Hodgkin’s Reed-Sternberg cell line (KM-H2) promotes a bidirectional differentiation of CD4⁺CD25⁺Foxp³⁺ T cells and CD4⁺ cytotoxic T lymphocytes from CD4⁺ naive T cells

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Abstract: A recent report revealed that a large population of Hodgkin’s lymphoma-infiltrating lymphocytes (HLILs) consisted of regulatory T cells. In this study, we cocultured CD4⁺ naive T cells with KM-H2, which was established as a Hodgkin’s Reed-Sternberg cell line, to clarify their ability to induce CD25⁺Foxp³⁺ (Foxp³⁺) T cells. The characteristic analyses of T cells cocultured with KM-H2 revealed the presence of CD4⁺CD25⁺ T cells. They expressed CTLA-4, glucocorticoid-induced TNFR family-related gene, and Foxp3 and could produce large amounts of IL-10. Conversely, KM-H2 also generated CD4⁺ CTLs, which expressed Granzyme B and T cell intracellular antigen-1 in addition to Foxp3⁺ T cells. They exhibit a strong cytotoxic effect against the parental KM-H2. In conclusion, KM-H2 promotes a bidirectional differentiation of CD4⁺ naive T cells toward Foxp3⁺ T cells and CD4⁺ CTLs. In addition to KM-H2, several cell lines that exhibit the APC function were able to generate Foxp3⁺ T cells and CD4⁺ CTLs. Conversely, the APC nonfunctioning cell lines examined did not induce both types of cells. Our findings suggest that the APC function of tumor cells is essential for the differentiation of CD4⁺ naive T cells into CD25⁺ Foxp3⁺ T cells and CD4⁺ CTLs and at least partly explains the predominance of CD25⁺Foxp3⁺ T cells in HLILs and their contribution to a better prognosis. Therefore, in APC-functioning tumors, including classical Hodgkin lymphomas, which generate Foxp³⁺ T cells and CD4⁺ CTLs, these T cell repertories play a beneficial role synergistically in disease stability. J. Leukoc. Biol. 82: 576–584; 2007.

Key Words: antigen-presenting cell · tumor surveillance · regulatory T cell · neoplastic cell

INTRODUCTION

Hodgkin and Reed-Sternberg (H-RS) cells are malignant neoplastic cells of classical Hodgkin lymphoma (cHL). Since the discovery of H-RS cells, they have continued to be an enigma in terms of their origin and nature. However, recent progress in molecular biology has highlighted the germinal center B cell origin of H-RS cells [1]. Another controversial issue concerning cHL is the unique histological architecture of the neoplastic lesions, in which only a small number of H-RS cells are scattered among much more abundant, reactive, nonneoplastic lymphocytes. Currently, the mechanism underlying this peculiar histology of cHL and the biological nature of Hodgkin’s lymphoma-infiltrating lymphocytes (HLILs) attract a great deal of attention. It has been shown that most HLILs are CD45RO⁺CD25⁺CD4⁺T cells, indicating that HLILs represent a memory subset [2]. Previous studies suggested that based on the profile of cytokine production, HLILs are Th2 cells [3, 4]. Conversely, recent studies have indicated that HLILs produce hardly any Th2 cytokines (IL-4 or IL-13) [5, 6]. Moreover, another study showed that HLILs produce IL-10 but not IL-4, raising the possibility that HLILs might be regulatory T (Treg) cells [7].

Recent studies by Marshall et al. [8] revealed that HLILs contain a large number of immunosuppressive CD4⁺CD25⁺ Treg cells, and studies by Alvaro et al. [9] have revealed that abundant Treg cells are present in HLILs. These findings may lead to the assumption that Treg cells surrounding H-RS cells participate in the immune-defense mechanism of cHL. However, it remains unclear how Treg cells become mobilized around H-RS cells. One possible mechanism is that H-RS cells themselves induce CD4⁺ naive T cells to differentiate into Treg cells. This hypothesis is postulated partly in view of the finding of our previous study, wherein we demonstrated that a H-RS cell line (KM-H2) has a dendritic cell (DC)-like phenotype and exhibits T cell stimulatory functions [10, 11].

