-specific $CD8^+$ T cells) did not result in inhibition of proliferation.

When HO212-reactive T cells were tested, they inhibited proliferation of $CD4^+$ T cells by 70% and of $CD8^+$ T cells by 88%. Importantly, this stronger inhibitory effect was already observed at a regulatory to responder cell ratio of 1:100, i.e., only 500 HO212-reactive T cells had to be added to 50,000 responder cells to achieve such an efficient inhibition of T cell activity. Figure 5E illustrates our gating strategy for isolation of $CD4^+CD25^{hi}CD127^-$ cells.

The inhibitory activity of supernatant from HO-1–reactive cells. Next, we determined whether we could detect a known cytokine release from HO-1–specific cells. However, we could not detect either IL-2, perforin, IL-10, or TGF-β release in HO212-stimulated PBL cultures by ELISPOT (data not shown). To determine whether the inhibitory activity of the HO-1–specific cells was indeed mediated by soluble factors, we isolated 200 HO-1–specific T cells from PBLs from a MM patient by FACS and cultured these cells overnight in 100 μl medium. The supernatant was removed and added to an ELISPOT with PBLs from either an RCC patient or another MM patient. An IFN-γ response against a TAA ($Survivin_{96–104}$) was detected in PBLs from both patients in a normal ELISPOT; however, the addition of supernatant to the ELISPOT completely suppressed the TAA-related IFN-γ secretion (Figure 7A). Likewise, we isolated between 200 and 400 specific HO-1 T cells from 3 cancer patients and cultured the cells overnight in 100 μl medium. The 3 different supernatants were added to 3 cultures of a RhoC-specific T cell clone. In 1 of the cultures, we observed that the addition of supernatant completely abrogated the killing by this clone in a chrome release assay (Figure 7B). As a control, we examined the TAA-specific ($MART\text{-}1_{27–35}$ or $Survivin_{96–104}$) IFN-γ release in PBLs from 4 different cancer patients, 2 MM patients (MM50, MM48) and 2 RCC patients (Ur27, Ur25), in ELISPOT performed with either 1 of 2 different HO-1 supernatants or with supernatants from CMV-reactive cells and bulk $CD8^+$ cells. One of the HO-1 supernatants suppressed TAA-specific IFN-γ release in all patients ($P = 0.02$), whereas the other suppressed IFN-γ in 3 out of 4 cultures compared with the control supernatants ($P = 0.04$) (Figure 7C).

Discussion

Tregs are a highly specialized subset of immune cells capable of specifically suppressing autoreactive cells and thereby preventing autoimmunity. In the context of malignancies, however, accumulation of Tregs occurs in the tumor microenvironment and has been associated with prevention of antitumor immunity (43–45). The generation and maintenance of Tregs is believed to require the presence of the right target antigens (30, 31); however, the identity of Treg-specific antigens remains unknown. Besides local production of chemokines that attract Tregs to the site of the tumor (46), ligands or antigens expressed by cancer cells may play a crucial role in the recruitment, maintenance, and expansion of Tregs in the tumor microenvironment, thereby leading to increased numbers of Tregs at the tumor site. It is likely that the same antigenic peptides can stimulate either effector cells or Tregs depending on antigen dose, peptide-MHC avidity, and presence of costimulatory molecules and cytokines (47, 48). Although the thymic signals that confer lineage specificity have not been fully determined, it is known that at least some of the naturally occurring Tregs develop in the thymus (32) (in contrast with thymus-independent peripheral "conversion" of T cells to Tregs); thus, it can be hypothesized that explicit Treg-specific antigens do exist. Based on the data presented within this report, it is likely that such Treg-specific antigens are proteins that are expressed in the late phase of inflammatory reactions to inhibit or terminate inflammation, such as HO-1. The HO-1 protein plays an important role in immune regulation and, importantly, is protective against diseases mediated by effector T cells. Moreover, HO-1 is highly expressed in many tumors of different tissue origin. Initially, we set out to determine whether HO-1 is a target for specific $CD8^+$ T cells in cancer patients. Indeed, we detected a significant number of HO-1–reactive T cells circulating in peripheral blood ex vivo and among tumor-infiltrating lymphocytes in situ of cancer patients. In fact, up to 0.2% of the total $CD8^+$ T cells in circulating blood were specific for a single epitope from HO-1 in cancer patients diagnosed with metastatic BC, RCC, and MM. The frequencies of the HO-1–reactive T cells were noticeably higher than

