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Identification and Clinical Relevance of Naturally Occurring Human CD8$^+$HLA-DR$^+$ Regulatory T Cells

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The lack of responsiveness to self and non-self Ags is normally maintained by multiple mechanisms, including the suppressive activities of several T cell subsets. In this study, we show that CD8$^+$ T cells from both adult peripheral blood and umbilical cord blood mononuclear cells constitutively expressing HLA-DR represent a natural human CD8$^+$ regulatory T cell subset. Their suppressive effect appears to be cell-to-cell contact dependent and may involve CTLA-4 signaling between neighboring T cells. These regulatory T cells can be expanded in vitro and exhibit a suppressive capacity similar to that observed in ex vivo CD8$^+$HLA-DR$^+$ T cells. The high frequency of CD8$^+$HLA-DR$^+$ T cells that we detected in patients with non–small cell lung cancer deserves further work to confirm their putative suppressor effect within the tumor. ***The Journal of Immunology*****, 2014, 193: 4469–4476.**

The existence of T cells with suppressive capacity was first proposed in the early 1970s (1). However, their existence was questioned, until CD4$^+$CD25$^+$ T cells were characterized as the most potent suppressor population among the T lymphocytes (2–8).

In 1980, it was reported that a T cell subset induced after T cell activation had very strong suppressor activity, and this function was restricted to T cells expressing HLA-class II DR Ags (9). Subsequently, we confirmed that these suppressor cells were CD8$^+$ T cells.

The expression of HLA-DR on human T cells has been regarded primarily as a marker of activated T cells. However, as recently reported, HLA-DR expression by human CD4$^+$ regulatory T cells (Tregs) also defined a functionally distinct population of mature Tregs (10).

A high level of tumor-infiltrating CD4$^+$FOXP3$^+$ Tregs was correlated with a shorter time to recurrence in non–small cell lung cancer (NSCLC) (11). Similarly, a higher ratio of CD4$^+$FOXP3$^+$ Tregs/T cells is correlated with shorter disease-specific survival in stage I NSCLC (12), and it has been suggested that CD4$^+$FOXP3$^+$ Tregs impinge upon antitumor immune responses in patients with cancer (13). In this context, it is also possible that Treg populations other than CD4$^+$FOXP3$^+$ Tregs mediate the suppression of antitumor responses.

Results from the current study indicate that CD8$^+$HLA-DR$^+$ T cells constitute a natural subset of Tregs that may represent a separate lineage of CD8$^+$ T cells. Their putative role in tumor immune responses was analyzed in patients with NSCLC by comparing their tumor infiltration with that of the classic CD4$^+$FOXP3$^+$ Tregs.

Materials and Methods

Subjects

Peripheral blood (PB) samples were obtained from healthy adult donors (HD). Cord blood (CB) samples were obtained from umbilical cord veins of full-term healthy neonates. None of the HD, neonates, or their mothers had any hereditary disorders, hematologic abnormalities, or infectious complications.

Twelve patients with NSCLC who had not received neoadjuvant therapy were recruited for the study with the approval of the local Ethics Committee and after their written informed consent was obtained. Tumor, lymph node, and metastasis staging was performed based on the 2012 National Comprehensive Cancer Network guidelines. All patients were diagnosed with stage I or II NSCLC, both of which are surgically treatable. We did not enroll patients who were at stage IIIa or stages IIIb to IV because they cannot be surgically treated. All donors underwent surgery between July 2013 and February 2014; 11 donors received pulmonary lobectomy, and one donor received pneumonectomy. Tumor tissues and adjacent normal tissues were collected from patients following surgical resection at Hospital de Clínicas "José de San Martín," and a histopathological diagnosis was obtained at the same center. The exclusion criteria were as follows: previous malignancies, secondary lung cancer, prior chemotherapy, and concomitant immunosuppressive diseases. The clinical characteristics of the patients with NSCLC are summarized in Table I.

This study was approved by the Investigation and Ethics Committee at the Hospital de Clínicas "José de San Martín" and Hospital de Pediatría S.A.M.I.C. "Prof. Dr. Juan P. Garrahan" in accordance with the Declaration of Helsinki.

Mononuclear cell isolation

PBMCs or CB mononuclear cells (CBMCs) were obtained from blood samples by Ficoll-Hypaque gradient centrifugation (GE Healthcare Life Sciences). Adjacent normal lung tissue and tumor specimens were transported and washed in complete medium (RPMI 1640 medium [Life Technologies] supplemented with penicillin, streptomycin, L-glutamine [Sigma-Aldrich], and 10% FCS [Natocor]). Within 30 min of resection from a patient, the samples were minced with blades in a petri dish and forced through 70-mm cell strainers to collect a single-cell suspension. The suspension was digested for ~40 min with collagenase type I (Sigma-Aldrich) and washed twice, and lymphocytes were isolated using a Ficoll-Hypaque gradient.

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Abbreviations used in this article: CB, cord blood; CBMC, CB mononuclear cell; DC, dendritic cell; HD, healthy donor; NSCLC, non–small cell lung cancer; PB, peripheral blood; Treg, regulatory T cell; TW, transwell.

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Table I. Clinical characteristics of patients with NSCLC

Number	12
Age (years ± SEM)	71 ± 10.2
Gender	
Male	8
Female	4
Smoking status	
Nonsmoker	3
Smoker	9
Histology	
Squamous carcinoma	4
Adenocarcinoma	8
Stage	
I	6
II	6
III	0
IV	0

Cell sorting

$CD8^+$ T cells from PB and/or CB were enriched by negative selection using RosetteSep Human $CD8^+$ T Cell Enrichment Cocktail (Stem Cell Technologies) following the manufacturer's protocols. Purified cells were isolated by staining purified $CD8^+$ T cells with anti-CD8 PerCP and anti–HLA-DR FITC Abs (all from BD Biosciences) and sorting with a FACSAria II flow cytometer (BD Biosciences), yielding two populations, as follows: $CD8^+HLA\text{-}DR^-$ and $CD8^+HLA\text{-}DR^+$ T cells. The cells were collected into RPMI 1640 medium containing 50% FCS and washed once prior to further studies. The purity determined by flow cytometry was always >95% in each subset.

Flow cytometry

Freshly isolated or in vitro cultured cells were stained with anti-CD8 (PerCP or FITC), anti–HLA-DR (FITC, allophycocyanin, or PE), anti-CD25 (PE or allophycocyanin-Cy7), anti-CD45RA (PE-Cy7), anti-CD56 (allophycocyanin), anti-CCR7 (FITC), anti-CD62L (allophycocyanin), anti-CD28 (allophycocyanin), anti-CD38 (FITC), and anti–CTLA-4 (PE), (all from BD Biosciences). Intracellular detection of FOXP3 with anti-FOXP3 (PE or Alexa Fluor 488) and Ki-67 Ag with anti-Ki-67 (FITC) Abs was performed using fixed and permeabilized cells following the manufacturer's instructions. Negative control samples were incubated with an isotype-matched Ab. Dead cells were excluded by forward and side scatter characteristics. Statistical analyses are based on at least 100,000 events gated on the population of interest. The data were acquired using a FACSAria II (BD Biosciences) and analyzed with FlowJo software.

