Disruption of T cell regulatory pathways is necessary for immunotherapeutic cure of T cell acute lymphoblastic leukemia in mice

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ABSTRACT. Acute lymphoblastic leukemia (ALL) is the most common cancer in children. In recent years, the outcome has been globally improved by current therapies, but it remains poor in patients with high, persistent residual disease following the first course of chemotherapy, prompting evaluation of the possible beneficial effects of immunotherapy protocols. In this study, we hypothesized that the disruption of two immunoregulatory pathways controlling the auto-reactive T cell response might synergize with dendritic cell-based immunotherapy of the disease, which is considered to be poorly immunogenic. In this study, we used TAL1xLMO1 leukemia cells adoptively transferred in mice, to generate murine leukemia with poorly immunogenic cells as a model for human T-ALL. Subsequently, these animals were treated with several different immunotherapeutic protocols. We compared the efficiency of a classical, dendritic cell-based immunotherapy (injection of dendritic cells loaded with tumor-derived antigenic products), to a combined treatment associating injection of antigen-loaded dendritic cells and disruption of the two immunoregulatory pathways: CD25+ suppressive T cells and cytotoxic T lymphocyte-associated antigens (CTLA-4). We show that this combined treatment resulted in cure, concomitantly with *in vivo* generation of immune memory, and TNF-alpha secretion. This study demonstrates that the disruption of these two immunoregulatory pathways synergized with immunostimulation by dendritic cells loaded with tumor-derived antigens, and paves the way for the testing of this combination in clinical trials.

Keywords: immunotherapy, leukemia, dendritic cells, T-ALL, regulatory T cells

In the last decades, therapeutic progress has dramatically improved the outcome of children with ALL. Fewer benefits were observed in adults [1]. The oncogene TAL1 is over-expressed by leukemia cells in about 50% of children and adults with T-ALL. In this disease, TAL1 over-expression is associated with an unfavourable outcome; the ectopic expression of the LMO1 oncogene is also found in a high percentage of TAL1-expressing cases [2]. The response of patients to chemotherapy has been identified as the major prognostic factor. Patients with high, persistent residual disease after the first course of chemotherapy have a high risk of relapse [3]. For these at-risk patients, there is hope that immunotherapy could elicit CTL responses capable of eliminating the residual leukemia cells after chemotherapy. The importance of T cell-mediated immune responses for eradication of residual disease is documented by the GVL effect that leads to lower relapse rates in AML patients who received allogeneic stem cell transplantation (ASCT) [4]. The fact that relapses are more frequent after T cell graft depletion further underscores the essential role of the immune system in controlling residual CML cells. This allogeneic effect is obviously weaker in ALL than in myeloid malignancies. Limited benefits of donor lymphocyte infusions were observed in patients with recurrent ALL after ASCT [5]. However, the relationship between these disparities and the poor immunogenicity of ALL blasts remains speculative.

One major obstacle to tumor-specific immunotherapy is the low efficiency of many cell types in antigen presenta-

Abbreviations:

ALL	acute lymphoblastic leukemia
LNB	leukemia necrotic bodies
CTLA-4	cytotoxic T lymphocyte-associated antigen

tion, but the systematic comparison of efficiency obtained by different antigen presentation systems within a unique tumor model has not been performed. It is believed that dendritic cells are the most efficient antigen-presenting cells, but their ability to generate anti-tumor immune responses *in vivo* has been documented in only a few therapeutic models. When tumor-specific antigens are not known, DC can be efficiently loaded with exosomes, tumor lysates, apoptotic or necrotic bodies, or with synthetic MHC class I-restricted peptides *i.e.* tumor-associated antigen-derived peptides. Many of these cancer antigen sources have been shown to induce tumor-specific immune responses [6-10].

A second obstacle to the generation of efficient tumor-specific immune response is the possibility of T cell tolerance towards tumor antigens. Of particular interest in this regard are the intensively studied sub-populations of CD4+ T cells, which have an inhibitory role in the control of autoreactive CD8+ T cells [11]. The tumor regression induced by injection of anti-CD25 antibodies has been documented [12, 13]. However, in these studies, the effect of CD25 depletion was only analyzed over a short period of 30 days. On the other hand, the role of CD4+CD25+ suppressive T cells in limiting autoreactive immune responses during anti-tumor therapy has been shown in mice, but the effect of CD25 depletion was studied in experiments with GM-CSF-secreting tumor cells as antigen-presenting cells (APC), not with DC [14]. Recent results have shown that it is important to separate the role of CD4+ CD25+ suppressive T cells and of CD4+ CD25- helper T cells because CD4 CD25+ T cells have been shown to suppress anti-tumor CTL responses and to permit the progressive growth of tumor cells [15, 16].

Efficient T-cell activation also depends on various co-stimulatory molecules on the surface of DC that enhance interactions with the corresponding molecules on T cells. The most efficient costimulatory molecule expressed by T cells is CD28, which shares its ligand with the "down-regulator" of T cell responsiveness, the CTL-associated antigen (CTLA-4) [17 1198]. CTLA-4 is expressed by antigen-presenting cells such as DC [18]. Anti-CTLA-4 antibodies have been successfully used for murine melanoma therapy [19]. However, the combined effect of CD25 depletion and CTLA-4 blockade was investigated in this paper with GM-CSF-secreting tumor cells, as opposed to potent APC such as DC [14].

Since no efficient immunotherapy protocol has been published for T-ALL up to now, we first defined an immunotherapy protocol based upon injection of DC loaded with leukemia necrotic bodies, and demonstrated that it elicited a partial elimination of T-ALL cells. We then compared this DC-based immunotherapy protocol to a combined treatment associating tumor antigen-pulsed DC with anti-CD25 depletion and CTLA-4 blockade. We deliberately selected this mAb combination since it had been shown to be the most efficient in association, in the B16 melanoma mouse model [14]. We show here that the disruption of those regulatory mechanisms, synergized with DC-based immunotherapy for eliminating poorly immunogenic T-ALL.

