View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Phase- and Stage-Related Proportions of T Cells Bearing the Transcription Factor FOXP3 Infiltrate Primary Melanoma

Giuseppe De Panfilis[1], Nicoletta Campanini[2], Marcello Santini[1], Giovanni Mori[3], Elena Tognetti[1], Roberta Maestri[2], Mara Lombardi[1], Elisabetta Froio[2], Donata Ferrari[1] and Roberto Ricci[2]

Although tumor-infiltrating lymphocytes (TILs) of primary cutaneous melanoma (PCM) include cytolytic T cells able to exert anti-PCM immunity, progression of PCM most frequently occurs, raising the hypothesis that the PCM microenvironment may also exert suppressive forces, for example, possibly developed by regulatory T (T_{REG}) lymphocytes. The aim of this study was to investigate whether TILs of PCMs include lymphocytes bearing the transcription factor forkhead box protein P3 (FOXP3), which is the T_{REG} lineage specification molecule in mice, and is debated to have a similar role in humans. Fourteen patients with PCM were selected, of which four had radial growth phase (RGP) stage I melanoma, five had vertical growth phase (VGP) stage I melanoma, and five had VGP stage III–IV melanoma. Formalin-fixed, paraffin-embedded sections were utilized for immunohistochemical single and double stainings. TILs of PCMs included FOXP3-bearing lymphocytes, which predominantly were CD20- and CD8-negative, but CD3-, CD4-, and CD25-positive, thus consistent with the standard immunophenotypical characteristics of "natural" T_{REG} cells. Further, the proportions of FOXP3-bearing lymphocytes were higher in vertical than in RGP ($P=0.001$), as well as in late than in early melanoma stages ($P<0.001$). Should these FOXP3-bearing lymphocytes actually exert regulatory capabilities within the PCM microenvironment, they may suppress "*in vivo*" the local anti-PCM immune response, thus favoring melanoma progression.

Journal of Investigative Dermatology (2008) **128,** 676–684; doi:10.1038/sj.jid.5701046; published online 13 September 2007

INTRODUCTION

It is well established that primary cutaneous melanoma (PCM) is nearly always associated with a chronic inflammatory infiltrate including various amounts of tumor-infiltrating lymphocytes (TILs) (Yazdi *et al.*, 2006). Melanoma, in fact, can induce immune responses that include the activation of melanoma-specific T cells (Romero *et al.*, 1998), which can interfere with the growth of melanoma (Yee *et al.*, 2000). On the other hand, ''*ex vivo*'' analysis of TILs revealed T-cell anergy (Guilloux *et al.*, 1994) and lack of cytolytic activity by $CD8^+$ TILs (Mortarini *et al.*, 2003; Zippelius *et al.*, 2004), suggesting that factors in the PCM microenvironment can lead to dysfunction of melanoma-specific activated TILs and to melanoma escape from immune-mediated destruction: this concept has led to a renewed search for immune resistance mechanisms in the PCM microenvironment (reviewed in Gajewski, 2006). To this respect, although such ''antitumor lymphocyte quiescence'' may have a lot of causes, an important role can be played by regulatory T (T_{REG}) cells (Coulie and Connerotte, 2005; Gajewski, 2006).

Indeed, ''natural'' that is, naturally arising T_{REG} cells, contrary to ''adaptive'' T_{REG} cells (Liu *et al.*, 2006), are a small subpopulation of T cells, canonically coexpressing the CD4 and the CD25 molecules, critically able to prevent autoimmunity against ''self'' antigens (Sakaguchi *et al.*, 1995, 2001; Asano *et al.*, 1996; Roncarolo and Levings, 2000; Shevach, 2000). Since antitumor immune responses are elicited to ''quasi-self'' antigens (Sakaguchi, 2005), natural T_{REG} cells can inhibit antitumor immunity (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999).