Suppression assay

Suppressor effect on responder cells was tested by two different methods, as follows: CFSE dilution and/or [^{3}H]thymidine uptake.

CFSE dilution method. Responder autologous PBMCs (1×10^5) labeled with 1 μM CFSE (Invitrogen) were cultured with highly purified unlabeled $CD8^+$ $HLA\text{-}DR^-$ or $CD8^+$ $HLA\text{-}DR^+$ T cells at decreasing responder: suppressor cell ratios (2:1, 4:1, and 8:1). Cells were stimulated with anti-CD3/CD28 Abs (1 μg/ml; BD Pharmingen) in 96-well round-bottom plate and cultured in complete medium. Proliferation of CFSE-labeled cells was assessed by flow cytometry after 4 d of culture. Percentage suppression was calculated by dividing the number of proliferating CFSE-diluting responder cells in the presence of different ratios of responder:suppressor cells by the number of proliferating responder cells when cultured alone, and multiplied by 100. Unlabeled cells and mytomicin-treated cells were used as control.

[^{3}H]thymidine uptake method. Responder autologous PBMCs (1×10^5) were cocultured with highly purified $CD8^+$ $HLA\text{-}DR^-$ or $CD8^+$ $HLA\text{-}DR^+$ T cells at different responder:suppressor cell ratios. Cells were stimulated with anti-CD3/CD28 Abs in 96-well round-bottom plate and cultured for 4 d in complete medium. On the last day of culture, proliferative response was tested by the addition of 1 μl [^{3}H]thymidine/well, followed by harvesting 18 h later. Cell proliferation was measured based on [^{3}H]thymidine uptake (Perkin Elmer Life Sciences). The percentage inhibition was calculated using the following formula: 1 − (cpm in the presence of $CD8^+HLA\text{-}DR^+$ Tregs/cpm in the absence of $CD8^+HLA\text{-}DR^+$ Tregs) × 100.

Transwell assay

To assess whether $CD8^+HLA\text{-}DR^+$ Tregs exert their regulatory function through direct cell contact or through release of soluble factors, we performed transwell (TW) experiments in which PBMCs were used as responder cells. Purified $CD8^+HLA\text{-}DR^-$ or $CD8^+HLA\text{-}DR^+$ cells were added at a ratio of 1:4 to autologous PBMCs seeded at 5×10^5 cells/well in the lower chamber of a 24-well plate. The 1:4 ratios were selected based on three preliminary experiments in which suppressor activity was detected up to ratios of 1:8. $CD8^+HLA\text{-}DR^+$ or $CD8^+HLA\text{-}DR^-$ T cells were cultured either in the lower chambers directly in contact with the responder cells or in the upper chambers separated from the responder cells by a 0.4-μm–pore membrane (Corning Life). After cells were cocultured with anti-CD3/CD28 during 4 d, cells from the lower chambers were collected and transferred to 96-well plates, at 1×10^5 cells/well, in which the proliferative response was assessed by the addition of 1 μl [^{3}H]thymidine/well, followed by harvesting 18 h later. The percentage inhibition was calculated as explained.

Neutralization assay

To investigate whether the suppressor function of $CD8^+HLA\text{-}DR^+$ Tregs is related to the release of regulatory cytokines, anti–IL-10 (13 μg/ml; BD Biosciences), anti–TGF-β (3.5 μg/ml; R&D Systems), or both were added to the suppression assay. To test the involvement of CTLA-4 in the suppression mechanism, the cells were incubated with decreasing doses of anti–CTLA-4 (15 and 5 μg/ml; BD Biosciences). All neutralization assays, including the use of anti–HLA-DR Ab (clone L243), were performed under the same culture conditions described above and using as control the corresponding isotype-matched mAb.

Cultures of PBMCs in the presence of IL-2

PBMCs were activated with anti-CD3 and anti-CD28 (1 μg/ml; BD Pharmingen). Three days after activation, IL-2 (10 ng/ml; PeproTech) was added every 72 h, and the PBMCs were maintained in culture for 15 d. The kinetics of cell proliferation was analyzed by flow cytometry at different time points using Ki-67 staining, and the expression of different markers was examined, which allowed the identification of different cell subsets.

Statistical analysis

The normality of variable distribution was assessed using the Kolmogorov-Smirnov goodness-of-fit test. Once the hypothesis of normality was accepted ($p < 0.05$), comparisons were performed using paired and unpaired Student t tests, as appropriate. If the hypothesis of normality was rejected, the analysis was performed using Wilcoxon's rank-sum test. A p value <0.05 was considered significant.

Results

Natural $CD8^+HLA\text{-}DR^+$ T cells have immunosuppressive properties

$CD8^+HLA\text{-}DR^-$ and $CD8^+HLA\text{-}DR^+$ T cell subsets from peripheral blood of HD were identified by flow cytometry. It was established that $CD8^+HLA\text{-}DR^+$ T cells account for ∼14.4% ± 3.5 of the total $CD8^+$ cells (n = 10). We next assessed the in vitro suppressive capacity of purified $CD8^+HLA\text{-}DR^-$ and $CD8^+HLA\text{-}DR^+$ T cells by the extent of CFSE dilution of labeled autologous PBMCs (hereafter called responder cells) cocultured at different ratios with each $CD8^+$ T cell subset and stimulated for 4 d with anti-CD3/CD28. As illustrated in Fig. 1A, the results of CFSE staining demonstrated that purified $CD8^+HLA\text{-}DR^+$ Tregs are able to suppress the proliferative response of activated responder cells up to a responder:suppressor cell ratio of 4:1.

The suppressive capacity of $CD8^+HLA\text{-}DR^+$ T cells was also assessed by measuring their capacity to suppress the proliferation of responder cells determined by [^{3}H]thymidine uptake. Results from one representative experiment are depicted in Fig. 1B. The suppression occurred in a dose-dependent manner, and 50% inhibition was observed at a responder:suppressor cell ratio of 2:1. The addition of $CD8^+HLA\text{-}DR^-$ T cells did not exert any suppressor effect and effectively increased the proliferative response (Fig. 1C; n = 4).