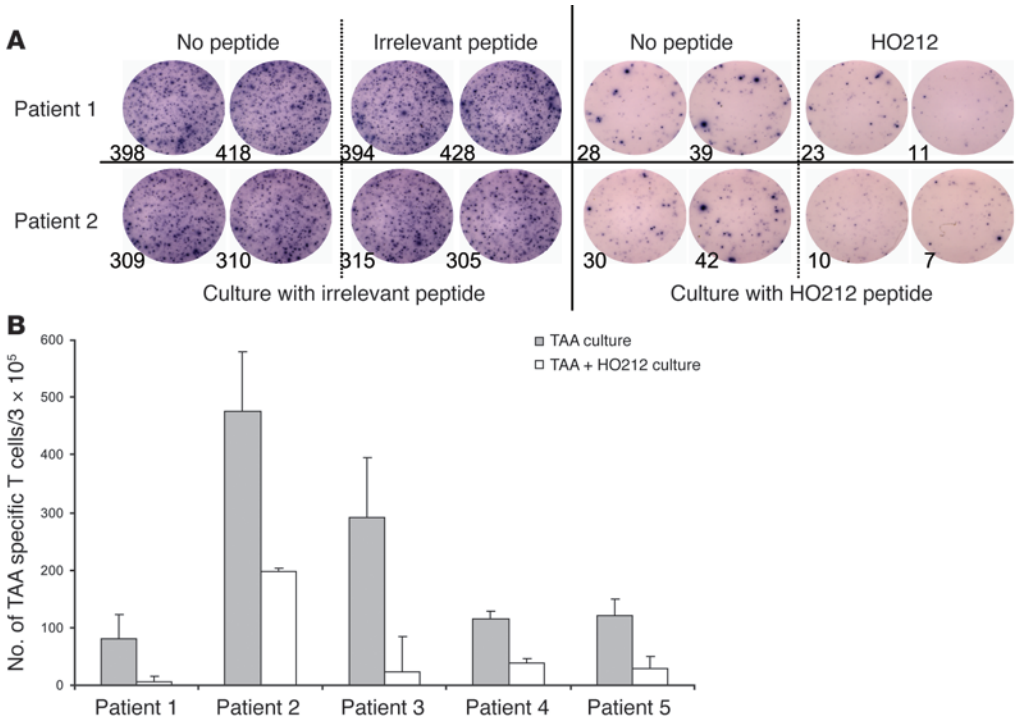

Figure 4

The peptide HO212 suppresses IFN-γ release. (**A**) PBLs from 2 BC patients was stimulated with HO212 peptide or irrelevant peptide (HIV $pol_{476–484}$) as well as IL-2 for a week. Cells that were stimulated with irrelevant peptide were plated at 10^5 cells per well in duplicates either without or with irrelevant peptide (left). Cells that were stimulated with HO212 peptide were likewise plated without or with HO212 peptide (right). Number of IFN-γ spots was counted using the ImmunoSpot Series 2.0 Analyzer (CTL). Number represent counted spots. (**B**) PBL T cells from 3 MM patients (patients 1, 2, and 3) as well as 2 BC patients (patients 4 and 5) were cultured with a TAA, i.e., MART-$1_{27–35}$ (patients 1, 2, and 3), Bcl-$X_{L173–182}$ (patient 4), or $Survivin_{96–104}$ (patient 5) alone or with the same TAA in coculture with HO212 peptide. Cells were plated at 13×10^5 cells per well in triplicate either without or with the relevant TAA peptide. The average number of peptide-specific spots (after subtraction of spots without added peptide) was calculated for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL). Error bars indicate SD.

for previously described TAA. Thus, with very few exceptions, it is not possible to detect TAA-specific T cells in PBLs from cancer patients directly ex vivo without in vitro–peptide stimulation (39). The functional characterization of these T cells revealed that these exerted immune suppression rather than antitumor functions. Thus, HO-1 seems to be immune suppressive per se. We conducted a series of experiments to study the inhibitory function of these cells: already, the presence of the HLA-A2–restricted peptide epitope HO212 in IL-2–activated PBL cultures from cancer patients decreased IFN-γ release of T cells; likewise, coculturing of the HO-1 epitope with the well-established immunogenic epitopes derived from different TAAs, i.e., MART-1, BCL-X_L, or survivin, significantly inhibited the immune response against these. Due to the relative high frequency of the HO-1–specific cells, it was possible to isolate HO-1–specific T cells from PBLs from cancer patients using HLA-A2/HO212 pentamers and examine the inhibitory function ex vivo. This analysis revealed that the presence of HO-1–specific $CD8^+$ T cells inhibited the antigen-specific cytokine release, proliferation, and cytotoxicity of T cells. Notably, these effects were not only observed for freshly isolated bulk cultures but also for antigen-specific T cell lines and clones. Even more importantly, only 500 HO-1–specific $CD8^+$ T cells had a superior suppressive effect on $CD4^+$ and $CD8^+$ lymphocytes compared with between 25,000 and 50,000 conventional $CD4^+CD25^{hi}CD127^-$ Tregs isolated from the same patients.

It seems that the HO-1–specific T cells mediate suppression on both "effector" cells and "target" cells. Hence, HO-1–specific T cells suppressed proliferation of CD8 as well as CD4 after direct stimulation with anti-CD3 antibodies, i.e., without target cell stimulation. Additionally, coculture of HO-1–specific T cells with MM cells rendered the MM cells partially resistant to T cell–mediated killing, suggesting a direct effect on the target cells.