In this study, we show that KM-H2-primed CD25⁺ T cells from CD4⁺ naive T cells without the cooperation of any other
factors. KM-H2-primed T cells contain the cells, which strongly express the Forkhead box p3 (Foxp3) gene and produce the immunosuppressive cytokine IL-10. In addition to Foxp3+ T cells, KM-H2 generates CD4+ CTLs, which exhibit a cytotoxic effect against the parental KM-H2. The APC function of tumor cells was essential to the generation of these CD25+Foxp3+ cells and CD4+ CTLs.

MATERIALS AND METHODS

Media and cells

RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin, and heat-inactivated 10% or 20% FCS was used for the cell culture throughout the experiments. In this study, we used the cell lines that were KM-H2, L-428, L-540, Raji, lymphoblastoid cell lines (LCL), Jurkat, HL-60, THP-1, or Hela. PBMCs from healthy volunteer donors were prepared by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation of heparinized venous blood. Allogeneic CD4+/CD45RA- naive T cells were isolated from PBMCs by negative selection to deplete CD14 (M5E2, Becton Dickinson), positive CD19 (HIB19, Becton Dickinson)-positive, or CD56 (B159, Becton Dickinson, San Diego, CA, USA)-positive, CD16 (3G8, Becton Dickinson)-positive cells, and then analyzed by FCM. The result clearly showed the presence of CD4+Foxp3+ cells in PBMCs from healthy donors.

Immunostaining

Cells were stained with FITC-labeled mAb against CD25 (M-A251, Becton Dickinson) and CD45RO (UCHL1, Becton Dickinson), PE-labeled mAb against CD69 (CH/11, Becton Dickinson), or CD122 (Mik-b2, Becton Dickinson), PE-labeled mAb (4S.B3, Becton Dickinson) and then analyzed by a FACScan (Becton Dickinson). Immunostaining was performed on cryostat sections of PBMCs, which were collected after 8 days of coculture with KM-H2 or Hela as target cells.

Dual staining of intracellular IFN-γ and intranuclear Foxp3

After 8 days of coculture, KM-H2/T cells were washed and restimulated with PMA (25 ng/ml) and ionomycin (2 μg/ml) for 6 h, and brefeldin A (10 μg/ml) was added during the final 2 h (all from Sigma Chemical Co., St. Louis, MO, USA). KM-H2/T cells were stained with FITC-labeled anti-CD25 mAb or PC5-labeled anti-CD30 mAb (BerH8, Becton Dickinson) and then analyzed by FCM. The result clearly showed the presence of CD4+Foxp3+ cells in PBMCs from healthy donors.

CFSE labeling

CD4+ naive T cells were resuspended in PBS at a final concentration of 1 × 10^6 cells/ml and were labeled with 500 nM 5 (and 6) CFSE (Molecular Probes, Eugene, OR, USA) for 10 min at 37°C in 5% CO2. The cells were then washed twice in culture medium. The CFSE-labeled cells were cultured with or without KM-H2 for 4 days, and these T cells were then stained with PE-labeled anti-CD25 (4 E3, Miltenyi Biotec) or Foxp3 mAb and analyzed by flow cytometry (FCM).

ELISA for cytokines

After 8 days of coculture, 2 × 10^6 cells of CD25+ KM-H2/T fraction, CD25- KM-H2/T fraction, or medium-activated T (MAT) cells were restimulated with plate-bound anti-CD3 mAb (4 μg/ml) for 48 h, and the supernatants were then collected. The concentrations of IL-10 or IFN-γ were assessed using ELISA kits (all from Endogen, Woburn, MA, USA) according to the manufacturer’s instructions. The ODs of individual wells were determined at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

KM-H2 survival

On Day 5, KM-H2, cultured with or without primed T cells, was washed with PBS containing 2 mM EDTA, and viable cells were counted in triplicate with trypan blue exclusion of the dead cells. Remaining cells were incubated with FcR-blocking reagent and then stained with FITC-labeled anti-CD3 mAb (BeH8, Becton Dickinson) and PC5-labeled anti-CD3 mAb. Cells were analyzed by FCM to detect CD3+ CD30+ T cells and CD3+ CD300 KM-H2. Dead cells were excluded on the basis of side- and forward-scatter characteristics. The number of viable CD30+ KM-H2 was calculated on the basis of the total viable cell counts. To determine apoptosis, KM-H2 was stained with FITC-labeled annexin-V mAb (Bender MedSystems, Vienna, Austria) and PE-labeled anti-CD83 mAb (HB15e, Becton Dickinson) to detect KM-H2.