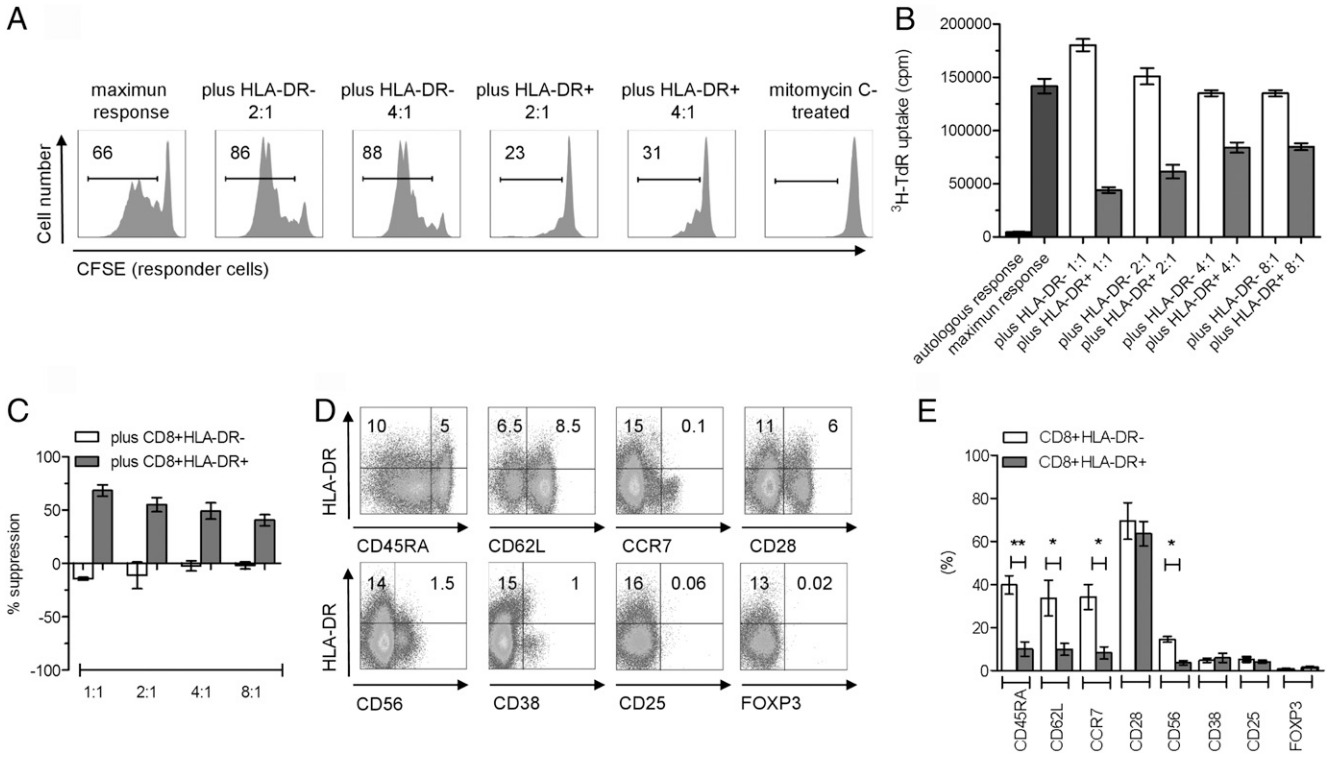

FIGURE 1. Naturally occurring human CD8$^+$HLA-DR$^+$ T cells with a suppressor function can be found in HD. (**A**) CFSE dilution by 1×10^5 labeled PBMC responder assessed after 4 d of TCR-stimulated coculture with indicated CD8$^+$ T cell subset at a 2:1 and 4:1 ratio. Cell number and percentage of dividing cells per well are indicated. Representative FACS profile of four independent experiments. (**B**) Figure shows one representative experiment of four that compares the capacity of CD8$^+$HLA-DR$^-$ and CD8$^+$HLA-DR$^+$ T cells to suppress the cell proliferation. In each experiment, 1×10^5 PBMCs were cultured with decreasing numbers of sorted CD8$^+$HLA-DR$^-$ or HLA-DR$^+$ T cells (ratios of responder cells:Tregs = 1:1, 2:1, 4.1, 8:1) and activated with anti-CD3/CD28. The proliferative response was measured by [^{3}H]thymidine uptake, and the percentage of inhibition was calculated as described. Bars represent the mean ± SEM of the replicates of this particular experiment. (**C**) Data are presented as the mean ± SEM of four independent experiments described in (B). (**D**) Representative FACS profile showing the coexpression of HLA-DR with different markers on CD8$^+$ gated T cells. Data are expressed as a percentage of all CD8$^+$ T lymphocytes. (**E**) Data show the mean ± SEM frequency of the different markers analyzed. Results are expressed as percentage on each CD8$^+$ T cell subset analyzed (n = 10). *$p < 0.05$, **$p < 0.01$.

Phenotypic characterization of CD8$^+$HLA-DR$^+$ Tregs

Because HLA-DR is upregulated during T cell activation, it was important to determine whether the CD8$^+$HLA-DR$^+$ Tregs isolated from the peripheral blood of healthy adults were associated with additional activation markers. Representative dot plots are shown in Fig. 1D. CD8$^+$HLA-DR$^+$ Tregs showed bimodal expression levels of CD45RA (10% ± 3.4 versus 40% ± 4.2; $p < 0.05$), CCR7 (8.2% ± 2.8 versus 34.2% ± 5.8; $p < 0.05$), and CD62L (9.8% ± 3 versus 33.6% ± 8; $p < 0.05$). Both CD8$^+$ T cell subsets showed similar expression levels of the CD28 costimulatory molecule. However, the expression of CD56, which is correlated with a more differentiated CD8$^+$ T cell phenotype (14), showed significantly higher expression in CD8$^+$HLA-DR$^-$ T cells (14.5% ± 1.3 versus 3.7% ± 1 in CD8$^+$HLA-DR$^+$ T cells; $p < 0.05$). The expression of CD38, which is a marker of cell activation, was almost not detectable in both subsets. In addition, the classic regulatory CD4$^+$ T cell markers, CD25 and FOXP3, were almost not detectable on both subsets of CD8$^+$ T cells (n = 10; Fig. 1E).

Suppression mediated by CD8$^+$HLA-DR$^+$ Tregs requires cell-to-cell contact

To investigate the potential requirement for cell-to-cell contact, purified CD8$^+$HLA-DR$^-$ or CD8$^+$HLA-DR$^+$ T cells were added to PBMCs either directly or in the upper well of a TW chamber. These experiments were performed at a responder:suppressor ratio of 4:1. In the TW experiments, autologous PBMCs were seeded in the lower chambers of a 24-well plate, whereas purified CD8$^+$ HLA-DR$^-$ or CD8$^+$HLA-DR$^+$ T cells were added either in the lower chamber or in the upper chamber to assess the need of cell contact with the responder cells. The cells were cultured for 4 d, in the presence of anti-CD3/CD28. As illustrated in Fig. 2A, CD8$^+$ HLA-DR$^+$ Tregs that were separated from the responder cells by the semipermeable membrane completely lost their suppressor effect (n = 3).

From preliminary experiments, we knew that anti–HLA-DR Ab blocks alloactivation or activation through mitogens such as Con A, but does not block the response of PBMCs to PHA activation. In this context, we observed that anti–HLA-DR Ab had no effect on the suppressive activity of CD8$^+$HLA-DR$^+$ Tregs on responder cells activated with PHA (data not shown).