MATERIALS AND METHODS

Mice

6-week-old (C57BL/6xC3H) F1 mice were purchased from Harlan, France. TAL1 and LMO1 transgenic mice were generated as described [20]. All animal manipulations and housing were in accordance with our Research Institute Animal Care Committee guidelines.

Murine model of T-ALL: in vivo adoptive transfer of leukemia cells; leukemia challenge

For short term experiments, 2 x 10^6 T-ALL 72C18 cells, a subcloned line from a previously described leukemia tumor clone [21], were adoptively transferred by *i.v.* injection into the retro-orbital sinus of normal (C57BL/6xC3H) F1 mice. We verified that leukemia cells were detected *in vivo* at the time of DC-based immunotherapy by RQ-PCR with a TCRβ clonotype-specific probe. T-ALL 72C18 cells that expressed Vβ9 TCR were detected by quantitative PCR in the lungs, bone marrow and kidney, 3 and 6 days after injection.

For combined treatment experiments, we transferred T-ALL lines expressing undetectable levels of CD25 to avoid interference with the *in vivo* depletion protocol. Two T-ALL lines H39 and H535, fresh *ex vivo* leukemia cells from leukemic transgenic mice, were selected. They were $CD4^+8^+$-, $CD25^{--}$ $CD44^{Low}$-, $CD3^+$-, $CD95^+$- and MHC class I- low. They were used respectively for primary adoptive leukemia transfer (2 x 10^6 H39 cells injected *i.v.* at day 0), and challenge injections to demonstrate anti-leukemia long term memory (8 x 10^6 H535 cells injected *i.v.* at 32 weeks). When necessary, they were maintained for less than 24h in RPMI supplemented with 10% FCS, 2mM glutamine, penicillin (0.1 U/mL), and streptomycin (0.1 mg/mL), before the *in vivo* injections.

RQ-PCR analysis

BM and kidney samples were obtained after euthanasia of the mice. DNA was extracted as previously described. Primer and probe were designed using the Primer Express 1.0 software (Applied Biosystems, Foster City CA, USA).
Forward Vβ9 primer sequence: 5'-TACATTGGCTCTGCA GGCCTA-3'
Reverse Jβ2.5 primer sequence: 5'-GAGTGCCT GGCCCAAAGTAC-3'
Clonotypic probe sequence: 5'-Fam-TGTGCTAC GAGTAGAGGGACAGGGGGCCAA-Tamra-3'
Forward GAPDH primer sequence: 5'-GGGAAG CCCATCACCATCTT-3'
Reverse GAPDH primer sequence: 5'-GCCTTCTCC ATGGTGGTGAA-3'
GAPDH probe sequence: 5'-CAGGAGCGAGACCC CACTAACATCAAATG-3'
RQ-PCR was performed using standard procedures. Efficiency and specificity of the Vβ9-Jβ2.5 RQ-PCR system, was assessed on diagnostic DNA (50 ng/μL) serially diluted from 10^{-1} to 10^{-5} into spleen cell DNA. Calibration

curves were performed using 10-fold serial dilutions of diagnostic DNA into organ-related DNA. No-template controls (H_2O) as well as non-amplification controls (irrelevant leukemia cell line DNA) were included in each assay. Cell numbers were calculated using the ratio 670 ng of DNA for 10^5 cells.

Biological reagents

Antibodies for CD25 T cell depletion: PC61 (anti-CD25) and 4F10 (anti-CTLA4) hybridomas were kindly provided by Dr A. Bandeira (Pasteur Institute, Paris, France) and PC61 and 4F10 mAbs were purified from culture supernatants with Econopack 10 DG (BIORAD, Hercules, CA, USA). 400µg of PC61 mAb were injected *i.p.* 5 days before tumor cell injection. $2x10^5$ H39 lymph node leukemia cells were injected *i.v.* into F1 mice at day 0; 100 µg 4F10 anti-CTLA4 mAb were injected *i.p.* at days 3 and 6 post-tumor injection.

GM-CSF-rich supernatants were obtained from cultures of the GM-CSF-transfected J558 cell line provided by D. Gray (London, UK) [22]. Cells were maintained in IMDM (Sigma, St Louis, MI, USA) supplemented with 10 % heat-inactivated foetal calf serum (Biowest, Nuaillé, France), 2 mM glutamine, penicillin (0.1 U/mL), and streptomycin (0.1 mg/mL) (Invitrogen, Carlsbad, CA, USA).

Immunophenotype of fresh murine T-ALL. CD4, CD8, CD3, CD25, CD44, CD95 antibodies were purchased from BD Biosciences (San Diego, CA, USA). Phenotype analyses were performed on 10^5 cells using APC-, PE-, FITC-conjugated monoclonal antibodies.

Generation of dendritic cells (DC) *in vitro*, leukemia antigens preparation, and loading DC.

Murine femur, bone marrow cells were plated in untreated plastic Petri dishes at 4 x 10^5/mL in IMDM, supplemented with 10 % FCS and 30 % of GM-CSF-transfected J558 cell culture supernatant for at least 18 days before use. The activation capacity of each DC preparation was tested as previously described [23].

Loading DC with leukemia necrotic bodies was as follow: necrotic bodies were prepared by freezing cell suspensions in liquid nitrogen followed by thawing at 37°C. After 4 freeze-thaw cycles, necrotic bodies were centrifuged at 1500 rpm for 10 minutes and supernatants were sterilized using a 0.22 µm filter(Millipore, Billerica, MA, USA).
DC loading was achieved by incubating *in vitro*-generated DC with necrotic body supernatants in a 1:1 cell number equivalent ratio for 20 hours [24].