Unfortunately, we have not been able to expand HO-1–specific T cells successfully in vitro, since they apparently need different conditions for growth than conventional $CD8^+$ cytotoxic T cells. This also implies that all assays have been performed with a natural subset of HO-1–specific T cells directly isolated from cancer patients. Naturally, this has complicated our attempts to reveal the phenotype and mechanism of action of these cells. However, the inhibitory activity of HO-1 cells seems at least partly to be mediated by soluble factors, although these cells did not release perforin, IL-10, or TGF-β. Naturally, it cannot be ruled out that cell-to-cell contact might increase the effect of suppression.

HO-1–specific T cells appear to have a rather naive or central memory-like phenotype, a notion corroborated by the fact that they did not release perforin upon activation. It has been suggested that in response to antigen exposure, $CD8^+$ cells switch from naive to memory cells followed by a gradual transformation into an "effector type" (reviewed in ref. 49). Hence, the phenotype is rather surprising, considering the frequency of HO-1 peptide–specific T

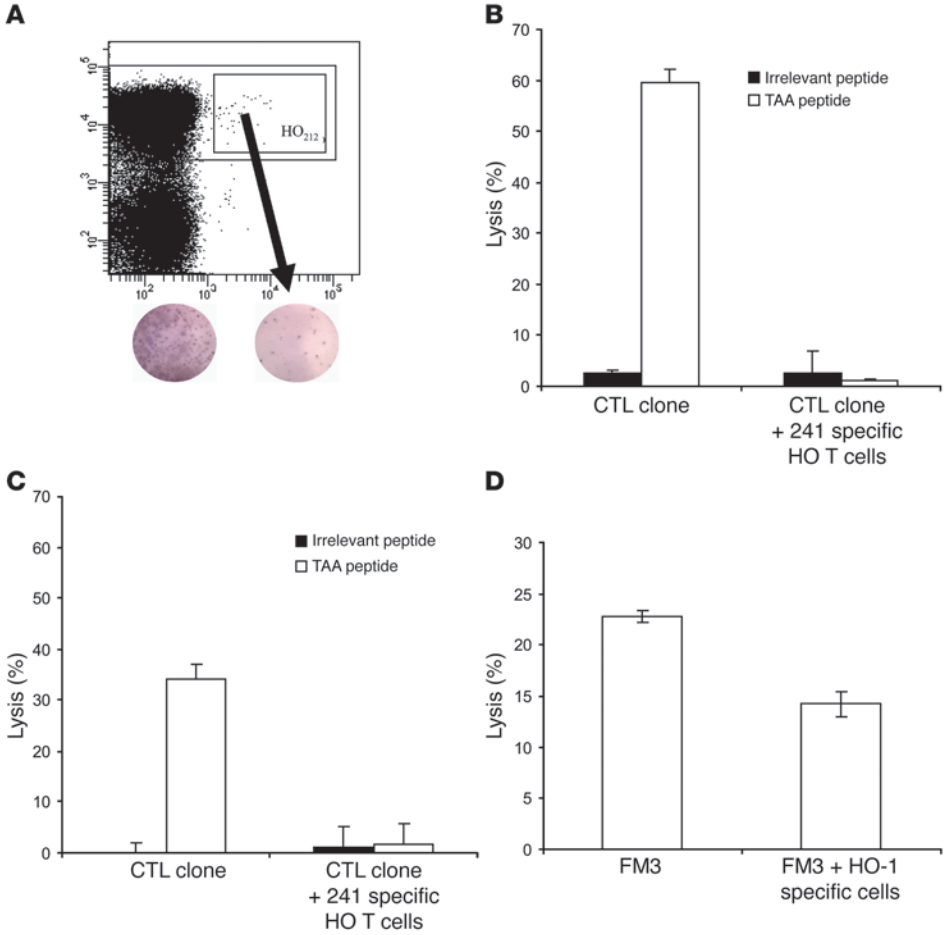

Figure 5

HO212-reactive T cells inhibit effector T cells and protect target cells. (**A**) 4×10^5 $CD8^+$ cells from an MM patient were assayed in ELISPOT for reactivity against the MART-$1_{27–35}$ epitope after culture alone (left ELISPOT well) or after culture with 100 autologous HLA-A2/HO212 pentamer$^+$ T cells (right ELISPOT well) (**B**) 189 isolated HO212-reactive T cells were added to 3×10^5 cells from a specific T cell bulk culture generated from the same patient that harbored cells that recognized an HLA-A3–restricted TAA, i.e., RhoC (RAGLQVRKNK); after a 5-day incubation, the RhoC-specific T cells were analyzed in a conventional 51Cr-release assay using peptide-pulsed T2-A3 cells as target cells (white bars) or unpulsed T2-A3 cells (black bars). The same T cell bulk culture without the addition of HO-1–reactive T cells was used as control. The effector/target ratio was 60:1. (**C**) 241 isolated HO212-reactive T cells were added to 1.5×10^4 cells from a specific T cell clone generated from the same patient that harbored cells that recognized an HLA-A3 restricted TAA, i.e., RhoC (RAGLQVRKNK); after a 5-day incubation, the RhoC-specific T cells were analyzed in a conventional 51Cr-release assay using peptide-pulsed T2-A3 cells as target cells (white bars) or unpulsed T2-A3 cells (black bars). The same T cell clone without the addition of HO-1–reactive T cells was used as control. The effector/target ratio was 3:1. (**D**) FM3 MM cells either cultured with 433 HO-1–specific T cells or $CD8^+$ T cells were used as target cells in a conventional 51Cr-release assay. Effector cells were an autologous MART-1–specific T cell clone. All measurements were made in duplicate. Error bars indicate SD.