In addition, we investigated whether regulatory cytokines, such as IL-10 and TGF-β, might also be implicated in the suppressive effects. Blocking experiments using neutralizing anti–IL-10 and/or TGF-β Abs only moderately inhibited the suppressor effect of CD8$^+$HLA-DR$^+$ Tregs, even when these two Abs were added together (n = 3; Fig. 2B), suggesting that these soluble factors do not play an essential role in mediating the suppressive function of CD8$^+$HLA-DR$^+$ Tregs.

Involvement of CTLA-4 in the suppressor mechanism induced by CD8$^+$HLA-DR$^+$ Tregs

Direct interactions between Tregs and dendritic cells (DCs) involving the inhibitory molecule CTLA-4, which is expressed constitutively by CD4$^+$FOXP3$^+$ Tregs, were ascribed as the mechanism responsible for the regulatory function of Tregs (15, 16). Because

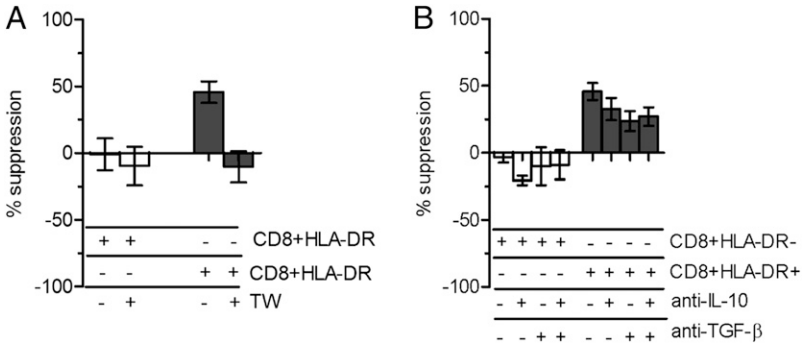

FIGURE 2. TW experiment shows abrogation of the suppressor effect due to loss of cell contact. (**A**) Purified $CD8^+HLA\text{-}DR^-$ T cells and $CD8^+HLA\text{-}DR^+$ Tregs were added at a ratio of 1:4 to autologous PBMCs seeded in the lower chambers of a 24-well plate. Tregs were either added directly or separated by a TW. The cells were cultured for 4 d in the presence of a CD3/CD28 Ab. On the last day of culture, the cells were collected, transferred to 96-well plates, and tested for [^{3}H]thymidine incorporation. Figure shows the percentage suppression of each $CD8^+$ T cell subset ($CD8^+HLA\text{-}DR^-$ T cells, white bars; $CD8^+HLA\text{-}DR^+$ T cells, black bars) in the presence (TW +) or absence (TW −) of membrane ($n = 3$). (**B**) Blocking experiments. Figure shows that $CD8^+HLA\text{-}DR^+$ T cells moderately decreased the suppressor effect when the assay was performed in the presence of anti–IL-10 or anti–TGF-β separated or in combination (ratio responder:suppressor cell, 4:1). Data are presented as the mean ± SEM ($n = 3$).

we confirmed that the suppressor effect of $CD8^+HLA\text{-}DR^+$ Tregs required cell-to-cell contact, we investigated the role of CTLA-4 in this subset of Tregs. We first analyzed the kinetics of CTLA-4 expression on $CD8^+HLA\text{-}DR^+$ Tregs after PBMC activation with anti-CD3/CD28. Cell surface CTLA-4 expression was analyzed by flow cytometry on gated $CD8^+HLA\text{-}DR^-$ and $CD8^+HLA\text{-}DR^+$ T cells at 0, 24, and 48 h. Compared with the $CD8^+HLA\text{-}DR^-$ T cells, $CD8^+HLA\text{-}DR^+$ Tregs showed a significantly higher baseline level of surface CTLA-4 expression (3.4% ± 0.2 in $CD8^+HLA\text{-}DR^+$ versus 0.14% ± 0.01 in $CD8^+HLA\text{-}DR^-$ T cells; $p < 0.02$). Additionally, the postactivation upregulation of this molecule was more pronounced in the $CD8^+HLA\text{-}DR^+$ subset (Fig. 3A, 3B; $n = 3$). Of note, blocking with an anti–CTLA-4 mAb resulted in a dose-dependent inhibition of the suppressor effect induced by $CD8^+HLA\text{-}DR^+$ Tregs. In contrast, anti–CTLA-4 Ab had no effect on $CD8^+HLA\text{-}DR^-$ T cells ($n = 3$; Fig. 3C).

$CD8^+HLA\text{-}DR^+$ Tregs are present in CBMCs from healthy newborn infants

The analysis of 12 independent samples confirmed the presence of $CD8^+HLA\text{-}DR^+$ Tregs in CBMCs at a frequency of 3.7% ± 0.8. Despite their low number, we were able to identify markers that were expressed on gated CB $CD8^+$ T cells. As illustrated at the dot plot of Fig. 4A, most $CD8^+HLA\text{-}DR^+$ T cells expressed CD45RA, with an intermediate expression of CCR7, CD62L, CD28, and CD38. In contrast, CB $CD8^+HLA\text{-}DR^+$ T cells show a very low or absent expression of CD25, FOXP3, and CD56 (which was completely restricted to $CD8^+HLA\text{-}DR^-$ T cells). Fig. 4B summarizes the different mean frequency of these markers expressed on both $CD8^+HLA\text{-}DR^+$ and $CD8^+HLA\text{-}DR^-$ T cell subsets. As expected, most CB $CD8^+HLA\text{-}DR^-$ and $CD8^+HLA\text{-}DR^+$ T cells exhibited a $CD45RA^+$ phenotype (94.3% ± 2.1 and 91% ± 4, respectively). In comparison with $CD8^+HLA\text{-}DR^-$ T cells, CB $CD8^+HLA\text{-}DR^+$ Tregs showed decreased expression of CCR7 (22.4% ± 8.8 versus 72.1% ± 13; $p < 0.05$) and a very low expression of CD25 and FOXP3, which are classic $CD4^+$ Treg markers. The mean frequency of all 12 samples analyzed revealed a significantly higher expression of surface CTLA-4 in the $CD8^+HLA\text{-}DR^+$ Tregs (5.2% ± 1.3 versus 1.05% ± 0.6, in $CD8^+HLA\text{-}DR^-$ T cells; $p < 0.02$).