In the function of $CD4^+CD25^+$ T_{REG} cells, an important involvement is currently agreed of the transcription factor forkhead box protein P3 (FOXP3) (reviewed in Fontenot and Rudensky, 2005). In fact, FOXP3 belongs to a large family of functionally diverse transcription factors based on its winged helix-forkhead DNA-binding domain (Kaestner *et al.*, 2000). Murine $CD4^+CD25^+$ T_{REG} cell development is critically

[1]*Section of Dermatology, Department of Surgical Sciences, Parma University, Parma, Italy;* [2]*Section of Pathology, Department of Pathology and Laboratory Medicine, Parma University, Parma, Italy and* [3]*Department of General and Inorganic Chemistry, Analytical Chemistry and Physical Chemistry, Parma University, Parma, Italy*

Correspondence: Professor Giuseppe De Panfilis, Clinica Dermatologica Università di Parma, Via Gramsci 14, Parma I-43100, Italy. E-mail: giuseppe.depanfilis@unipr.it

Abbreviations: FOXP3, forkhead box protein P3; PCM, primary cutaneous melanoma; RGP, radial growth phase; T_{REG}, regulatory T cell; TIL, tumor-infiltrating lymphocyte; VGP, vertical growth phase

Received 27 March 2007; revised 15 June 2007; accepted 5 July 2007; published online 13 September 2007

© 2007 The Society for Investigative Dermatology

dependent on FOXP3 expression (Fontenot *et al.*, 2003). Moreover, FOXP3 overexpression in mice results in the acquisition of suppressive properties even by CD4$^+$ CD25$^-$ and by CD8$^+$ T cells (Fontenot *et al.*, 2003; Hori *et al.*, 2003; Khattri *et al.*, 2003): thus, FOXP3 functions as the T_{REG} lineage specification factor in mice (Fontenot and Rudensky, 2005). Whether FOXP3 can function as the T_{REG} lineage specification factor even in man, is currently a matter of debate: in fact, not all patients diagnosed with an autoimmune pathology analogous to a FOXP3 mutation-linked murine pathology have FOXP3 mutations (Owen *et al.*, 2003). In addition, induction of FOXP3 expression can occur ''*in vitro*'' following antigen stimulation in humans (Walker *et al.*, 2003; Morgan *et al.*, 2005; Roncador *et al.*, 2005), but not in mice (Fontenot *et al.*, 2003; Hori *et al.*, 2003), and such induced FOXP3 can either activate a T_{REG} program (Walker *et al.*, 2003; Morgan *et al.*, 2005; Roncador *et al.*, 2005) or not (Gavin *et al.*, 2006). It is still clear, however, that, as in the mouse, even in man FOXP3 appears to be a good marker for CD4$^+$ T cells with regulatory functions (reviewed in Ziegler, 2006).

The aim of this study was to investigate whether the TIL population infiltrating PCMs, scrutinized at the single-cell level in radial growth phase (RGP) *versus* vertical growth phase (VGP), as well as in early *versus* late melanoma stages, includes T lymphocytes expressing the molecules CD4, CD25, and especially the transcription factor FOXP3, namely a putative T_{REG} subset possibly negatively controlling ''*in vivo*'' the local immune response against PCMs.

RESULTS

TILs in the PCM microenvironment consist of different subpopulations of t cells

As expected, T cells constituted the vast majority of the TIL population of PCMs. Specifically, CD3$^+$ cells represented 92.36±1.09 (mean±SD) of the lymphocytic population infiltrating PCMs. In fact, CD20$^+$ B lymphocytes were virtually absent. On the other hand, the percentage of CD4$^+$ TILs was 53.55±5.85 (mean±SD) in CD3$^+$ TILs; CD25$^+$ TILs were 14.58±6.49 (mean±SD) in CD3$^+$ TILs.

TILs in the PCM microenvironment include a subset of FOXP3-positive lymphocytes

The transcription factor FOXP3 was revealed immunohistochemically at the single-cell level by intracellular, mainly nuclear (Lopes *et al.*, 2006), staining (Figure 1a). The overall proportion of FOXP3$^+$ lymphocytes in CD3$^+$ TIL (Figure 1b) was 13.39±1.65 (mean±SD) (Figure 1c) (Lombardi M, Campanini N, Ricci R, Tognetti E, Maestri R, De Panfilis G. Primary cutaneous melanoma is infiltrated by a T-cell population which includes FOXP3-bearing lymphocytes (abstr.). International Meeting ''Molecular targets in cancer therapy: mechanism and therapeutic reversal of immune suppression in cancer'', Clearwater Beach, FL, USA, January 25–28, 2007).