Maturation of loaded DC was induced by incubation with lipopolysaccharide (LPS) (100 ng/mL) (Sigma) 12 hours before injection. Maturation by LPS induced a strong activation of the Th1 response by DC with secretion of IL2, IL12 and INFγ [25]. Before injection, matured, loaded DC were washed, and 2 x 10^5 cells, resuspended in 100 µL of serum-free RPMI, were injected subcutanously in the flank of each mouce.

For anti-CD25 depletion experiments, pulsed DC were injected at day 3 and 6 post-tumor injection.

Th1/Th2 cytokine secretion levels in immunized animals

Each serum was tested with the BD Mouse th1/Th2 Cytokine CBA Kit (BD Biosciences) as recommended.

RESULTS

Description of our murine model of T-ALL, obtained by adoptive transfer of leukemia cells, in normal mice in vivo

One of us had previously developed a transgenic mouse model that demonstrated the synergic oncogenic role of the TAL1 and LMO1 genes [20]. After 4 months, 100% of the transgenic mice developed clonal T cell tumors, with diverse phenotypes that represent all stages of thymocyte development. However, since very little is known about a possible role of these two oncogenes in mature T cell functions in transgenic animals, we decided to evaluate our immuno-intervention protocols using a murine model of T-ALL obtained by adoptive transfer of leukemia cells in normal mice. T-ALL cells from TAL1 x LMO1 transgenic animals were transferred into immunocompetent, syngeneic, immuno-competent (B6xC3H) F1 mice. To determine the invasion pattern in these mice, hyperleukocytosis (> 10 x 10^6 cell/mL) was measured in the blood of mice after 3 weeks *(figure 1A)*. Leukemia cells were observed in many locations, including lymph nodes, brain and spleen *(figure 1B-D)*. The results showed that our leukemia transfer model reproduced the invasion pattern seen in double transgenic mice, which in turn is very similar to the invasion pattern observed in patients.

Injections of DC loaded with leukemia-derived antigens alone induced a partial regression of T-ALL as evaluated at 30 days

Having tested various protocols with different sources of antigens for DC loading and co-stimulatory signals, we selected the most efficient protocol against T-ALL (not shown), which was based on DC loaded with leukemia necrotic bodies (LNB) and then incubated with LPS. Indeed, maturation by LPS induced a DC activation that ensured a better activation of Th1 response by DC, with secretion of IL2, IL12 and INF-γ [25].
We generated immature DC that retained their phenotype, their antigen internalisation capacity and their sensitivity to activation, for about two months in culture [23]; 48h after loading and LPS treatment, DC-LNB maturation was revealed by their high levels of CD40 molecule expression, the ligand of the costimulatory molecule CD40L that is required for cytotoxic T cell generation [26] *(figure 2A)*. We also observed increased expressions of MHC class II, CD80 and CD86 molecules (not shown). Antigen-loaded, LPS-matured DC were injected at days +3 and +6 post-tumor injection. LPS-activated unloaded DC were injected as a negative control.
Our first experiments monitored short-term leukemia cell invasion in recipient animals using RQ-PCR with a T-ALL cell-specific Vβ-Dβ-Jβ probe. We first injected cells of the T-ALL clone 72C18, and then treated the animals with two injections of the immunotherapeutic DC at days +3 and +6.

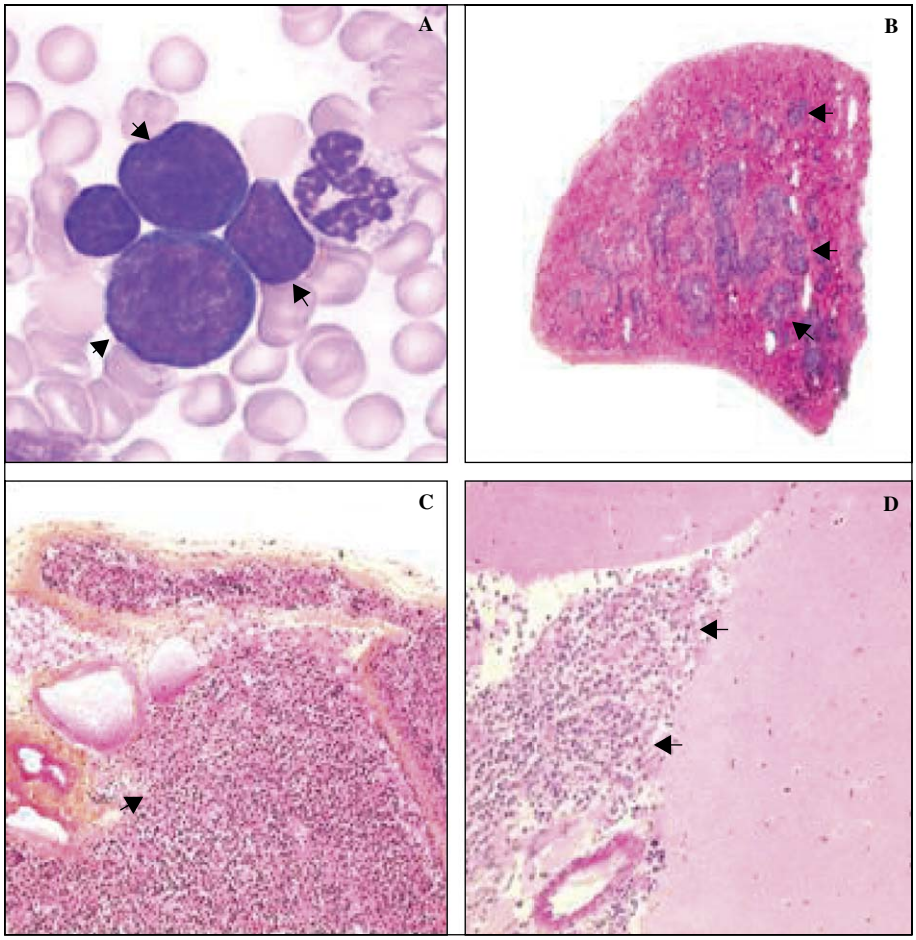

Figure 1

Invasion pattern of TAL1 x LMO1-expressing T-ALL cells in immunocompetent mice and induction of short term T-ALL regression by leukemia necrotic body-loaded DC.