Despite their naive phenotype ($CD45RA^+$), six independent experiments demonstrated that highly purified CB $CD8^+HLA\text{-}DR^+$

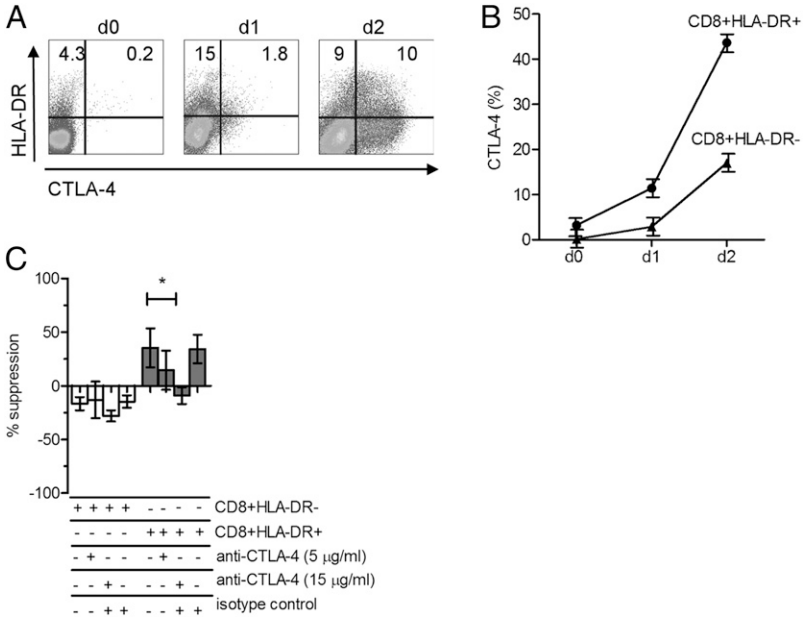

FIGURE 3. CTLA-4 is involved in the suppression mechanism of $CD8^+HLA\text{-}DR^+$ Tregs. PBMCs were activated with anti-CD3/CD28 for 48 h. The cell surface expression of CTLA-4 was analyzed by flow cytometry on gated $CD8^+HLA\text{-}DR^-$ and $CD8^+HLA\text{-}DR^+$ T cells at 0, 1, and 2 d of culture. (**A**) Representative dot plots showing the percentage of cell surface CTLA-4 detected on both subsets at different time points. Data are expressed as percentage of all $CD8^+$ T cells. (**B**) Data show the kinetics of CTLA-4 expressed as the mean ± SEM of three different experiments. Results are expressed as percentage of CTLA-4 expression on each $CD8^+$ T cell gated subset. (**C**) The suppressor effect induced by $CD8^+HLA\text{-}DR^+$ Tregs at ratio responder:suppressor cells 4:1 was inhibited by anti–CTLA-4 in a dose-dependent manner ($n = 3$). Isotype control for anti–CTLA-4 blocking Ab is also included. Data are presented as the mean ± SEM ($n = 3$), *$p < 0.05$.

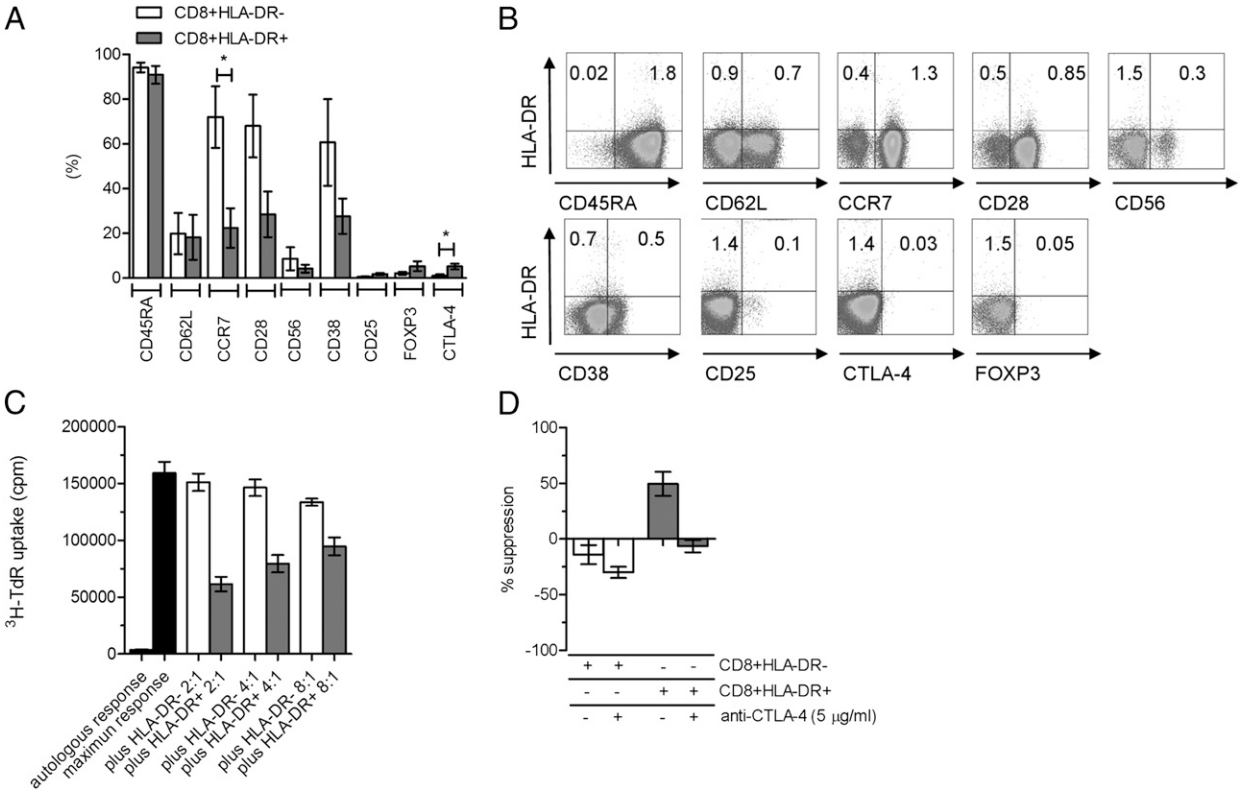

FIGURE 4. CD8$^+$HLA-DR$^+$ Tregs from umbilical CB are similar to those from adult PB. CBMCs were isolated and stained with a panel of mAbs. (**A**) Representative dot plots showing the coexpression of HLA-DR with different markers on CD8$^+$ gated T cells. Data are expressed as a percentage of all CD8$^+$ T lymphocytes. *$p < 0.05$. (**B**) Data show the mean ± SEM frequency of the different markers analyzed. Results are expressed as percentage on each CD8$^+$ T cell subset analyzed ($n = 12$). (**C**) Figure shows one representative experiment of six that compares the capacity of CD8$^+$HLA-DR$^-$ and CD8$^+$HLA-DR$^+$ T cells to suppress the cell proliferation. In each experiment, 1×10^5 PBMCs used as responder cells were cocultured with decreasing numbers of sorted CD8$^+$HLA-DR$^-$ or CD8$^+$HLA-DR$^+$ T cells at different ratios of responder:suppressor cells (1:1, 2:1, 4.1, 8:1) and activated with anti-CD3/CD28. The proliferative response was measured by [^{3}H]thymidine uptake, and the percentage of inhibition was calculated, as described in *Materials and Methods*. Bars represent the mean ± SEM of the replicates of this particular experiment. (**D**) Figure shows that the suppressor effect induced by CB CD8$^+$HLA-DR$^+$ Tregs was inhibited when the assay was performed in the presence of anti–CTLA-4. Data are presented as the mean ± SEM of three independent experiments ($n = 3$).