Characterization of FOXP3-positive TIL

Double staining experiments also showed that virtually 100% tumor-infiltrating FOXP3$^+$ lymphocytes coexpressed CD3

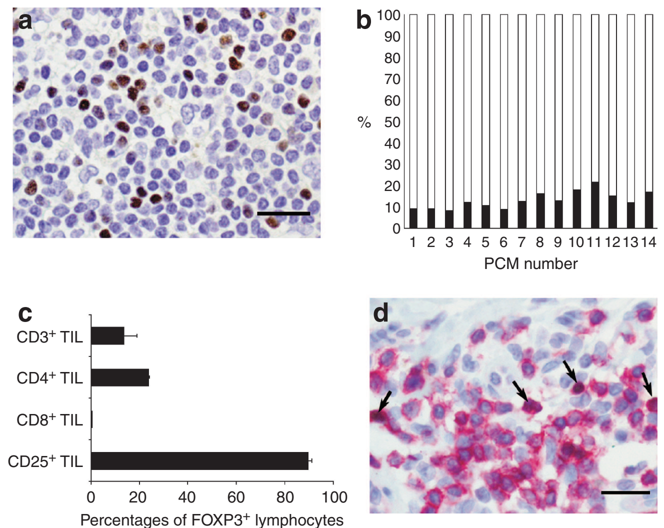

Figure 1. TILs in the PCM microenvironment include a subset of FOXP3-bearing T lymphocytes. (**a**) PCM infiltration of FOXP3-bearing cells. PCM tissue was taken and stained with anti-FOXP3 mAb. Bar = 25 μm. (**b**) Percentages of CD3$^+$FOXP3$^+$ cells (■) in CD3$^+$ lymphocytes infiltrating 14 PCMs. Tissues were stained with mAb to CD3 and to FOXP3, and CD3$^+$FOXP3$^+$ double stained cells were scored as a percentage of CD3$^+$(single plus double stained) cells. (**c**) Percentages of FOXP3-bearing lymphocytes, infiltrating 14 PCMs, in the considered T subpopulations. The percentages of FOXP3-bearing lymphocytes infiltrating PCMs were quantified by double staining analysis of FOXP3$^+$CD3$^+$ cells in CD3$^+$ population, FOXP3$^+$CD4$^+$ cells in CD4$^+$ subpopulation, FOXP3$^+$ CD25$^+$ cells in CD25$^+$ subpopulation (mean±SD). (**d**) FOXP3-bearing cells infiltrating PCMs are also CD3-positive. Tissue was stained with mAb to CD3 (red positivity) and to FOXP3 (brown nuclear positivity). Note that all the lymphocytes bearing FOXP3 within the nucleus are also CD3-positive at the periphery (arrows), whereas many CD3-positive cells are FOXP3-negative. Bar = 25 μm.

(Figure 1d), CD4 (Figure 2a), and CD25 (Figure 2b); by contrast, FOXP3$^+$ cells did not coexpress CD8 (Figure 2c) and CD20.

A subset of TIL with the ''natural'' T_{REG} lymphocyte phenotype infiltrate PCMs

Double labeling experiments also identified 25.64±1.96 (mean±SD) CD4$^+$CD25$^+$ cells in CD4$^+$ lymphocytes infiltrating PCMs. Since the CD4$^+$CD25$^+$ population is well known, as mentioned above, to include CD4$^+$CD25$^+$ FOXP3$^+$ cells, which act as T_{REG} (Ziegler, 2006), we directly compared by double stainings the CD25 *versus* FOXP3, as well as the CD4 *versus* FOXP3, expressions within the TIL population. A large subset (89.61±1.64, mean±SD) of infiltrating CD25$^+$ T cells coexpressed FOXP3 (Figure 1c), whereas the remaining CD25$^+$ cells were FOXP3$^-$; on the other hand, merely occasional CD25$^-$ T cells expressed FOXP3 in the examined samples. In the context of the CD4$^+$ TIL subpopulation, a subset (23.48±0.53, mean±SD) coexpressed FOXP3 (Figure 1c), whereas CD8$^+$ TIL did not virtually express FOXP3 (Figure 1c). Since, FOXP3$^+$ cells almost exclusively were both CD4$^+$ and CD25$^+$, rather than CD8$^+$ and CD25$^-$, these results strongly suggest that a subset of CD4$^+$ CD25$^+$ TIL infiltrating PCMs coexpresses FOXP3, thus showing the canonical phenotype of ''natural'' T_{REG}