Cytotoxic assay

The cytotoxic activity of the KM-H2/T cells was quantitated according to the manufacturer’s instructions (Cytotox96, Promega, Southampton, UK). Following 8 days of coculture, the CD25+ KM-H2/T fraction or CD25- KM-H2/T fraction was isolated as effector cells and was incubated with fresh KM-H2 or Hela as target cells. The E:T ratios were 5:1 or 1:1. After 4 h of incubation, the released lactate dehydrogenase (LDH) was measured at 450 nm using a microplate reader (Bio-Rad). Percent cytotoxicity = [experimental LDH release – (effector spontaneous LDH release) – (target spontaneous LDH release)]/[target maximum LDH release] × 100.

RT-PCR

Total RNA was extracted from 1 × 10^6 cells using TriZol reagent (Gibco-BRL, Grand Island, NY, USA) according to the manufacturer’s instructions. For RT-PCR, cDNA was synthesized from 2 μg total RNA using ReverTraAce (Toyobo, Tokyo, Japan) and oligo-dT18 primer. The primers were designed with fresh cycles of PCR in 20 μl reaction buffer containing BlendTaq DNA polymerase (Toyobo) and 10 pmoles each primer. PCRs were performed on a GeneAmp 2400-programmable thermal cycler (Perkin Elmer, Wellesley, MA, USA). The individual cycles consisted of 20 s at 90°C and 2 min at 65°C. The sequences of oligonucleotide primers were as follows: for human Foxp3, 5′-ATGTCCTCCTCA-GAGAGAGTGAAGGTCTCAAACATGATCTGGGTC-3′ and 5′-TGAAACCCGTGTAATGTCTCAGAAGATGC-3′; for human Tbet, 5′-AAGTTTAATCAGCACCAGACAGAGATGGTACAGTC-3′ and 5′-AACAGATGTGTACATGGACTCAAAGTTCTC-3′; for human Foxp3, 5′-GTGGTGAAAGCCTCTCAACATGCATTGGTCTC3′- and 5′-CAAGATCATTGCTCCTCTCAGCCAAATGAA3′.

RESULTS

KM-H2 up-regulated the expression level of CD25 and CTLA-4 in CD4+ naive T cells

We first assessed whether KM-H2 could promote the proliferation of CD4+ naive T cells. After 8 days of coculture, we counted the absolute cell numbers of KM-H2-primed T cells (KM-H2/T cells) using trypan blue staining. CD4+ naive T cells were cultured with or without KM-H2 for 8 days, and these T cells were then stained with PE-labeled anti-CD25 (4 E3, Miltenyi Biotec) or Foxp3 mAb and analyzed by flow cytometry (FCM). The result clearly showed the presence of two distinct populations: vigorously expanding cells and completely quiescent cells (Fig. 1B).
We next assessed whether KM-H2 could up-regulate the expression levels of Treg cell-related surface molecules in CD4^+ naive T cells. As CD25 and CTLA-4 were recognized as the major hallmarks of CD25^+ Treg cells, we examined their expression levels during the coculture period [12, 13]. A remarkable increase in the proportion of KM-H2/T cells, which were CD25-positive, was observed in a time-dependent manner (Fig. 1C). After 8 days of coculture, the proportion reached 66%. However, the proportion was still less than 10% when CD4^+ naive T cells were cultured without KM-H2 (data not shown). Likewise, the percentage of intracellular CTLA-4-positive cells also increased in a time-dependent manner (Fig. 1C). We also measured the expression level of CD69 as an activation marker. It is unexpected that all the T cells converted rapidly to positive staining for CD69, regardless of their CD25 staining (Figs. 1C and 2). Thus, we concluded that CD25^+ and CD25^- KM-H2/T fractions had achieved a terminal differentiation.