cells in the examined patients. Furthermore, HO-1–specific T cells did not express FoxP3 as analyzed by PCR on isolated T cells. Our data suggest that the HO-1–specific $CD8^+$ T cells represent a new subset of Tregs which — at least so far — is characterized by specificity rather than phenotype. Obviously, a more detailed characterization of the phenotype of HO-1–specific T cells could potentially aid sorting of larger populations of $CD8^+$ Tregs with properties similar to those associated with the HO-1–specific T cells described herein. However, as judged by the phenotype data included here based on rather few surface molecules, HO-1–specific T cells appear not to be characterized by a distinct phenotype.

It has been shown that HO-1 expression in conventional $CD4^+CD25^+$ Tregs is significantly higher than that in $CD4^+CD25^-$ T cells (50) and that the induction of FoxP3 and acquisition of regulatory activity in $CD4^+CD25^-$ T cells by activation coincides with the induction of HO-1 expression (51). Hence, it could be speculated that HO-1–specific T cells might — at least in part — function by activating such conventional Tregs. However, since HO-1–specific cells had direct functional impact on $CD8^+$ and $CD4^+$ T cells as well as on target cells, this suggests different mechanisms of action.

It has previously been shown that overexpression of HO-1 may protect tissues and organs from immunological destruction by a local immunomodulatory influence on infiltrating inflammatory cells (50, 52). In murine models, overexpression of HO-1 results in severe suppression of T cell– and NK cell–mediated effector functions (53). The HO-1–specific $CD8^+$ Tregs described here may play a vital role in this suppression. Our results suggest the HO-1–specific $CD8^+$ Tregs might interact specifically