T-ALL cells were obtained from transgenic mice lymph nodes. 2 x 10^5 cells were injected *i.v.* into the retro-orbital sinus in (C57BL/6xC3H) F1 mice. **A**) Peripheral blood smear showing a hyperleucocytosis (48G/L) mainly composed of lymphoid blasts (arrow head) of various size, basophilic cytoplasma and fine chromatin. A few segmented neutrophils (right) and lymphocytes (left) are shown. MGG x 3000. Mice were sacrificed 3 months after injection, and several organs were analyzed; **B**) Spleen: low magnification of the spleen, showing enlargement of lymphoid areas (arrowheads), hematoxylin-eosin x 25; **C**) Lymph node: disruption of the lymph node capsule, with invasion of the adipose tissue of the perinodal area (arrowhead), hematoxylin-eosin x 200; and **D**) the brain: invasion of the arachnoidal space by lymphoid cells (arrowheads) hematoxylin-eosin x 250.

Then, we measured residual disease one month later. 72C18 T-ALL cells expressed CD3, CD4 and CD8 molecules, and Vβ9-Jβ2.5 gene segments. Its clonality was verified by RT-PCR for the 23Vβ in Vβ-Cβ PCR, followed by direct sequencing of the PCR products (not shown). Since there were no residual leukemia cells circulating in the blood, their detection was performed using RQ-PCR and the DNA from the bone marrow and from a cryptic site, the kidneys.

We observed that the immunotherapeutic protocol with two injections of DC-LNB induced partial regression of T-ALL, with 1 % of leukemia cells, or near complete regression with less than 0.01 % of leukemia cells,. The overall anti-leukemia efficiency was one or two logs better in the bone marrow than in a cryptic site such as the kidneys *(figure 2B)*. Consistent results were obtained in duplicate evaluation of kidney and bone marrow samples (not shown). In view of these encouraging, but only partially successful results, we set up a more efficient immunotherapy protocol to induce long term survival.

Disruption of the CTL-A4 pathway and depletion of CD25+ suppressive T cells synergized with DC-based therapy in the long term cure of T-ALL

We attempted to improve the efficiency of the protocol by combining anti-CD25 depletion and blockade of CTLA-4 with DC-LNB injections (combined treatment) and compared its efficiency to DC-LNB treatment alone. To perform this experiment, we used as leukemia, cells the T-ALL H39 cell line, which does not expressed significant levels of CD25 molecule at the cell surface. Also, CD25 depletion was carried out before leukemia cell injection to avoid tumor cell depletion. We also randomly examined the efficacy of the CD25 cell depletion protocol. The number of CD4+CD25+ cells was measured by flow-cytometry in the blood of 6 out of 38 mice that had received CD25-depleting antibody, 5 days before tumor injection. The depletion was not total, but there was a five-fold reduction in the CD4+CD25+ T cell population in the blood *(figure 3A)*.

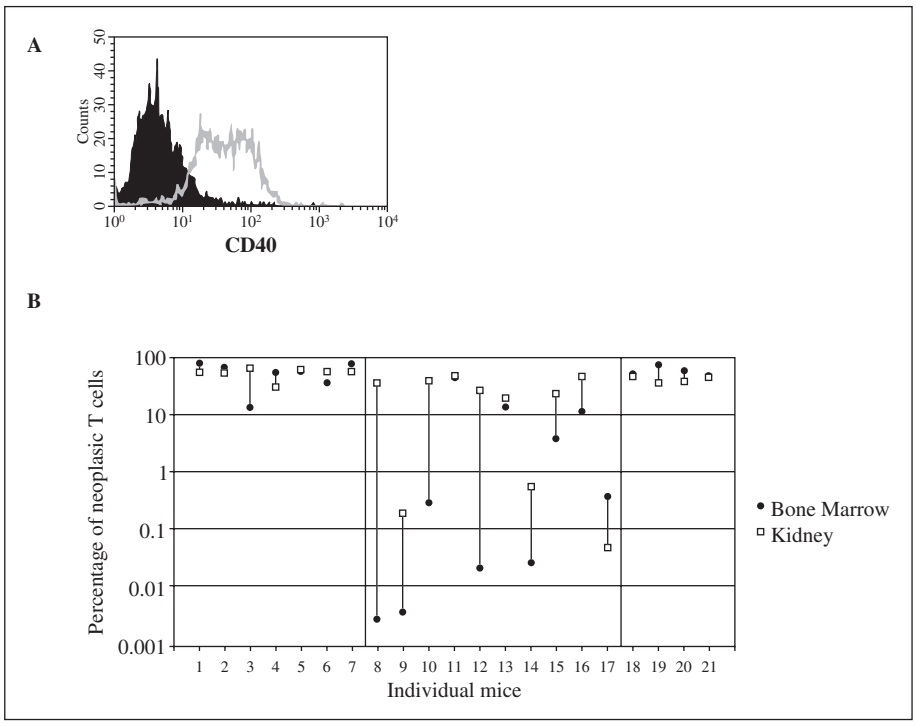

Figure 2

Induction of short term T-ALL eradication by leukemia necrotic body-loaded DC. **A**) Phenotypic analysis of bone marrow-derived DC after 48h exposure to LNB. LNB-activated DC expressed high levels of CD40 molecules compared to immature DC. **B**) Quantification of Vβ9-Jβ2.5 rearrangements by real time quantitative PCR in bone marrow and kidneys one month after injection. DNA of organs was amplified using forward Vβ9 and reverse Jβ2.5 specific primers as well as a clonotypic-specific Taqman probe. Mice received either DC+LPS (n = 7), DC-LNB-LPS (n = 10), or PBS (n = 4), (circle) bone marrow DNA and (circle) kidney DNA.