Fig. 1. KM-H2 induced CD4^+ naive T cells to proliferate and differentiate. (A) CD4^+ naive T cells were cultured with or without irradiated KM-H2 for 8 days. The cultured cells were harvested, and the number of viable cells was counted using trypan blue. Results are shown as the mean ± SEM of five independent experiments. Statistical analysis was performed using the Mann-Whitney test. (B) CFSE-labeled CD4^+ naive T cells were cultured with or without irradiated KM-H2 for 8 days. The cultured cells were stained for CD3 and analyzed for CFSE. (C) CD4^+ naive T cells were cocultured with irradiated KM-H2 for 2, 4, 6, or 8 days following analyses by FCM. All histograms were gated on CD3^+ cells and showed the lymphocyte staining positive for CD25, intracellular CTLA-4, or CD69. Representative data from five independent experiments are shown.

Fig. 2. CD25^+ KM-H2/T fraction expressed the surface markers associated with naturally occurring Treg cells. After 8 days of coculture with irradiated KM-H2, T lymphocytes were isolated for a CD25-positive or -negative fraction. Each fraction was stained for CD45RO, CD122, GITR, CD134, or CD69. Shaded histograms show the lymphocytes staining positive for respective antibodies, and open histograms show the lymphocytes staining positive for isotype control antibodies. Data represent one of three independent experiments.
CD25+ KM-H2/T fraction shared the phenotypical features of CD25+ Treg cells

We next analyzed the cell surface expression of various molecules on CD25+ or CD25− fractions of KM-H2/T cells. Freshly isolated CD4+ naive T cells were CD25+/CD45RAhigh/CD45RO−/CD122+/GITR−/CD134− at a purity of more than 95%. The CD25− KM-H2/T fraction kept the character of CD45RO−/CD122+/GITR−/CD134−, although they were converted into CD69-positive cells. Nevertheless, the CD25+ KM-H2/T fraction altered their surface phenotype to CD45RO+/CD122+/GITR+/CD134+, whereas the CD25− KM-H2/T fraction never expressed IFN-γ simultaneously. As described, the CD25− KM-H2/T fraction contained Foxp3+ and Foxp3− T cells. Therefore, we used the dual-staining method for intracellular cytokines and intranuclear Foxp3 to confirm the cytokine production of the Foxp3+ expressing cells. We were able to show clearly that the CD25+ Foxp3+ T cells produced IFN-γ but that the CD25+ Foxp3− T cells did not (Fig. 4C). As T-bet is an essential transcription factor for IFN-γ

CD25+ KM-H2/T fraction produced IFN-γ and IL-10, but CD25− KM-H2/T fraction did not

We investigated cytokine production for CD25+ and CD25− KM-H2/T fractions by ELISA. In vitro, the restimulated CD25+ KM-H2/T fraction secreted large amounts of IL-10, but the CD25− KM-H2/T fraction never produced IL-10 (Fig. 3A). We also demonstrated that the restimulated CD25+ KM-H2/T fraction was capable of releasing IFN-γ in addition to IL-10 (Fig. 3B). In addition to these cytokines, we analyzed a membrane-bound type of TGF-β on KM-H2/T cells, but the result revealed that the whole KM-H2/T cell fraction stained negative for TGF-β (data not shown).

KM-H2/T cells were composed of three distinct subpopulations, which could be distinguished by CD25 and Foxp3 expression