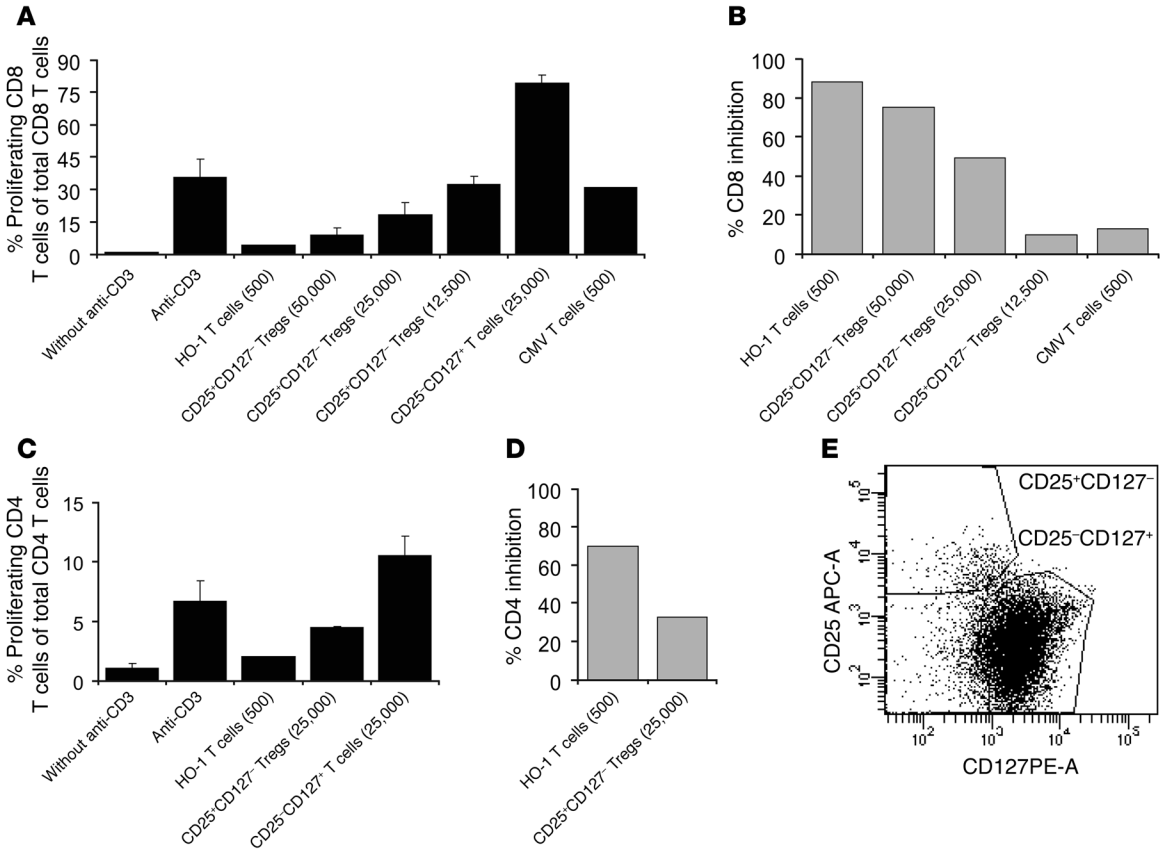

Figure 6

Direct comparison of inhibitory capacity of HO212-reactive CD8+ T cells and conventional Tregs. Fifty-thousand PKH26-labeled autologous PBMCs were cocultured with $CD4^+CD25^{hi}CD127^-$ Tregs, $CD4^+CD25^-CD127^+$ T cells, HO212-reactive $CD8^+$ T cells, or CMV-specific $CD8^+$ T cells for 6 days (cell numbers are indicated in the figure). Cells were stained with anti-CD4 and anti-CD8 antibodies and proliferation was determined by dilution of PKH26. All measurements were made in duplicate. (**A**) Proliferation of $CD8^+$ T cells either without stimulation or after CD3 stimulation alone or in cocultures with conventional Tregs, HO212-reactive $CD8^+$ T cells, or relevant controls. Data depict triplicates ($CD4^+CD25^{hi}CD127^-$ Tregs or $CD4^+CD25^-CD127^+$ T cells) and single experiment (HO212-reactive $CD8^+$ T or CMV-specific $CD8^+$ T cells). Shown are representative results of 2 independent experiments. (**B**) Percentage inhibition of $CD8^+$ T cell proliferation induced by conventional Tregs or HO212-reactive $CD8^+$ T cells. (**C**) Proliferation of $CD4^+$ T cells either without stimulation or after CD3 stimulation alone or i cocultures with conventional Tregs, HO212-reactive $CD8^+$ T cells, or relevant control cells. Error bars indicate SD. (**D**) Percentage inhibition of $CD4^+$ T cell proliferation induced by conventional Tregs or HO212-reactive $CD8^+$ T cells. (**E**) Sorting strategy for $CD4^+CD25^{hi}CD127^-$ Tregs and $CD4^+CD25^-CD127^+$ T cells.

with HO-1–expressing cells at sites of inflammation and veto further $CD8^+$ driven cytotoxicity. Hence, the HO-1–specific $CD8^+$ Tregs described here may be an important part of this effect and play a general role in protecting cells from an immune attack. Although we were not able to detect HO-1–specific T cells in healthy individuals, it seems likely that such cells play a role in controlling inflammation in general, thereby supporting the protective function of HO-1. As HO-1 is a stress-responsive gene, the suppressive effects of HO-1–specific $CD8^+$ Tregs might be restricted to tissues undergoing oxidative stress where cells expressing HO-1 and presumably presenting peptides derived from HO-1 can be found. The interaction of HO-1–specific $CD8^+$ Tregs with HO-1–expressing cells should dampen further $CD8^+$ T cell cytotoxicity, thus limiting "bystander" injury associated with $CD8^+$ T cell responses. This effect is desirable in preventing autoimmunity but may be deleterious in the case of cancer. Thus, the presence of HO-1–reactive $CD8^+$ T cells in the tumor microenvironment must be regarded as an obstacle for the development of effective anticancer immune responses (45). On the other hand, in the clinical setting, the induction of HO-1–specific immune responses could suppress rejection of transplanted organ or graft-versus-host disease after allogenic stem cell transplantation. Similarly, such therapeutic measures could be employed to treat autoimmune diseases.

Recent evidence suggests that expression of HO-1 in DCs may further enhance the tolerogenic effects of HO-1 expression (54). HO-1 expression in DCs inhibits both their maturation and proinflammatory function but conserves their IL-10 expression. The latter is particular important as IL-10 induces HO-1 expression in macrophages (55, 56). Thus, HO-1–expressing APCs may activate HO-1–specific suppressor T cells, thereby boosting suppression of local immune responses. Accordingly, adenoviral transduction of DCs with HO-1 in rats receiving heart transplants resulted in long-term allograft survival associated with an inhibition of cellular antigraft immune responses (57). Furthermore, for human ovarian cancer, it was demonstrated that