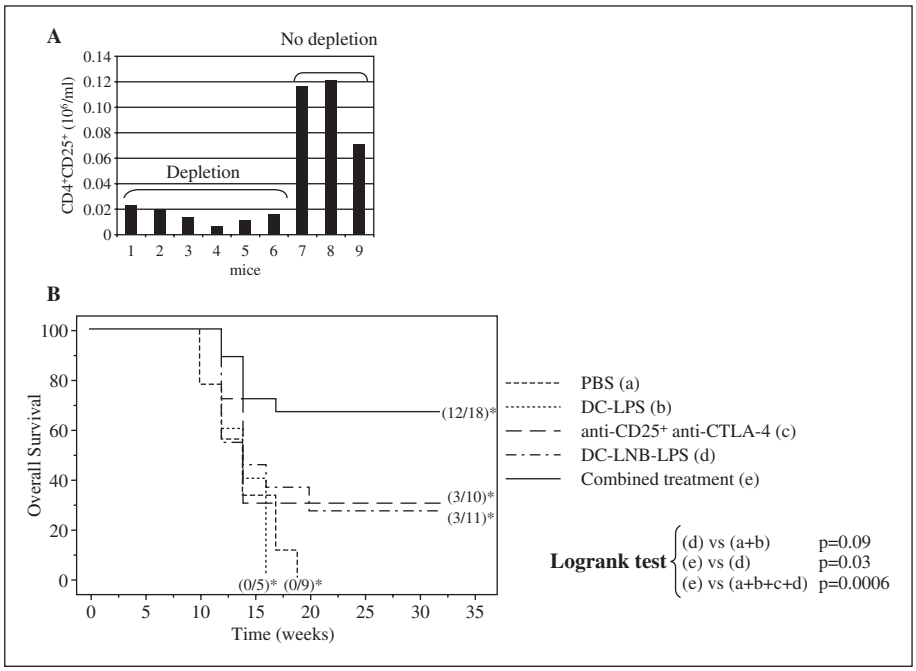

Figure 3

Efficiency of DC-based therapy combined with suppressive T cell depletion in T-ALL. **A**) CD4+CD25+ T cell population depletion in anti-CD25 mAb-treated mice. PBL taken at day 5 from mice injected *i.p.* with 400 µg of anti-CD25 mAb or with PBS were gated on live cells in the presence of PI and CD4+CD25+ positive T cells were analysed and the $CD4^+CD25+$ cell concentration was determined. **B**) Survival curves for leukemia-bearing mice treated with combination treatment. Mice received either PBS (n = 9), or DC-LPS (n = 5), DC-LNB-LPS (n = 11), anti-CD25 on day (–5) and anti-CLTA-4 at day 3 and 6 (n = 10), DC-LNB-LPS and depleting anti-CD25 on day (–5) and anti-CLTA-4 at day 3 and 6 (n = 18).

The combined treatment was as follows: five days after the CD25-depleting antibody injection, T-ALL H39 leukemia cells were adoptively transferred in CD25-depleted recipient mice, and then DC-LNB were injected at days +3 and +10 post-tumor injection. CTLA-4 antibodies were injected at day 3 with DC-LNB, and alone at day +6. Control groups of animal received either PBS injection alone, or unloaded LPS-matured DC (DC-LPS), or antigen-loaded and LPS-matured DC alone (DC-LNB-LPS) or treatment with depleting antibodies only (anti-CD25+ anti-CTLA-4),

We observed that depletion of CD25 T cells, combined with blockade of CTLA-4 without DC injection, resulted in a small increase in the number of surviving mice *(figure 3B)*, indicating either a marginal effect of antibody treatment on the growth of T-ALL cells, or that treatment with these two antibodies improved the natural immune response of these mice. DC-LNB injections alone resulted in survival of 50% of the mice at 13 weeks, then of 30% of the mice at 20 weeks post-injection. This result indicated that although DC-LNB could induce a therapeutic effect at one month post-injection *(figure 2B)*, this effect did not last long enough to ensure sufficient leukemia cell elimination ($p = 0.09$ at 20 weeks). Most interestingly, combined treatment, associating DC-LNB and mAb-induced disruption of suppressive pathways, resulted in a striking increase in treatment efficacy, with 65% of mice capable of rejecting the T-ALL cells and surviving over 32 weeks ($p = 0.003$ and $p = 0.0006$) *(figure 3B)*. This suggests that combined treatment, significantly improved the overall, long-term survival of mice when compared to DC-LNB-LPS treatment ($p = 0.03$) and when compared to unloaded DC or to antibodies ($p = 0.0006$).

Our results therefore clearly show that efficient immunotherapy against T-ALL can be obtained by the disruption of immunoregulatory mechanisms that synergize with DC-based immunotherapy.