For the reason that forkhead transcription factor Foxp3 has been considered to be a master control gene of CD25+ Treg cells and has allowed CD25+ Treg cells to be distinguished from activated CD25+ T cells, we examined the expression level of Foxp3 mRNA in isolated CD25+ and CD25− KM-H2/T fractions using the RT-PCR technique [14]. The result of RT-PCR clearly showed the expected size product of Foxp3 mRNA in the CD25+ KM-H2/T fraction after 8 days of coculture, whereas it was not detectable in the CD25− KM-H2/T fraction (Fig. 4A). Conversely, as T-bet is an essential transcriptional factor in T cells during IFN-γ production, we also determined the expression level of T-bet [15]. It is interesting that abundant expression of T-bet was observed as well as Foxp3 in the CD25+ KM-H2/T fraction (Fig. 4A). Based on this expression profile, we subsequently tried to visualize the Foxp3+ expressing cells in the KM-H2/T fraction using an intranuclear staining technique. Before the coculture with KM-H2, Foxp3 expressing cells were not detectable in CD4+ naive T cells. After 8 days of cocultivation, only a small number of Foxp3-expressing cells were present in the CD25+ T cell fraction of MAT cells, indicating that a spontaneous conversion of CD4+ naive T cells to CD25+ T cells was not associated with an induction of Foxp3+ T cells. On the contrary, a large proportion of the CD25+ KM-H2/T cell fraction strongly expressed Foxp3,
production, we considered the T-bet-expressing cells to be CD25+Foxp3+ T cells.

CD25+Foxp3+ KM-H2/T cells were converted from CD4+ naive T cells, subsequently expanding by cell proliferation.

To assess whether Foxp3+ KM-H2/T cells have a proliferative potential, we stained CD25 or Foxp3 for KM-H2/T cells using CFSE labeling. Again, CD25- and Foxp3-expressing cells were not observed in CD4+ naive T cells. We found that after cocultivation with KM-H2, a minor population of CD25+ T cells was induced without cell expansion, and the majority of CD25+ T cells was generated by cell expansion (Fig. 5, upper panels). We confirmed that the CD25− KM-H2/T fraction was completely quiescent. We also found that the major population of Foxp3+ T cells was generated by cell division and that a fraction of Foxp3+ T cells was induced without expansion (Fig. 5, lower panels). These findings suggest that the CD25+Foxp3+ KM-H2/T fraction is converted from naive T cells and generated by cell proliferation.

CD25+ KM-H2/T fraction had the cytotoxic function.

To assess whether the CD25+ KM-H2/T fraction has an effect on the viability of parental KM-H2, the CD25+ KM-H2/T fraction and CD25− KM-H2/T fraction were cultured for another 5 days with nonirradiated KM-H2. We found that unlike the CD25− KM-H2/T fraction, the CD25+ KM-H2/T fraction could suppress the proliferation of KM-H2 (Fig. 6A). Moreover, the CD25+ KM-H2/T fraction induced the up-regulation of Annexin V expression on KM-H2, indicating induction of apoptosis (Fig. 6B). Consequently, we speculated that the CD25+ KM-H2/T fraction involved a cytotoxic component such as CD4+ CTLs. As CD4+ CTLs have been reported recently to express Granzymes A and B, Perforin, and TIA-1, we investigated these markers in the CD25+ KM-H2/T fraction [16, 17]. The CD25− KM-H2/T fraction expressed none of these, whereas most of the CD25+Foxp3+ KM-H2/T cells and CD25+Foxp3− KM-H2/T cells expressed Granzyme B and TIA-1 but not Granzyme A and Perforin (Fig. 6C). We also found in the cytotoxicity assay that the CD25+ KM-H2/T fraction had a marked killing ability for KM-H2 but that the CD25− KM-H2/T fraction did not. This cytotoxic activity of the CD25+ KM-H2/T fraction was dependent on the E:T ratio (Fig. 6D, right panel). It is interesting that the CD25+ KM-H2/T fraction did not show a killing ability for Hela (Fig. 6D, left panel).

Fig. 4. CD25+ KM-H2/T fraction expressed Foxp3 and T-bet. (A) CD4+ naive T cells were cultured with or without irradiated KM-H2. Following 8 days of culture, a CD25+ KM-H2/T fraction or CD25− KM-H2/T fraction was isolated from the culture mixture. Simultaneously, fresh CD25+CD4+ T cells or CD25−CD4+ T cells were sorted from a healthy volunteer’s PBMC. The expression of Foxp3 or T-bet was analyzed using RT-PCR. Lane 1: MAT cells; Lane 2: CD25+ KM-H2/T fraction; Lane 3: CD25− KM-H2/T fraction; Lane 4: freshly isolated CD25+CD4+ T cells; Lane 5: freshly isolated CD25−CD4+ T cells. A representative result of three independent experiments is shown. (B) CD4+ naive T cells, MAT cells, or KM-H2/T cells were stained for CD25 and intranuclear Foxp3. Contour plots show the percentage of the indicated marker. Data represent one of five independent experiments. (C) After 8 days of coculture, KM-H2/T cells were restimulated for 6 h by PMA, ionomycin, and brefeldin A. The stimulated T cells were dual-stained for Foxp3 and IFN-γ. Contour plots show the percentage of the indicated marker. Data represent one of three independent experiments.