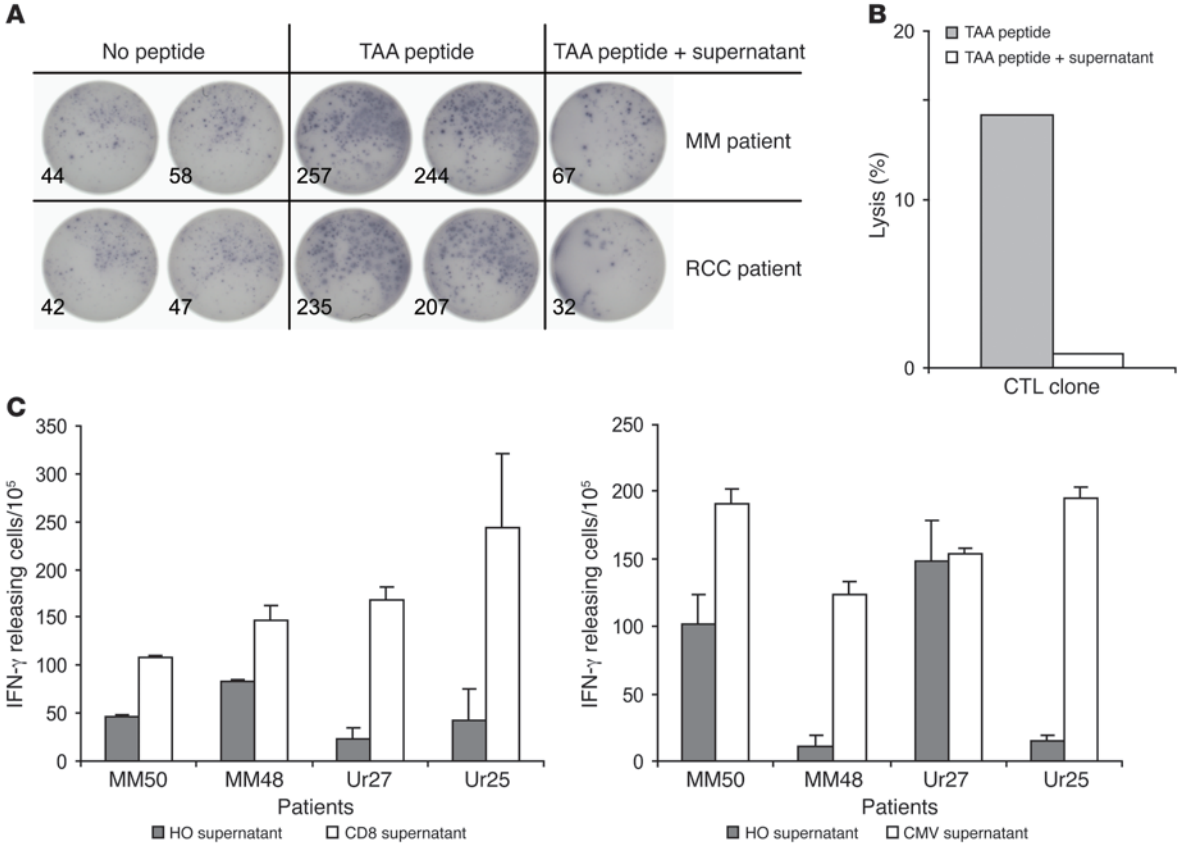

Figure 7

The inhibitory activity of supernatant from HO-1–reactive cells. (**A**) 200 HLA-A2/HO212–pentamer$^+$ T cells were directly isolated by FACS sorting from PBLs from an MM patient and cultured overnight in 100 μl medium. The supernatant was removed and added to an ELISPOT with PBLs from either an RCC patient or another MM patient. The TAA (Survivin$_{96–104}$) was added to the wells. The same ELISPOT was performed with and without TAA in regular medium with no supernatant. Numbers represent counted spots. (**B**) 300 HLA-A2/HO212 pentamer$^+$ T cells were directly isolated by FACS sorting from PBLs from an RCC patient and cultured overnight in 100 μl medium. The supernatant was removed and added to a chrome release assay. The effector cells were a RhoC-specific T cell clone; target cells were T2-A3 cells pulsed with RhoC (RAGLQVRKNK) peptide. The effector/target ratio was 3:1. (**C**) The supernatants from either directly isolated HO-1–specific T cells, CMV-specific T cells, or bulk CD8$^+$ cells were added to an ELISPOT with PBLs from 4 different cancer patients; 2 MM patients (MM50, MM48) and 2 RCC patients (Ur27, Ur25), as well as TAA (MART-1$_{27–35}$ or Survivin$_{96–104}$). All measurements were made in duplicate. Error bars indicate SD.

plasmacytoid DCs induce IL-10–secreting CD8$^+$ Tregs capable of suppressing antitumor immunity through IL-10 (3, 24).

In conclusion, the outstanding capacity of HO-1–specific Tregs to efficiently suppress T cell responses opens new opportunities for therapeutic intervention to modulate immune responses. However, a more detailed understanding of the mechanism(s) of action will be a prerequisite before the full potential of this new class of antigen-specific Tregs can be exploited. Moreover, we believe that the detection of HO-1–specific regulatory CD8$^+$ Tregs may represent a general phenomenon. Hence, it is likely that other proteins of protective function similar to that of HO-1 are targets for naturally occurring Tregs.

Methods

Patients. PBLs were collected from cancer patients (RCC, MM, and BC), and healthy controls. Blood samples were drawn a minimum of 4 weeks after termination of any kind of anticancer therapy. The majority of RCC patients had previously been treated with IL-2 and IFN-α, and most MM patients had received high-dose IL-2 and IFN-α, while all BC patients were pretreated with several kinds of chemotherapy (e.g., epirubicin, docetaxel, capecitabine), trastuzumab, and/or endocrine therapy. PBMCs were isolated using Lymphoprep separation, HLA-typed (Department of Clinical Immunology, University Hospital, Copenhagen, Denmark), and frozen in FCS with 10% DMSO. The protocol was approved by the Scientific Ethics Committee for the Capital Region of Denmark and conducted in accordance with the provisions of the Declaration of Helsinki. Written informed consent from the patients was obtained before study entry.