Long-lasting, specific resistance of mice, cured of leukemia by combined treatment, to high leukemia burden: generation of specific anti-leukemia memory

We have shown in a previous study that mice, surviving after immunotherapy, could be challenged with a high burden of live tumor cells, and reject them, if they had developed a specific T or B cell-mediated response [10]. In the present study, a selected group of 6/12 mice, previously cured of leukemia following combined treatment, and 5 control mice, were challenged with a high dose of T-ALL H535 T cells (8 million) at 32 weeks. The group of combined treatment-cured mice was followed for signs of leukemia for about 60 additional weeks. We observed that, 6/6 of those mice survived the leukemia challenge, while no control mice survived, euthanasia or death occurring by 42 weeks in the 5 control mice. Our results show that high numbers of leukemia cells could not establish a tumor in mice treated with the combined treatment, which strongly suggests a T cell-mediated immune memory. Since T-ALL H535 and H39 cell lines were from distinct, individual mice, these data indicate that the immune response was not clonotype-specific and did not involve antigens other than TCR. The fact that DC-based immunotherapy, together with CD25 and CTLA-4 depletion, induced the eradication of leukemia with a memory response, strongly suggests the induction of a specific, T cell-cytotoxic response.

Injection of high dose leukemia cells to mice cured of leukemia by combined treatment, induced TNFα secretion in vivo

To measure cytokine production after tumor challenge with T-ALL cells, 6/12 mice previously cured of leukemia following combined treatment, and 5 untreated control mice, were challenged with the same, high dose of T-ALL H535 T cells at 32 weeks. One week after T-ALL challenge, Th1/Th2 cytokine protein levels were measured in the blood serum of injected mice. We measured interleukin-2 (IL-2), IL-4, IL-5, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα). In the serum of treated mice, TNFα protein levels reached 26.3 - 93. 9 pg/mL, while it stayed below detection level (< 20 pg/mL) in serum of control mice ($p = 0.002$). The increase of TNFα protein in the serum was probably due to secretion by CD8-expressing T lymphocytes [28]. In contrast, the other cytokines were not differentially produced (not shown).

We further investigated the effect of TNFα on the mitochondrial membrane of H535 cells, by measuring the mitochondrial damage with the potentiometric dye $Dioc_6$. We observed a partial depolarization after 24h of culture, indicating that the tumor cells were sensitive to TNFα apoptosis induction (not shown).

To analyze the immunogenic properties of our H535, H72C18 and H39 tumor cell lines in CTL assays, we used allogeneic T cells as effectors and syngeneic or allogeneic conA blasts as negative and positive control targets respectively. While allogeneic blasts could be lysed, syngeneic and tumor cells were resistant to allogeneic, T cell -mediated lysis (not shown). Furthermore, we observed that tumor cell lysis could be induced by perforin-granzyme purified from IL2-activated LAK cells indicating that their recognition by CTL could be the limiting reaction, and not their lysis (not shown).

Auto-immune manifestations in T-ALL therapy

Since LMO1 is expressed in the brain [30], and both TAL1 and LMO1 are necessary for primary hematopoiesis [31, 32], autoimmune responses were a potential risk of the combined treatment. We analyzed red blood cell numbers for the anemia, and the behavior of mice in their cage for neurological disorders. We did not observe any evidence of auto-immune manifestations in this model as has been reported with anti-CTLA-4-based therapy in both prostate tumor and melanoma treatment [14, 33].

DISCUSSION

Immune control of tumor regression is dependent on specific interactions between T lymphocytes and host tumor. One limitation to these immune responses is that tumor cells might express mainly self antigen, but very low levels of tumor-specific antigens, except when malignant transformation is due to viruses. Thus, according to the self-non-self model [34], the thymic selection would generate self-tolerance, with peripheral T cells having a low avidity for these self-antigens. Nevertheless, studies in bone

marrow-transplanted patients have shown that leukemia can be eliminated by T cells (graft-*versus*-leukemia effect, GVL). Based upon this GVL effect, it is possible that a vaccine inducing autologous T cells to kill leukemia cells, might have therapeutic potential. Several publications report vaccine induction of Wilm's tumor antigen (WT1)-specific CTL associated with clinical effects [35, 36]. However, WT1 is expressed in acute myelocytic leukemia and chronic myelocytic leukemia, but not in T-ALL. Thus, when cancer antigens are unknown, it is necessary to develop efficient immunotherapy by using bulk sources of antigens. T-ALL represent a particularly difficult target for immunotherapy protocols because they are poorly immunogenic, and they share many molecules with normal effector T cells since they result from the malignant transformation of their thymic precursors.

Previous studies have shown that DC are essential for the initiation of tumor immunity since they are capable of activating "low avidity" autoreactive T cells [24]. When DC are resting, they have high endocytosis capacities, which allow them to internalize antigens. Activation stimuli are then crucial for the production of more effective adjuvants for use in vaccines against pathogens. In contrast, immune responses to tumors can overcome T cell tolerance in the presence of co-stimulatory signals such as CD40 molecules [37]. Proof of efficiency of dendritic cell-based immunotherapy has been shown in animal models and with varying degrees of success in human clinical trials [38, 39]. In this study, we show that therapeutic injections of dendritic cells loaded with, and activated by, leukemia necrotic bodies induced an immune response against poorly immunogenic T-ALL. Apoptotic bodies and necrotic bodies can be endocytosed, but only the latter can activate DC to become co-stimulatory. Our own experiments are consistent with these observations since only leukemia necrotic body internalization by DC (DC-LNB) was capable of inducing an increased level of CD40 expression, while apoptotic body-loaded DC did not induce tumor regression. Several factors could contribute to the efficiency of DC-LNB, including the presence of heat shock proteins that participate in the maturation of DC, and various chaperone molecules bound to tumor-derived peptides which could facilitate peptide transfer to immature DC by receptor-dependent uptake [40-44].