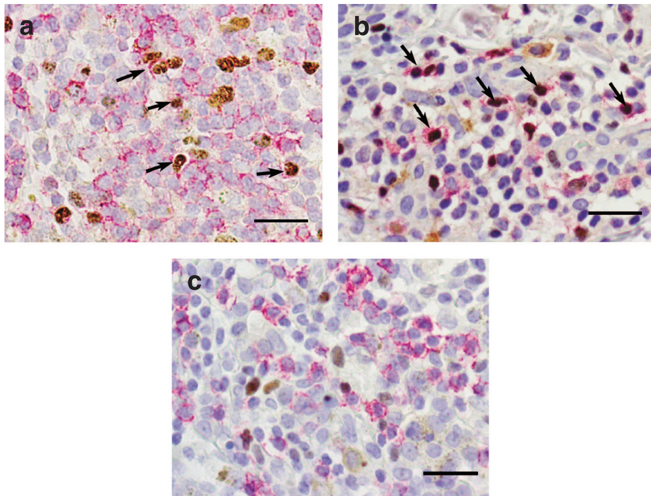

Figure 2. FOXP3-bearing cells harbored within the PCM microenvironment show a "natural" T_{REG} lymphocyte phenotype. (**a**) FOXP3-bearing cells infiltrating PCMs are also CD4-positive. PCM tissue was stained with mAb to CD4 (red membrane positivity) and to FOXP3 (brown nuclear positivity). Note that all the lymphocytes bearing FOXP3 within the nucleus are also CD4-positive at the periphery (arrows), whereas some CD4-positive cells are FOXP3-negative. Bar = 25 μm. (**b**) FOXP3-bearing cells infiltrating PCMs are also CD25-positive. PCM tissue was stained with mAb to CD25 (red positivity) and to FOXP3 (brown nuclear positivity). Note that all the lymphocytes bearing FOXP3 within the nucleus are also CD25-positive at the periphery (arrows), whereas scarce CD25-positive cells are FOXP3-negative. Bar = 25 μm. (**c**) FOXP3-bearing cells infiltrating PCMs are CD8-negative. PCM tissue was stained with mAb to CD8 (red membrane positivity) and to FOXP3 (brown nuclear positivity). Note that all the lymphocytes bearing FOXP3 within the nucleus are negative at the periphery, whereas all the CD8-positive cells at the periphery are not immunostained within the nucleus. Bar = 25 μm.

lymphocytes (Campanini N, Lombardi M, Ferrari D, Maestri R, Tognetti E, Ricci R, De Panfilis G. A consistent proportion of the melanoma cellular microenvironment is represented by FOXP3$^+$ lymphocytes coexpressing CD4 and CD25 (abstr.). "*4th international conference on tumor microenvironment: progression, therapy and prevention*", Florence, Italy, March 6–10, 2007). Considering that no functional data are presented in this study, it is possible to refer to these lymphocytes as phenotypic T_{REG} cells.

The proportions of FOXP3$^+$ TILs are higher in PCMs with VGP than in PCMs with RGP

The proportions of FOXP3$^+$ T lymphocytes infiltrating PCMs in different melanoma phases (Balch *et al.*, 2004) are shown in Figure 3a. PCMs with RGP of stage I melanoma patients were infiltrated by 9.96 ± 4.45 (mean ± SD) FOXP3$^+$ cells in CD3$^+$TIL, whereas PCMs with VGP of stage I melanoma patients were infiltrated by 12.53 ± 3.59 (mean ± SD) FOXP3$^+$ cells in CD3$^+$ TIL, and such a difference was significant ($P = 0.001$).

The proportions of FOXP3$^+$ lymphocytes infiltrating PCMs are higher in stages III–IV than in stage I melanoma

PCMs of stage I melanoma patients ($n = 9$) were infiltrated by a lower number of FOXP3$^+$ TIL than that infiltrating PCMs of