Assembly assay for peptide binding to MHC class I molecules. The binding affinity of the synthetic peptides (Invitrogen) to HLA-A2 molecules, metabolically labeled with [^{35}S]-methionine, was measured in the assembly assay, as described previously. The assay is based on peptide-mediated stabilization of empty HLA molecules released upon cell lysis from the TAP-deficient cell line T2. Stably folded HLA molecules were immunoprecipitated using the HLA class I–specific, conformation-dependent mAb W6/32 and separated by isoelectric focusing (IEF) gel electrophoresis. MHC heavy-chain bands were quantified using the ImageGauge PhosphorImager program (Fujifilm). The intensity of the band is directly related to the amount of peptide-bound class I MHC recovered during the assay. The C_{50} value was calculated for each peptide as the peptide concentration sufficient for half-maximal stabilization.

Antigen stimulation of PBLs. To determine the ability of HO-1 peptide to suppress IFN-γ release in cultures, PBLs were stimulated with peptide and IL-2 in vitro prior to analysis (58). At day 0, PBLs were thawed and diluted to 2×10^6 cells/well in X-Vivo Medium (BioWhittaker) containing 5% heat-inactivated human serum. These cells were plated at 2 ml/well in 24-well plates (Nunc); 10 μM of the relevant peptide and 40–120 IU/ml recombinant IL-2 (Chiron) were added to the cultures.

ELISPOT assay. The ELISPOT assay was used to quantify peptide epitope–specific effector cells that release cytokine (i.e., IFN-γ, IL-10, TGF-β, or perforin) as described previously (38). In some experiments, PBMCs were stimulated once in vitro with peptide prior to analysis as described (59). In brief, nitrocellulose-bottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated with the appropriate capture mAb (i.e., anti–IFN-γ, anti–IL-10, or anti-perforin mAb; Mabtech) or anti–TGF-β (BD Biosciences — Pharmingen)). The wells were washed and blocked by X-Vivo Medium, and the effector cells were added in duplicate at different cell concentrations, with or without 10 μM peptide. The plates were incubated overnight. The following day, medium was discarded, and the wells were washed prior to addition of the relevant biotinylated secondary Ab. The plates were incubated at room temperature (RT) for 2 hours and washed; avidin-enzyme conjugate (AP-avidin; Calbiochem, Invitrogen) was added to each well. Plates were incubated at RT for 1 hour, and the enzyme substrate NBT/BCIP (Invitrogen) was added to each well and incubated at RT for 5–10 minutes. Upon the emergence of dark purple spots, the reaction was terminated by washing with tap water. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL).

FACS. PBLs were analyzed by flow cytometry using FACSAria (BD Biosciences). The surface expressions of phenotype markers on HO212-specific T cells were analyzed after staining with PE-conjugated HLA-A2 Pro5 pentamers (ProImmune) with HO212 peptide followed by antibody staining with the following fluorochrome-coupled mAbs: 7-AAD–PerCP, CD3-FITC or CD3-APC-Cy7, CD8-APC, CD8-PerCP or CD8-APC-Cy7, CD28-APC or CD28-FITC, CD127-FITC, CD62L-FITC, CD25-APC, CD45RO-FITC, and CCR7-PE-Cy7 (BD Biosciences — Immunocytometry Systems). HLA-A2/HIV-1 $pol_{476–484}$, HLA-A2/HIV-1 $gag_{76–84}$, and HLA-A2 MART-$1_{26–35}$ (which were included for non-MM patients) pentamer stainings were performed as negative controls. Stainings were performed in RPMI 1640 medium (Gibco BRL; Invitrogen) for 20 minutes (4°C for mAb and RT for pentamers) in the dark. Pentamer-positive cells were sorted for direct use in coculture assays.

RNA preparation and reverse transcription–coupled PCR. RNA from FACS-sorted cells was prepared by centrifugation of the cells followed by lysis by freeze/thawing in a 5 to 10 μl volume of first-strand buffer for the cDNA synthesis, carried out in the in the presence of RNAse inhibitor using the manufacturer's instructions for the SuperScript Choice System (Invitrogen). Resulting cDNA was tested using primers for GAPDH (5′-AGGGGGGAGCCAAAAGGG-3′, 5′-GAGGAGTGGGTGTCGCTGTTG-3′, positions 440 and 980 in NM_002046.3, respectively; product size, 541 bp). Primers suited for amplification were as follows: CD28 (5′-CAACTGTGATGGGAAATTGGG-3′; 5′-CACCACCAGCACCCAAAAG-3′, positions 474 and 678, respectively, in access NM_006139; product size, 223 bp), CD62L (5′-CCAGCTTCAGCTTTACCTCTGC-3′; 5′-GATAAATGCCAACCCAGAGAATG-3′, positions 1028 and 1229, respectively, in NM_000655; product size, 222 bp), and CD127 (5′-CCCAGTCTCCCCGATCATAAG-3′; 5′-CATCCCCTCCAAGCCTCTG-3′, positions 909 and 1101, respectively, in NM_002185; product size, 211 bp).