Fig. 5. CD25+Foxp3+ KM-H2/T cell and CD25+Foxp3− KM-H2/T cell have a proliferative ability. CFSE-labeled CD4+ naive T cells were cultured with irradiated KM-H2 for 8 days. The CFSE-labeled T cells were stained for CD3 and analyzed for CD25 or Foxp3 expression before and after the coculture. Data represent one of five independent experiments.
findings revealed the presence of CD4<sup>+</sup> CTLs, which had the ability of specific cell lyses for KM-H2 in the CD25<sup>+</sup> KM-H2/T fraction. Unfortunately, we could not determine whether Foxp3<sup>+</sup> cells or Foxp3<sup>−</sup> cells were CD4<sup>+</sup> CTLs, as they could not be separated from the others. However, we have already shown that CD25<sup>+</sup>Foxp3<sup>−</sup> KM-H2/T cells were able to produce IFN-γ, which was secreted preferentially by cytotoxic immunocomponents. We therefore consider that CD25<sup>+</sup>Foxp3<sup>−</sup> KM-H2/T cells might be CD4<sup>+</sup> CTLs rather than CD25<sup>+</sup>Foxp3<sup>+</sup> KM-H2/T cells.

**APC function was required for the induction of CD25<sup>+</sup>Foxp3<sup>+</sup> T cells and CD4<sup>+</sup> CTLs**

To determine whether the induction of Foxp3<sup>+</sup> T cells and CD4<sup>+</sup> CTLs was specific for KM-H2, we further analyzed other cell lines derived from various malignant tumors. L-428 and L-540, both of which were derived from cHL, converted CD4<sup>+</sup> naïve T cells into Foxp3<sup>+</sup> T cells and CD4<sup>+</sup> CTLs as efficiently as KM-H2 (Fig. 7). Similarly, the cell lines derived from Burkitt’s lymphoma (Raji), acute myeloid leukemia (HL-60), acute monocytic leukemia (THP-1), and B lymphoblastoid cell line established by EBV infection (LCL) were all able to induce Foxp3<sup>+</sup> T cells and CD4<sup>+</sup> CTLs. Conversely, the cell lines derived from T cell leukemia (Jurkat) or cervix cancer (Hela) failed to generate either type of cells. KM-H2, L-428, Raji, HL-60, THP-1, and LCL are known to exhibit the APC function [11, 18–22], and all of them express CD86. By contrast, Jurkat and Hela do not exhibit the APC function, and they lack the expression of CD86 (data not shown). Thus, we concluded that the APC function of tumor cells played a crucial role in generating CD25<sup>+</sup>Foxp3<sup>+</sup> T cells and CD4<sup>+</sup> CTLs.

Fig. 6. CD25<sup>+</sup> KM-H2/T cells have a cytotoxic ability. (A, B) CD4<sup>+</sup> naïve T cells were cultured with irradiated KM-H2 for 8 days. After extensive washing, the primed T cells were then cultured for another 5 days with fresh KM-H2 at a T:KM-H2 ratio of 5:1. (A) The number of viable CD30<sup>+</sup> KM-H2 was calculated as described in Materials and Methods. (B) The CD83-gated KM-H2 were stained with Annexin V to determine apoptosis. (C) After 8 days of coculture with irradiated KM-H2, T lymphocytes were isolated and were stained for Granzyme A, Granzyme B, T cell intracellular antigen-1 (TIA-1), or Perforin in addition to CD25 and Foxp3. Histogram shows the lymphocytes staining positive for respective antibodies. (D) Cytotoxicity assay was performed as described in Materials and Methods. (Left panel) The CD25<sup>+</sup> KM-H2/T fraction or CD25<sup>−</sup> KM-H2/T fraction was incubated with fresh KM-H2 or Hela. The E:T ratio was 5:1. (Right panel) The CD25<sup>+</sup> KM-H2/T fraction was incubated with fresh KM-H2 at the indicated E:T ratio. Data (A, D) are mean ± SEM of quintuplicate wells. (A–D) A representative result from three independent experiments is shown. Statistical analyses were performed using Student’s t-test.
DISCUSSION