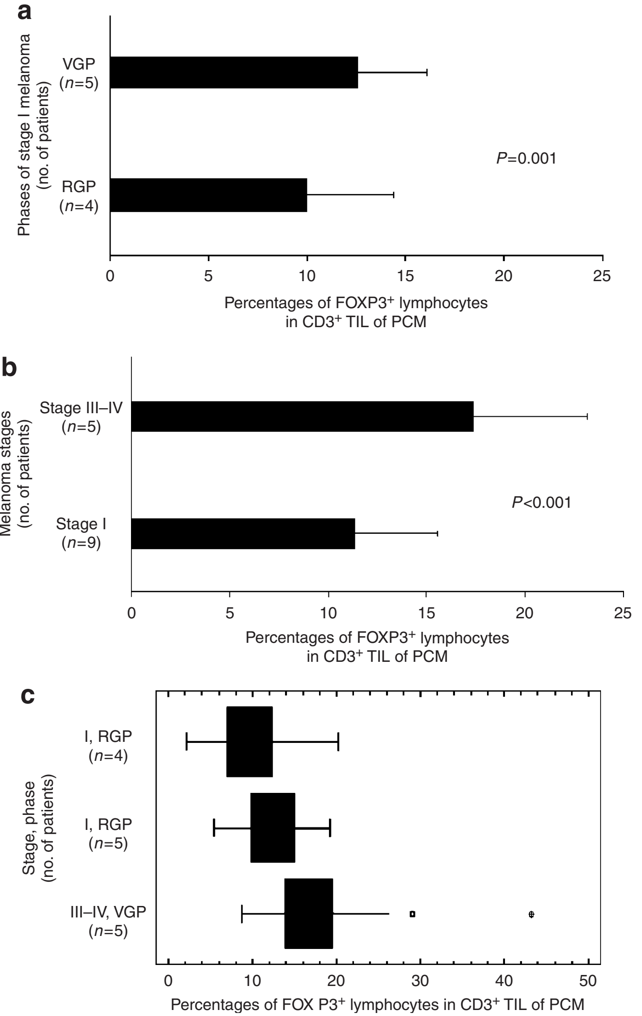

Figure 3. The proportions of FOXP3-bearing TILs are higher in VGP than RGP, as well as in late- than in early-stage melanoma. (**a**) The proportions of FOXP3-bearing cells infiltrating PCMs are higher in VGP melanoma than in RGP melanoma. The percentages of FOXP3-bearing lymphocytes in CD3$^+$ lymphocytes were quantified by CD3/FOXP3 double staining analysis performed on tissue sections of nine stage I PCMs, five of them showing VGP, and four showing RGP (mean ± SD). Fifty-six microscopic fields were counted for five VGP specimens, and 48 microscopic fields for four RGP specimens. $P = 0.001$ VGP *versus* RGP. (**b**) The proportions of FOXP3-bearing cells infiltrating PCMs are higher in late-stage melanoma than in early-stage melanoma. The percentages of FOXP3-bearing cells in CD3-positive lymphocytes were quantified by CD3/FOXP3 double staining analysis performed on tissue sections of 14 PCM, nine of them stage I melanoma, and five stage III–IV melanoma (mean ± SD). One hundred and four microscopic fields were counted for nine stage I melanoma specimens, and 57 microscopic fields for five stage III–IV melanoma specimens. $P < 0.001$ stage I *versus* stages III–IV. (**c**) Synthetic overview of the data obtained in this study, showing that phase- and stage-related proportions of FOXP3-positive TILs occur within the PCM microenvironment. Box and Whisker plot of the percentages of FOXP3-positive lymphocytes in CD3-positive TILs of the samples of the three considered PCM groups are shown. The two dots on the right of the whisker correspond to two points that are more than 3/2 times the interquartile range from the end of the box (Weisstein, 1999). The level of significance comparing RGP stage I melanoma ($n = 4$) *versus* VGP stage I melanoma ($n = 5$) was $P = 0.001$; the level of significance comparing VGP stage I melanoma ($n = 5$) *versus* VGP stage III–IV melanoma ($n = 5$) was $P < 0.001$; the level of significance comparing pooled data of stage I melanoma ($n = 9$) *versus* stage III–IV melanoma ($n = 5$) was $P < 0.001$.

stage III–IV melanoma patients ($n=5$). When CD3/FOXP3 double stained specimens of stage I melanoma patients ($n=9$) were compared with that of stage III–IV patients ($n=5$), stage I melanoma patients had PCMs infiltrated by 11.35 ± 4.19 (mean$\pm$SD) FOXP3$^+$ cells in CD3$^+$ TIL, whereas stage III–IV melanoma patients had PCMs infiltrated by17.12 ± 5.78 (mean$\pm$SD) FOXP3$^+$ cells in CD3$^+$ TIL, and such a difference was highly significant ($P<0.001$) (Figure 3b). Even when only the stage I melanoma patients having PCM with VGP ($n=5$) were considered, the number of FOXP3$^+$ TIL in CD3$^+$ TIL of this population (12.53 ± 3.59, mean$\pm$SD) was highly significantly ($P<0.001$) lower when compared to that of stage III–IV melanoma patients.