T cells possess a potent suppressor capacity that was not observed for CD8$^+$HLA-DR$^-$ T cells (Fig. 4C). Similar to the effect observed in adults, the suppressor effect of CB CD8$^+$HLA-DR$^+$ Tregs was completely abrogated by the addition of anti–CTLA-4 Ab, with no effect on CD8$^+$HLA-DR$^-$ T cells (Fig. 4D; $n = 3$).

Clinical relevance of natural CD8$^+$HLA-DR$^+$ Tregs

Increased expansion of CD8$^+$HLA-DR$^+$ Tregs with anti-CD3 and IL-2. Because of the therapeutic potential of CD8$^+$HLA-DR$^+$ Tregs, we attempted to expand these cells in vitro with anti-CD3 and IL-2, and their phenotype was analyzed at 0, 4, 8, and 15 d of cell culture. As depicted in Fig. 5A, up to 8 d after activation, CD8$^+$HLA-DR$^+$ Tregs and CD8$^+$HLA-DR$^-$ T cells showed similar levels of Ki-67 expression. In contrast, after 15 d of culture, the expression of Ki-67 remained constant in CD8$^+$HLA-DR$^+$ Tregs, but decreased in CD8$^+$HLA-DR$^-$ T cells, indicating a higher proliferative rate ($n = 4$). As a consequence of their proliferation, the frequency of CD8$^+$HLA-DR$^+$ T cells increased from 14.4% ± 3.5 at day 0 to 37% ± 7 at day 15. To explain these previous results, we explored the expression of CD25 along the cell culture. In Fig. 5B, a representative dot plot shows how the expression of CD25 is induced during the first 4 d that follow cell activation, reaching similar expression levels in CD8$^+$HLA-DR$^+$ and CD8$^+$HLA-DR$^-$ (Fig. 5C). However, the expression of CD25 on CD8$^+$HLA-DR$^-$ T cells shows a substantial decrease 8 d postactivation, with a sustained expression of the IL-2R on CD8$^+$ HLA-DR$^+$ Tregs. These results may explain the preferential proliferation of CD8$^+$HLA-DR$^+$ Tregs detected with Ki-67 at day 15. Interestingly, 8 d after cell activation, CD8$^+$HLA-DR$^+$ T cells began to express FOXP3, which remained stably expressed until day 15, probably associated with the addition of IL-2 (Fig. 5C).

After being activated with anti-CD3 and IL-2 for 8 d, purified CD8$^+$ T cells were sorted as CD8$^+$HLA-DR$^-$ and HLA-DR$^+$ T cells, and their suppressor activity was assayed as described above. Similar to ex vivo CD8$^+$HLA-DR$^+$ Tregs, day 8 CD8$^+$HLA-DR$^+$ Tregs showed a very powerful suppressor effect (Fig. 5D).

Increased frequency of CD8$^+$HLA-DR$^+$ Tregs in NSCLC. It was reported that CD8$^+$ Tregs and CD4$^+$FOXP3$^+$ Tregs (17), which infiltrate tumors, may contribute to tumor immune escape and were associated with advanced tumor growth and poor prognosis (18–23). More recently, it was found that intratumoral accumulation of CD4$^+$FOXP3$^+$ Tregs has prognostic potential in NSCLC (24). Thus, having access to matched lung tumor tissue, adjacent unaffected lung tissue, and peripheral blood from 12 patients diagnosed with NSCLC enabled us to compare the frequency of CD4$^+$FOXP3$^+$ Tregs and CD8$^+$ Tregs derived from these different compartments. The clinical characteristics of the patients with NSCLC are shown in Table I. In contrast to the numerous reports describing increased Treg levels in advanced cancers, few reports have been issued on Treg levels in early cancers. In this context, it is important to note that all patients included in this analysis were diagnosed with stage I or II NSCLC.

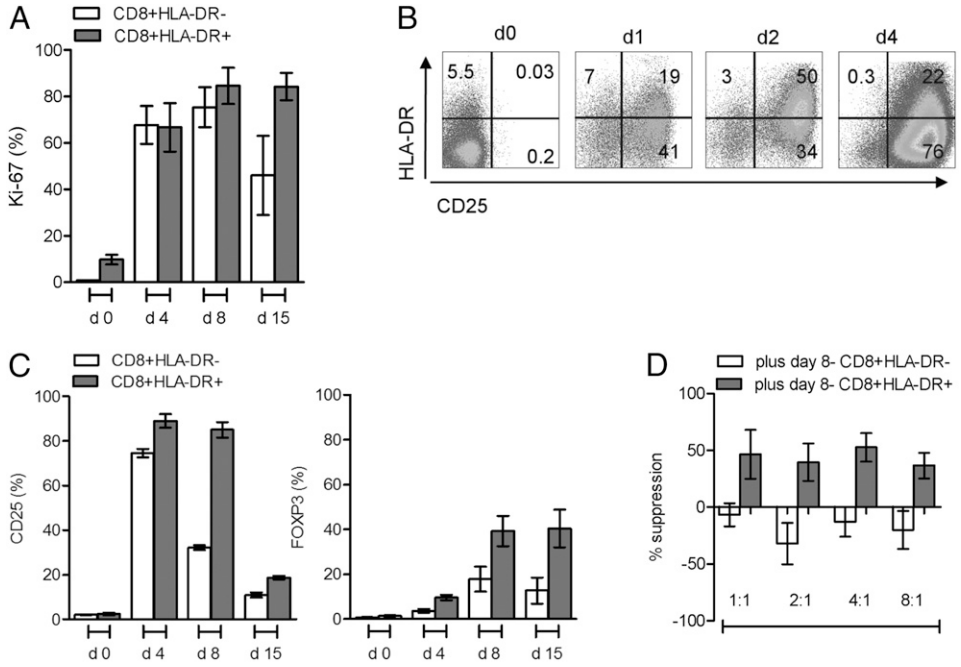

FIGURE 5. CD8$^+$HLA-DR$^+$ Tregs expanded in vitro retain their suppressive capacity. PBMCs were activated with anti-CD3 and IL-2, and expression of different markers was analyzed by flow cytometry at 0, 4, 8, and 15 d of cell culture. (**A**) Data show the mean ± SEM of Ki-67 percentage on each CD8$^+$ T cell subset analyzed ($n = 4$). (**B**) Representative dot plots showing the percentage of CD25 detected on both subsets during the first 4 d after activation. Data are expressed as percentage of all CD8$^+$ T cells. (**C**) Shows the similar levels of CD25 expression detected at day 4 postactivation in CD8$^+$HLA-DR$^+$ and CD8$^+$HLA-DR$^-$. The expression of CD25 on CD8$^+$HLA-DR$^-$ shows a substantial decrease at day 8 postactivation, with a sustained expression of the IL-2R on CD8$^+$ HLA-DR$^+$ Tregs ($n = 4$). After 8 d of anti-CD3 plus IL-2 activation, FOXP3 started to be expressed on CD8$^+$HLA-DR$^+$ Tregs. This expression remains stable until day 15 of culture. Data are presented as the mean ± SEM ($n = 4$). (**D**) After 8 d of activation, CD8$^+$ T cells were sorted into HLA-DR$^-$ and HLA-DR$^+$ cells, and their suppressor effect was assayed as described above. After cell expansion, only CD8$^+$HLA-DR$^+$ Tregs (black bars) retained a very powerful suppressor effect ($n = 3$).