Neoplastic cells use several strategies to ensure their own survival. Especially immunosuppression via Treg cells is an important aspect of this unique mechanism. CHL is also a disease entity, which is involved in Treg cell-correlated malignant tumors. Marshall et al. [8] stated that CD25$^{+}$ Treg cells are enriched in HLILs. Alvaro et al. [9] also visualized the presence of abundant Treg cells in cHL-involved lymph nodes using immunohistochemistry for tissue microarray of cHL patients. However, the origin of these Treg cells in HLILs remains to be elucidated.

There has been a rapid accumulation of experimental evidence concerning the origin of conventional Treg cells in humans. Watanabe et al. [23] stated that thymic stromal lymphopoietin-primed CD11c$^{+}$ DCs can convert human neonatal thymocytes but not peripheral T cells into CD25$^{+}$Foxp3$^{+}$ Treg cells. In addition, some experimental results indicate the generation of Treg cells in the thymus and their migration toward the periphery [24, 25]. Therefore, it has been proposed that CD25$^{+}$Foxp3$^{+}$ Treg cells originate in the thymus. Furthermore, a recent experimental approach has shown that CCL22, which was secreted by H-RS cells, mediated trafficking of CCR4$^{+}$Foxp3$^{+}$ Treg cells to the tumor microenvironment [26]. Another study indicated that ovarian cancer cells used a similar recruitment mechanism [27]. These experimental data strongly suggest that the tumor-associated Foxp3$^{+}$ T cells are drawn from a preserved Treg cell pool in the thymus toward the tumor-localized area.

Despite these results, others have suggested that CD25$^{+}$-Foxp3$^{+}$ Treg cells are generated in the periphery as well [28–31]. Ashley Moseman et al. [32] revealed that CpG oligodeoxynucleotide-primed CD11c$^{+}$ plasmacytoid DCs can convert human peripheral, CD4$^{+}$ naive T cells into CD25$^{+}$Foxp3$^{+}$ T cells. These findings led us to consider the possibility that de novo generation of CD25$^{+}$Foxp3$^{+}$ T cells occurred in the tumor-localized area in addition to the retrieval of CD25$^{+}$Foxp3$^{+}$ T cells from a circulating pool of Treg cells. We therefore came up with a working hypothesis that H-RS cells, which harbor an APC function, directly convert peripheral CD25$^{+}$Foxp3$^{+}$ T cells into the CD25$^{+}$Foxp3$^{+}$ T cells. Based on our working hypothesis, we conducted cocultivation of KM-H2 and CD4$^{+}$ naive T cells and then succeeded in the in vitro generation of CD25$^{+}$Foxp3$^{+}$ T cells. Our in vitro-generated CD25$^{+}$Foxp3$^{+}$ KM-H2/T cells expressed several Treg cell-associated molecules, such as CTLA-4, GITR, or CD45RO. Moreover, they produced large amounts of IL-10, which is now recognized as a Treg cell-associated immunosuppressive cytokine [13, 33]. These results clearly show that human peripheral CD4$^{+}$ naive T cells could be a precursor of CD25$^{+}$Foxp3$^{+}$ T cells in eHL and that they could be generated by H-RS cells directly.