Vaccination with DC loaded with leukemia-derived antigens alone induced a partial regression of T-ALL as evaluated at 30 days. Noteworthy is our observation that such a vaccination with loaded DC only, led to a striking reduction of tumor cell numbers in the bone marrow, but did not eradicate tumor cells from the kidneys. This variation shows that cryptic organs are less easily accessible to immune effectors than bone marrow, and might represent a hidden source of leukemia cells for relapse. The tumor regression that we observed in this setting after one month did not necessarily predict long-term survival since it could be mediated by NK cells which would not generate a memory response. This option was examined by testing NK depletion *in vivo* with anti-NK1.1 mAb PK136, but the time course of leukaemia progression was not modified (not shown). Therefore, mechanisms, in addition to dendritic cell-based therapy, are greatly needed to enhance tumor elimination.

In the present paper, we tested the idea that inhibition of regulatory T cell activity would increase the efficiency of such a protocol. This DC+ anti-CD25 + anti-CTLA-4 combined treatment has not been used in mice or in human so far. It is therefore possible that therapy using DC and CTLA-4 blockage might have a synergistic effect, improving the anti-tumor activity of the innate response. Human data in melanoma are promising, with response rates of up to 30% when given with anti-melanoma vaccines [33, 45]. Interestingly, CTLA-4 blockade seems to participate in an increased inflammatory response, leading to inhibition of angiogenesis, and to tumor growth inhibition [46, 47]. Regarding anti-CD25 mAb, it has been shown that treatment with anti-CD25 mAb alone was not enough to induce an immune response against several, poorly immunogenic leukemias [12]. in addition, it has also been shown to be insufficient for inducing autoimmunity in mice because second signals (TCR activation, nonspecific proliferation, inflammation) are needed to induce autoimmune diseases [48]. Thus anti-CD25 antibodies might be used to augment vaccine-related immunity in the clinical setting

Although one must be cautious in extrapolating mouse studies to human trials, we have shown here that the combination of LNB-DC-based therapy with CTLA-4 blockade and elimination of CD25+ suppressive T cells resulted in a remarkably effective anti-T-ALL therapy, which could be correlated with TNFα secretion. TNFα is known to be secreted by CTL against target cells during anti-viral and anti-tumor immune responses [28]. Furthermore, circulating TNFα could also play an important role in enhancing the number and functional capacities of circulating DC, and their survival [49-51].

In conclusion, this is a preliminary report presenting a promising strategy for the induction of T cell immunity against tumor-associated antigens that should be tested as immunotherapy for many more tumor types.

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REFERENCES

1. Hoelzer D. Acute lymphoblastic leukemia-progress in children, less in adults. *N Engl J Med* 1993; 329: 1343.
2. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, *et al.* Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell* 2002; 1: 75.
3. Cave H, van der Werfften Bosch J, Suciu S, Guidal C, Waterkeyn C, Otten J, *et al.* Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer--Childhood Leukemia Cooperative Group. *N Engl J Med* 1998; 339: 591.
4. Horowitz M, Gale R, Sondel P, Goldman J, Kersey J, Kolb H, *et al.* Graft-versus leukemia reactions after bone marrow transplantation. *Blood* 1990; 75: 555.
5. Kolb H, Schattenberg A, Goldman J, Hertenstein B, Jacobsen N, Arcese W, *et al.* Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. *Blood* 1995; 86: 2041.

6. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, van den Eynde B, *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; 254: 1643.

7. van den Eynde B, Lethe B, van Pel A, De Plaen E, Boon T. The gene coding for a major tumor rejection antigen of tumor P815 is identical to the normal gene of syngeneic DBA/2 mice. *J Exp Med* 1991; 173: 1373.

8. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998; 392: 86.

9. Toes RE, van der Voort EI, Schoenberger SP, Drijfhout JW, van Bloois L, Storm G, *et al.* Enhancement of tumor outgrowth through CTL tolerization after peptide vaccination is avoided by peptide presentation on dendritic cells. *J Immunol* 1998; 160: 4449.

10. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, *et al.* Eradication of established murine tumors using a novel cell-free vaccine: dendritic-cell derived exosomes. *Nat Med* 1998; 4: 594.

11. Nagai H, Hara I, Horikawa T, Oka M, Kamidono S, Ichihashi M. Elimination of CD4+ T cells enhances anti-tumor effect of locally secreted interleukin-12 on B16 mouse melanoma and induces vitiligo-like coat color alteration. *J Invest Dermatol* 2000; 115: 1059.

12. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 1999; 59: 3128.

13. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 1999; 163: 5211.

14. Sutmuller RPM, van Duivendoorde LM, van Elsas A, Schumacher TNM, Wildenberg ME, Allison JP, *et al.* Synergism of cytotoxic T lymphocyte-associated Antigen 4 blockade and depletion of CD25+ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 2001; 194: 823.

15. Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H, Fu YX. Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 2005; 201: 779.

16. den Boer AT, van Mierlo GJ, Fransen MF, Melief CJ, Offringa R, Toes R. CD4+ T cells are able to promote tumor growth through inhibition of tumor-specific CD8+ T-cell responses in tumor-bearing hosts. *Cancer Res* 2005; 65: 6984.

17. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green J, *et al.* CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994; 1: 405.

18. Cassell DJ, Schwartz RH. A quantitative analysis of antigen-presenting cell function: activated B cells stimulate naive CD4 T cells but are inferior to dendritic cells in providing co-stimulation. *J Exp Med* 1994; 180: 1829.

19. van Elsas A, Sutmuller RPM, Hurwitz AA, Ziskin J, Villasenor J, Medema JP, *et al.* Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T Lymphocyte Antigen-4 blockade in combination with a B16 Melanoma vaccine: comparison of prophylaxis and therapy. *J Exp Med* 2001; 194: 481.

20. Aplan PD, Jones CA, Chervinsky DS, Chuanzen W, McGuire EA, Gross KW, *et al.* An SCL gene product lacking the transactivation domain induces bony abnormalities and cooperates with LMO1 to generate T-cell malignancies in transgenic mice. *EMBO J* 1997; 16: 2408.