The frequency of CD8$^+$ T cells within the tumor was ~10%, being ~3% in the adjacent lung tissue. Interestingly, we found that most CD8$^+$ T cells within the tumor were CD8$^+$HLA-DR$^+$ T cells (82.4% ± 4.2). During this analysis, we gated out cells with high expression of HLA-DR (such as B cells and monocytes/macrophages). Although the percentage of CD8$^+$HLA-DR$^+$ T cells decreased on the borders of the tumors, the frequency remained very high (61.4% ± 6.4; Fig. 6A), possibly reflecting a strong chemotactic effect generated by the tumor cells. In agreement with our findings in adult PBMCs and CB from newborn infants, FOXP3 expression was almost not detectable on lung resident CD8$^+$HLA-DR$^+$ Tregs as depicted in a representative dot plot (Fig. 6B). There is no remarkable difference between squamous carcinoma and adenocarcinoma, suggesting that the mechanism that promotes the expansion of CD8$^+$HLA-DR$^+$ Tregs in patients with different types of cancers is likely to be similar.

In agreement with previous reports, we detected increased infiltration of CD4$^+$FOXP3$^+$ Tregs within the tumor, and the proportion of these cells was significantly higher compared with that in the adjacent normal lung tissue (20.1% ± 3.1 versus 9.9% ± 2.1; $p < 0.05$; Fig. 6C). Of note, within the tumor, >80% of the CD8$^+$ T cells expressed the Treg phenotype, with only a few CD8$^+$ HLA-DR$^-$ T cells. The similar frequency of CD4$^+$FOXP3$^+$ and CD8$^+$HLA-DR$^+$ T cells in PBMCs of HD and NSCLC patients confirmed the relevance of detecting tumor-infiltrating lymphocytes (Fig. 6C).

Most tissue samples obtained after surgery were fixed in formalin for histopathological analysis essential for tumor staging of the patient. Thus, we had only access to a small size of the specimens obtained from the lung cancer. In addition, CD8$^+$ T cells represent only 10% of the cell sample. Therefore, the low numbers of Tregs isolated from these samples limited the possibility of performing functional studies. However, all experiments in the current study demonstrate that the suppressor activity of CD8$^+$ cells is completely restricted to CD8$^+$ T cells expressing HLA-DR.

Discussion

In this study, we describe a novel human regulatory CD8$^+$HLA-DR$^+$ T cell population present in adult and umbilical venous blood samples. Similar to CD4$^+$FOXP3$^+$ Tregs, these natural CD8$^+$HLA-DR$^+$ Tregs represent a small subset within PB or CBMCs. The comparison between CD8$^+$HLA-DR$^-$ and CD8$^+$HLA-DR$^+$ T shows lower expression of CCR7 and CD62L in this last subset, with similar expression of the costimulatory molecule CD28. The expression level of CD56, which is correlated with a more differentiated CD8$^+$ phenotype (14), was significantly higher in CD8$^+$HLA-DR$^-$ T cells. The expression of CD38, which is a marker of cell activation, showed lower levels. Notably, in contrast to classic CD4$^+$FOXP3$^+$ Tregs, the expression of CD25 and FOXP3 was almost undetectable in CD8$^+$HLA-DR$^+$ Tregs.

Similar to naturally occurring CD4$^+$FOXP3$^+$ Tregs, CD8$^+$HLA-DR$^+$ Tregs most likely originate from thymic emigrants and gradually increase over time. Their expansion is presumably induced through an encounter with environmental Ags that generate the memory-like phenotype we observed in adult CD8$^+$HLA-DR$^+$ Tregs. Of note, CB and PB CD8$^+$HLA-DR$^+$ Tregs have similar ability to suppress the proliferative response of effector T cells.

Concerning the mechanisms implicated in the regulatory effect of CD8$^+$HLA-DR$^+$ Tregs, we demonstrated a requirement for cell-to-cell-contact that mainly involved CTLA-4. We observed that surface expression of CTLA-4 is rapidly induced after T cell activation, and blocking this B7 ligand completely abrogates the