Conversely, the infiltration of a large number of Treg cells, as depicted by immunological studies of clinical specimens, resulted in the designation of various malignant tumors as Treg cell-correlated tumors. For example, ovarian, lung, breast, pancreatic, and gastrointestinal tract cancers were included in this disease entity [27, 34–37]. As stated previously, eHL and non-Hodgkin lymphomas (NHL) were also enriched with tumor-associated Treg cells [8, 38]. However, there is a discrepancy concerning these tumor-associated Treg cells from the viewpoint of their influence on the disease progression. Actu-
ally, in APC-nonfunctioning tumors (e.g., ovarian cancer), Treg cells play an important role in tumor surveillance, and their enrichment has been recognized as a poor prognostic factor [27, 39]. APC nonfunctioning tumors are thus thought to be an effective target for Treg-depleting therapy. In contrast, it has been reported that a Treg cell-rich status was a good prognostic factor for patients of cHL and NHL [9, 40]. We speculated that this discrepancy results from the distinct procedures for recruiting the Treg cells. We showed that the APC function of neoplastic cells is essential for the de novo generation of CD25+Foxp3+ T cells from peripheral CD4+ naive T cells. These APC-functioning tumor-generating Foxp3+ T cells were independent of the thymic differentiation program of the host, leading them to be distinct from thymus-derived Foxp3+ Treg cells, which are chemoattracted by APC nonfunctioning tumors. Consequently, they likely have a distinct biological function, which may make them reciprocal prognostic factors.

Despite the Foxp3+ T cells, KM-H2 also induced CD25+Foxp3+ T cells. The majority of them demonstrated the expression of TIA-1 and Granzyme B, which were recognized as a hallmark of CD4+ CTLs [16, 17, 41]. They also exhibited a powerful cytotoxic ability against a parental KM-H2, leading to the conclusion that they belonged to the blood relatives of CD4+ CTL. During the past two decades, the cytotoxic potential has been thought to be peculiar to CD8+ T cells and NK cells [42]. Even if this were true, the alternative CTL subsets were defined. CD4+ CTLs were distinguished as an independent T cell subset, which harbors a cytotoxic ability. However, such cytotoxic ability in CD4+ T cells has usually been observed only in cell lines and CD4+ T cell clones generated by long-term in vitro culture and has therefore been considered to be an artifact [43]. However, recent studies have revealed the existence of CD4+ CTLs in vivo. A significant increase of CD4+ CTLs was observed in the peripheral blood during an early phase of primary HIV-1 infection. These CD4+ CTLs express high levels of TIA-1 and Granzyme B but low levels of Perforin [16]. In addition to HIV-1, such CD4+ CTLs had also been observed accompanying several other viral infections [17, 41]. Although they exhibit a cytolytic ability in a Perforin-dependent or Perforin-independent manner, their biological role remains unclear. In this study, we demonstrated that CD4+ CTLs killed tumor cells directly, suggesting that CD4+ CTLs might act as an effector repertoire for anti-tumor immune surveillance in vivo.

We have also demonstrated experimental evidence that APC-functioning tumor cells directly promote a bidirectional differentiation into Foxp3+ T cells and CD4+ CTLs. Unfortunately, we could not clarify the interaction between Foxp3+ T cells and CD4+ CTLs under these experimental conditions. In any case, Foxp3+ T cells are always generated together with CD4+ CTLs. In other words, the identical stimulus certainly promotes such a bidirectional differentiation of T cells. Thus, one possibility is that de novo-generated Foxp3+ T cells, but not naturally occurring Treg cells, play a crucial role in the definitive differentiation toward CD4+ CTLs. We speculated that Foxp3+ T cells and CD4+ CTLs could synergistically inhibit the tumor progression in vivo, leading to a good prognosis in APC-functioning tumors, including cHL. Therefore, a depletion of Treg cells from a patient with an APC-functioning tumor could reduce the number of CD4+ CTLs and accelerate disease progression, resulting in a worse prognosis. In conclusion, we propose that an independent strategy for Treg cell-modulating immunotherapy against tumors is needed for APC-functioning tumors and others.

The most important result of this study is the finding that human peripheral, CD4+ naive T cells can be converted into CD25+Foxp3+ T cells by coculture with APC-functioning, neoplastic cells. This is also the first report indicating the direct recruitment of CD25+Foxp3+ T cells by neoplastic cells. This result strongly supports the notion of peripheral Treg cell generation without thymic involvement but accompanied by neoplastic transformation and is a milestone in the exploration of the mechanism of Treg cells.

REFERENCES