21. Chervinsky D, Lam D, Zhao X, Melman M, Aplan P. Development and characterization of T cell leukemia cell lines established from SCL/LMO1 double transgenic mice. *Leukemia* 2001; 15: 141.

22. Zal T, Volkmann A, Stockinger B. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J Exp Med* 1994; 180: 2089.

23. Regnault A, Lankar D, Lacabanne V, Théry C, Rodriguez A, Rescigno M, *et al.* Fc-gamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 1999; 189: 371.

24. Albert ML, Darnell JC, Bender A, Francesco L, Bhardwaj N, Darnell RB. Tumor-specific killer cells in paraneoplastic cerebellar degeneration. *Nat Med* 1998; 4: 1-4.

25. Morelli A, Zahorchak A, Larregina A, Colvin B, Logar A, Takayama T, *et al.* Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood* 2001; 98: 1512.

26. Bennett SRM, Carbone FR, Karamalis F, Flavell RA, Miller JFAP, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 1998; 393: 478.

27. Vantomme V, Boel P, De Plaen E, Boon T, van der Bruggen P. A new tumor-specific antigenic peptide encoded by MAGE-6 is presented to cytolytic T lymphocytes by HLA-Cw16. *Cancer Immun* 2003; 3: 17.

28. Bilsborough J, Uyttenhove C, Colau D, Bousso P, Libert C, Weynand B, *et al.* TNF-mediated toxicity after massive induction of specific CD8+ T cells following immunization of mice with a tumor-specific peptide. *J Immunol* 2002; 169: 3053.

29. Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, *et al.* CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* 2005; 174: 2591.

30. McGuire E, Hockett R, Pollock K, Bartholdi M, O'Brien S, Korsmeyer S. The t(11;14)(p15;q11) in a T cell acute lymphoblastic leukemia cell line activates multiple transcripts, including Ttg-1, a gene encoding a potential zinc finger protein. *Mol Cell Biol* 1989; 9: 2124.

31. Greenberg J, Boehm T, Sofroniew M, Keynes R, Barton S, Norris M, *et al.* Segmental and developmental regulation of a presumptive T-cell oncogene in the central nervous system. *Nature* 1990; 344: 158.

32. Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt F, Orkin SH. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* 1996; 86: 47.

33. Phan G, Yang J, Sherry R, Hwu P, Topalian S, Schwartzentruber D, *et al.* Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci USA* 2003; 100: 8372.

34. Janeway Jr. CA, Dianzani U, Portoles P, Rath S, Reich EP, Rojo J, *et al.* Cross-linking and conformational change in T-cell receptors: role in activation and in repertoire selection. *Cold Spring Harb Symp Quant Biol* 1989; 54 Pt 2: 657.

35. Mailander V, Scheibenbogen C, Thiel E, Letsch A, Blau IW, Keilholz U. Complete remission in a patient with recurrent acute myeloid leukemia induced by vaccination with WT1 peptide in the absence of hematological or renal toxicity. *Leukemia* 2004; 18: 165.

36. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, *et al.* Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci USA* 2004; 101: 13885.

37. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath W. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 1998; 393: 478.

38. Akiyama Y, Tanosaki R, Inoue N, Shimada M, Hotate Y, Yamamoto A, *et al.* Clinical response in Japanese metastatic melanoma patients treated with peptide cocktail-pulsed dendritic cells. *J Transl Med* 2005; 3: 4.

39. Caruso DA, Orme LM, Amor GM, Neale AM, Radcliff FJ, Downie P, *et al.* Results of a phase I study utilizing monocyte-derived dendritic cells pulsed with tumor RNA in children with stage 4 neuroblastoma. *Cancer* 2005; 103: 1280.

40. Tamura Y, Peng P, Liu K, Daou M, Srivastava PK. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* 1997; 278: 117.

41. Blachere NE, Li Z, Chandawarkar RY, Suto R, Jaikaria NS, Basu S, *et al.* Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J Exp Med* 1997; 186: 1315.

42. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 1999; 5: 1249.

43. Somersan SLM, Fonteneau JF, Basu S, Srivastava P, Bhardwaj N. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* 2001; 167: 4844.

44. Noessner E, Gastpar R, Milani V, Brandl A, Hutzler P, Kuppner M, *et al.* Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. *J Immunol* 2002; 169: 5424.

45. Sanderson K, Scotland R, Lee P, Liu D, Groshen S, Snively J, *et al.* Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and montanide ISA 51 for patients with resected stages III and IV melanoma. *J Clin Oncol* 2005; 23: 741.

46. Perrin P, Maldonado J, Davis T, June C, Racke M. CTLA-4 blockade enhances clinical disease and cytokine production during experimental allergic encephalomyelitis. *J Immunol* 1996; 157: 1333.

47. Hurwitz A, Foster B, Kwon E, Truong T, Choi E, Greenberg N, *et al.* Combination immunotherapy of primary prostate cancer in a transgenic mouse model using CTLA-4 blockade. *Cancer Res* 2000; 60: 2444.

48. McHugh RS, Shevach EM. Cutting Edge: Depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J Immunol* 2002; 168: 5979.

49. Caux C, Dambuyant CD, Schmitt D, Banchereau J. GM-CSF and TNF-alpha cooperate in the generation of dendritic cells. *Nature* 1992; 360: 258.

50. Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, Choi Y. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J Exp Med* 1997; 186: 2075.

51. Chen B, Shi Y, Smith JD, Choi D, Geiger JD, Mule JJ. The role of tumor necrosis factor alpha in modulating the quantity of peripheral blood-derived, cytokine-driven human dendritic cells and its role in enhancing the quality of dendritic cell function in presenting soluble antigens to CD4+ T cells in vitro. *Blood* 1998; 91: 4652.