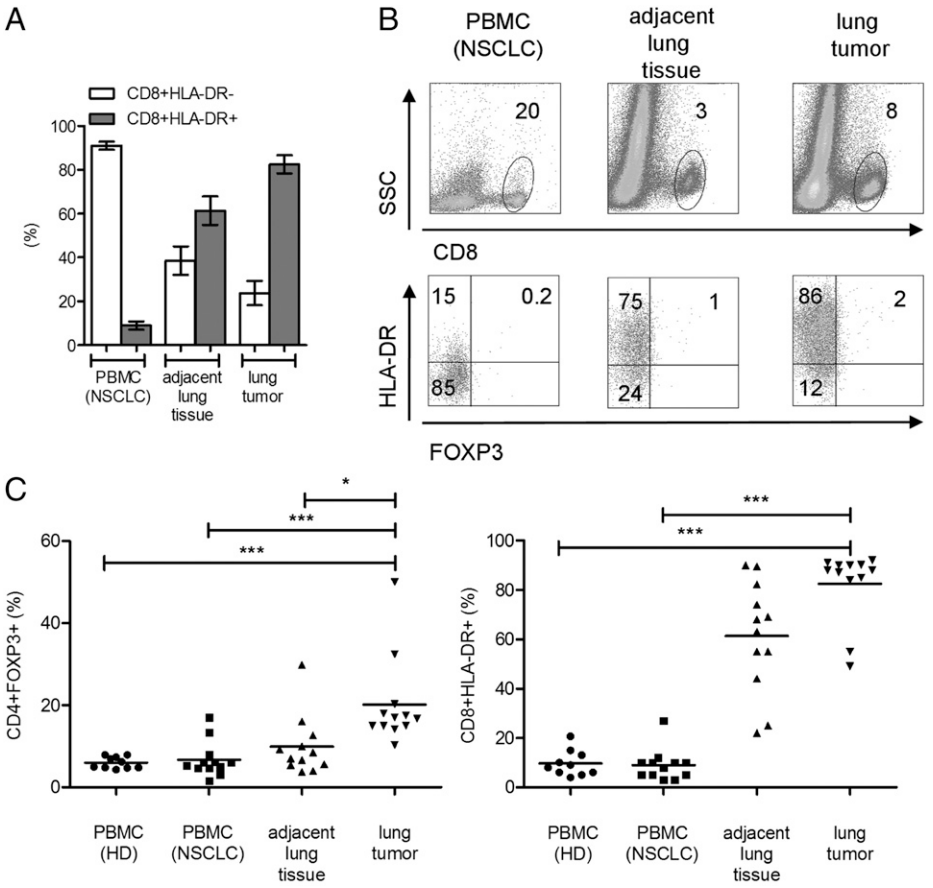

FIGURE 6. Ex vivo frequency of $CD4^+FOXP3^+$ Tregs and $CD8^+HLA\text{-}DR^+$ Tregs in NSCLC patients. (**A**) Frequencies of live $CD8^+HLA\text{-}DR^-$ T cells and $CD8^+HLA\text{-}DR^+$ Tregs obtained from PBMCs, NSCLC tumor samples, and adjacent normal tissues from 12 different patients. Data are presented as the mean ± SEM. Data are expressed as percentage of all $CD8^+$ T cells. (**B**) Representative dot plots showing the expression of HLA-DR and FOXP3 on live $CD8^+$ T cells obtained from matched PB, unaffected lung tissue, and lung tumor samples. Data are expressed as percentage of all $CD8^+$ T cells. (**C**) Percentage of live $CD4^+FOXP3^+$ Tregs and $CD8^+HLA\text{-}DR^+$ Tregs in PBMCs from age-matched HD (n = 10; mean age, 65 y) and PBMCs, adjacent normal lung tissue samples, and tumor samples from NSCLC patients (n = 12; mean age, 71 y). Data are presented as the mean ± SEM. Data are expressed as percentage of all $CD4^+$ and $CD8^+$ T cells. $*p < 0.05$, $*** p < 0.0001$.

suppressor capacity of the cells. It has not yet been determined whether CTLA-4 acts directly on the T cell that expresses it or acts on the APCs, either by binding the ligand B7, which leads to back-signaling to APCs (25, 26), or by downmodulating B7 expression (27). A recent report indicated that CTLA-4 may act on both conventional T cells and Tregs (28) and suggested that particular self-reactive T cells that escape negative selection must either express CTLA-4 themselves or become subject to peripheral control by Tregs that also depend on CTLA-4 for their function. Because we showed that blocking interactions between CTLA-4 and its ligands completely abrogate the suppressor effect of $CD8^+HLA\text{-}DR^+$ Tregs, it is possible that a high-affinity interaction between CTLA-4 and B7 molecules expressed on APCs affects the delivery of costimulatory signals from APCs to $CD4^+$ responsive cells.

IL-10 was reported to play an essential role in the immunosuppression mediated by $CD4^+CD25^+$ Tregs (29), either by itself or in association with CTLA-4 (30). In the current study, the role played by IL-10 and the potential role played by TGF-β appear to be secondary to the role involving CTLA-4.

The existence of a subset of human $CD8^+CD25^+$ thymocytes sharing phenotype, functional features, and mechanism of action with $CD4^+CD25^+$ Tregs was demonstrated, but the physiological meaning of this subset of $CD8^+CD25^+$ Tregs outside the thymus is not known (31). After polyclonal stimulation, the naturally occurring $CD8^+HLA\text{-}DR^+$ Tregs described in the current study acquire CD25 and FOXP3, which resemble those originally described in thymus.

FOXP3 was identified as the master regulator of $CD4^+$ Treg function (5, 32, 33), but detection of $CD8^+FOXP3^+$ T cells in peripheral blood is a rare event. However, $CD8^+FOXP3^+CTLA\text{-}4^+$ $CD45RO^+CD127^{low}$ and $CD25^-$ Tregs were detected in human tonsils (34). This finding is not unexpected because tonsil cells are in a continuously activated state. In fact, in the same report, a similar phenotype was induced in peripheral blood after polyclonal cell activation. Similarly, although our resting $CD8^+HLA\text{-}DR^+$ Tregs expressed minimal levels of FOXP3, this transcription factor was induced after polyclonal activation and remained stable during the 15 d of culture. Other inducible $CD8^+$ Tregs were obtained after anti-CD3 treatment. These cells, which do not express FOXP3, inhibit the $CD4^+$ proliferative responses to Ags and to superantigens (35, 36).

$CD8^+$ Tregs can also be induced by plasmacytoid DCs both in animal and human models (23, 37). In humans, Wei et al. (23) described $CD8^+$ Tregs induced by plasmacytoid DCs from tumor ascites that suppress tumor Ag-specific T cell effector function through IL-10 secretion.

Natural $CD8^+$ Treg population distinguished by the expression of CD122 (the β-chain of the IL-2R) (38) was described in mice. It should be stressed that mice T cells do not express MHC class II Ags, and these $CD8^+$ Tregs have not been identified to date in humans. These murine and human $CD8^+$ Tregs appear to exert their suppressor effect via different mechanisms. $CD8^+HLA\text{-}DR^+$ Tregs act through cell-to-cell contact, and $CD8^+CD122^+$ Tregs exert their regulatory effects through IL-10. Finally, because $CD8^+$ $HLA\text{-}DR^+$ Tregs express CD28, these Tregs can be differentiated from the previously described $CD8^+$ Tregs that lack the expression of this costimulatory molecule.

Although expression of HLA-DR is considered to be a marker of activation, their presence and suppressor activity in CB $CD8^+HLA\text{-}DR^+CD45RA^+$ T cells strongly support the concept that these cells represent naturally developed Tregs. Additionally, they do not express activation markers such as CD25 and CD38. Moreover, only activated and expanded $CD8^+HLA\text{-}DR^+$ T cells retain their suppressor activity.

We can conclude that CD8+ T cells from adult and CB samples constitutively expressing HLA-DR represent, to our knowledge, the first example of natural human CD8+ Tregs. The suppression appears to be cell-to-cell contact dependent and may involve CTLA-4 signaling between neighboring T cells. These Tregs can be expanded in vitro and exhibit a suppressive capacity similar to that observed in ex vivo CD8+HLA-DR+ Tregs. Concerning the biological implications of these natural CD8+ Tregs, our finding that CD8+HLA-DR+ T cells represent >80% of CD8+ T cells detected in patients with NSCLC opens a new avenue of research; particularly, it will be important to uncover the factors that recruit CD8+ HLA-DR+ Tregs to the tumor. Overall, the data presented in this study indicate that CD8+HLA-DR+ Tregs most likely control antitumor immune responses in the local environment during the early stages of lung tumor development. Thus, therapeutic strategies that aim to overcome Treg activity as a means of enhancing antitumor immune responses must take into account this novel intratumoral subset of highly suppressive CD8+HLA-DR+ Tregs.

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Disclosures

The authors have no financial conflicts of interest.

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