DISCUSSION

The present results show several new findings. Up to 13.39 ± 5.54 (mean$\pm$SD) lymphocytes in CD3$^+$ TIL within the microenvironment of 14 PCMs were represented by T cells bearing the transcription factor FOXP3. These FOXP3-bearing lymphocytes were predominantly CD20$^-$, CD3$^+$, CD4$^+$, CD8$^-$, and CD25$^+$, thus showing a phenotype analogous to that of ''natural'' T_{REG} cells (Fontenot *et al.*, 2005; Roncador *et al.*, 2005). In nine PCM of stage I melanoma patients (Clark *et al.*, 1989), VGP was associated to a significantly ($P=0.001$) higher number of FOXP3$^+$ TILs as compared with RGP. In a series of 10 PCMs showing VGP and having a ''brisk'' infiltrate (Elder *et al.*, 1985; Clark *et al.*, 1989; Clemente *et al.*, 1996), the percentage of FOXP3-bearing lymphocytes was highly significantly ($P<0.001$) higher in late melanoma stages than in early melanoma stages.

This study shows that a subset of TIL in the PCM microenvironment expresses the transcription factor FOXP3, which is both necessary and sufficient for the development and function of naturally arising CD4$^+$ CD25$^+$ T_{REG} in mice, and in men is expressed by CD4$^+$ CD25$^+$ T cells that act as suppressors (reviewed in Ziegler, 2006). Moreover, recent results show that FOXP3 dictates the program of T_{REG} differentiation (Gavin *et al.*, 2007), and down-modulates responses to TCR-mediated stimulation (Wang *et al.*, 2007; Ziegler, 2007). Indeed, other T_{REG} markers, such as CD25, CTLA-4, CD45RB, CD62L, NRP1, LAG-3, CD103, and GITR, are less specific than FOXP3 (Nakajima *et al.*, 2005; Banham, 2006); moreover, FOXP3$^+$ cells may show regulatory activity irrespective of their CD25 expression (Banham, 2006; Liu *et al.*, 2006; Seddiki *et al.*, 2006).

On the other hand, although in humans, FOXP3 is predominantly expressed in CD4$^+$ CD25$^+$ T_{REG} cells, smaller FOXP3$^+$ CD25$^-$ and FOXP3$^+$ CD8$^+$ subpopulations may also be identified (Roncador *et al.*, 2005; Banham, 2006). In this study, however, FOXP3$^+$ lymphocytes were virtually excluded from both CD25$^-$ and CD8$^+$ subsets; rather, they were entirely included in the CD3$^+$, CD4$^+$, and CD25$^+$ subpopulations. Thus, present results strongly suggest that FOXP3-bearing lymphocytes infiltrating PCMs herein observed, are included within a subset of the CD4$^+$ CD25$^+$ population, analogous to the canonical ''natural'' T_{REG} cells (Sakaguchi *et al.*, 1995; Shevach, 2002; Fontenot *et al.*, 2003; Hori *et al.*, 2003; Khattri *et al.*, 2003; Roncador *et al.*, 2005). If this was the case, these FOXP3$^+$ lymphocytes, although functional experiments were not performed in this study, might have regulatory activity, because CD4$^+$ CD25$^+$ T cells expressing FOXP3 act as suppressors (Ziegler, 2006).