Amplifications were carried out in a total volume of 15 μl containing 1× PCR buffer (50 mM KCl, 20 mM Tris, pH 8.4, 2.0 mM $MgCl_2$, 0.2 mM cresol red, 12% sucrose, 0.005% [w/v] BSA; Boehringer Mannheim), 1 pmol of each primer, 40 mM deoxyribonucleotide triphosphate (dNTPs) (Pharmacia LKB), and 1 unit of AmpliTaq Polymerase (Applied Biosystems). Parameters and conditions used for amplification were as follows: 94°C for 30 seconds, 60°C for 60 seconds, and 72°C for 60 seconds, for 36 cycles. *Taq* polymerase and dNTPs were added to the reaction tube at an 80°C step between the denaturation and annealing steps of the first cycle (hot-start PCR). For analysis of the PCR, aliquots were electrophoresed with 2% NuSieve agarose gel and stained with EtBr.

Immunohistochemistry stainings. Multimerized peptide/HLA complexes were used to identify antigen-specific T cells in situ in lesions from MM patients, as previously described (60). Tissue sections were dried overnight and subsequently fixed in cold acetone for 5 minutes. All incubation steps were performed in the dark at RT: (a) 45 minutes of the primary antibody (1:100 diluted); (b) Cy3-conjugated goat anti-mouse (1:500 diluted; code 115-165-100; Jackson ImmunoResearch Laboratories Inc.) for 45 minutes; and finally (c) the FITC-conjugated MHC-dextramer complexes HLA-A2/HO212 (QLFEELQEL) for 75 minutes. Between steps, slides were washed 2 times for 10 minutes each time in PBS/BSA 0.1%. Slides were mounted in VECTASHIELD and kept in the refrigerator until observed under the confocal microscope (Leica).

Cytotoxicity assay. Conventional ^{51}Cr-release assays for CTL-mediated cytotoxicity were carried out as described elsewhere (61). Target cells were peptide-pulsed T2 cells. Effector cells consisted of RhoC-specific T cell lines and clones (42) as well as a MART-1–specific clone (62) and a survivin-specific clone (63).

Sorting of conventional $CD4^+CD25^{hi}CD127^-$ Tregs. Cells were thawed in PBS buffer containing 0.5% BSA and 0.025 mg/ml pulmozyme (Roche) to prevent clotting of cells. Cells were stained with FITC anti-human CD4, PE anti-human CD127, and APC anti-human CD25 (eBioscience). Cells were sorted into $CD4^+CD25^{hi}CD127^-$ (conventional Tregs) and $CD4^+CD25^-CD127^+$ T cells (control cells). Cell sorting was performed on FACSAria (BD).

T cell proliferation assay (inhibitory assay). To compare the inhibitory capacity of conventional Tregs ($CD4^+CD25^{hi}CD127^-$) and HO-1–specific $CD8^+$ T cells, a PKH26 fluorescent dye–based proliferation assay was performed. Round-bottom 96-well microtiter plates were coated at 37°C with anti-CD3 antibodies (0.5 μg/ml in PBS) (OKT3; eBioscience). Prior to the assay, autologous PBMCs (responder cells) were stained with PKH26 (Sigma-Aldrich) following the protocol from the manufacturer. Fifty-thousand PKH26 responder cells were added to each well. Enriched from the same donor, 25,000 $CD4^+CD25^{hi}CD127^-$ T cells (conventional Tregs) or $CD4^+CD25^-CD127^+$ T cells (control cells) and 500 HO-1–specific $CD8^+$ T cells or CMV-specific $CD8^+$ T cells (control cells) were added to the responder cells in a final volume of 200 μl RPMI 1640 plus GlutaMAX plus 25 mM HEPES (Gibco; Invitrogen), supplemented with 10% FBS (Gibco; Invitrogen) plus penicillium-streptomycin (100 μg/ml). The plates were incubated for 6 days at 37°C in a humidified 5% CO_2 atmosphere. The cells were stained with PerCP anti-human CD4 and FITC anti-human CD8 and analyzed for proliferation (dilution of the PKH26 dye). PHK26-labeled PBMCs cultured with anti-CD3 antibody and without anti-CD3 antibody were included as positive and negative controls, respectively. Percentage of inhibition was calculated as follows: percentage proliferation of $CD4^+$ or $CD8^+$ responder T cells added to anti-CD3 antibodies minus percentage proliferation of $CD4^+$ or $CD8^+$ responder T cells added to either conventional Tregs or HO-1–specific $CD8^+$ T cells, divided by percentage proliferation of $CD4^+$ or $CD8^+$ responder T cells added to anti-CD3 antibodies.

Statistics. The significance of the presence of HLA-A2–restricted HO212-specific $CD8^+$ T cells in PBLs from MM patients, RCC patients, and BC patients compared with healthy individuals (Figure 1) was calculated using Mann-Whitney test, 2-sided (exact method). The significance of the suppression of TAA-specific IFN-γ release by the addition of HO-1 peptide

(Figure 4) or HO-1 supernatant (Figure 7) was calculated using 1-tailed paired Student's *t* test. $P < 0.05$ was considered significant.

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