Although this study provides early evidence of the presence of FOXP3$^+$ TIL in the human PCM microenvironment, previous studies have already shown T_{REG} (i) in mice affected with melanoma, (ii) in lymph node metastases, ascites fluid, and/or blood of patients with melanoma, and (iii) in the microenvironment of human primary tumors other than melanoma. (i) In transplantable murine melanoma models, melanoma rejection was increased upon elimination of CD4$^+$ CD25$^+$ T_{REG} cells (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999; Sutmuller *et al.*, 2001). Moreover, elimination of CD25$^+$ T cells from bulk T cells in adoptive transfer systems ''*in vivo*'' could lead to improved control of B16 melanoma (Nagai *et al.*, 2004; Gajewski, 2006), including poorly immunogenic B16 melanoma (Turk *et al.*, 2004). Even more intriguing, in an ''*in vivo*'' model of B16 melanoma, is that TIL were recently shown to contain CD4$^+$ FOXP3$^+$ T_{REG} cells within the melanoma mass (Quezada *et al.*, 2006), analogous to the present demonstration of such cells within human PCM mass. (ii) After pioneer studies demonstrating CD4$^+$ T-cell clones with suppressive activity from metastatic melanoma lymph nodes (Mukherji *et al.*, 1986, 1989), CD4$^+$CD25$^+$ FOXP3$^+$ T_{REG} cells were shown to be overexpressed in metastatic lymph nodes of patients with melanoma (Viguier *et al.*, 2004), and variable expression of FOXP3 was observed in melanoma metastases by real-time reverse transcription-PCR (Gajewski, 2006). In a patient with metastatic melanoma and ascites, the ascites fluid showed abundant CD4$^+$CD25$^+$ FOXP3$^+$ T cells (Harlin *et al.*, 2006). CD4$^+$CD25$^+$ T cells were shown in the blood of patients with melanoma undergoing tumor antigen immunization (Javia and Rosenberg, 2003); the number of CD4$^+$ CD25$^+$ T cells was markedly increased in the circulation of melanoma patients as compared with age-matched controls (Gray *et al.*, 2003), and CD4$^+$CD25$^+$FOXP3$^+$ CTLA-4$^+$ T_{REG} cells were significantly elevated in melanoma patients as compared with normal donors (Cesana *et al.*, 2006). (iii) The prevalence of T_{REG} was increased within the tumor microenvironment in several cancers, such as lung cancer (Woo *et al.*, 2001), pancreas or breast carcinoma (Liyanage *et al.*, 2002), gastric or esophageal cancers (Ichihara *et al.*, 2003), Hodgkin lymphoma (Marshall *et al.*, 2004), ovarian carcinoma (Woo *et al.*, 2001; Curiel *et al.*, 2004), colorectal cancer (Loddenkemper *et al.*, 2006), nasopharyngeal carcinoma (Lau *et al.*, 2007), and hepatocellular carcinoma (Kobayashi *et al.*, 2007).

The role played in human melanoma by the herein demonstrated FOXP3-bearing T cells, also expressing CD4 and CD25, infiltrating PCM is not known at present, because the presence of FOXP3-positive T cells without suppressive functions cannot be excluded (Gavin *et al.*, 2006). It is possible to hypothesize, however, that these TILs, since show, as above mentioned, an immunophenotype analogous to that of natural T_{REG}, may mediate suppression of

melanoma-associated antigen-reactive cytolytic TIL, thus downregulating melanoma-specific T-cell immunity and contributing to PCM growth. Interestingly, melanoma-specific human T_{REG} clones with anti-LAGE-1 (Wang *et al.*, 2004) or ARTC1 (Wang *et al.*, 2005) specificity, derived from TIL into a PCM, had potent suppressive activities, because they strongly inhibited both proliferation and cytokine secretion by autologous indicator T-cell clones. Further, CD4^{+}CD25^{+}FOXP3^{+} T_{REG} cells overrepresented in melanoma metastatic lymph nodes inhibited ''*in vitro*'' the proliferation and cytokine production of infiltrating CD4^{+} and CD8^{+} T cells (Viguier *et al.*, 2004).

In this study, we demonstrated that the proportions of FOXP3-bearing T cells in CD3^{+} TIL of the PCM microenvironment were higher in VGP stage I melanoma than in RGP stage I melanoma, as well as in late melanoma stages than in early melanoma stages (Figure 3c). In fact, it is well established that VGP melanomas may metastasize, whereas RGP melanomas are associated to a 100% survival (Clark *et al.*, 1989). On the other hand, survival rates of patients with stage I–IV melanoma are strongly different, because the differences between survival curves comparing localized melanoma (stages I and II) *versus* metastatic melanoma (stages III and IV) are highly significant (Balch *et al.*, 2004). Interestingly, in a recent study investigating metastatic melanoma patients, clinical post-therapy responses were associated to the frequency of T_{REG} cells in circulation, and T_{REG} frequency was suggested as a method for monitoring patients after initial positive response to immunotherapy (Cesana *et al.*, 2006). Even more intriguing, in a study of 70 patients affected with ovarian cancer, not only a stage-related difference in the numbers of T_{REG} TIL was demonstrated, but also a significant inverse correlation between the number of such T_{REG} TIL and survival (Curiel *et al.*, 2004). Although this aspect is debated for colorectal cancer (Clarke *et al.*, 2006; Loddenkemper *et al.*, 2006; Garajova *et al.*, 2007 (Garajova I, Fabian P, Svoboda M, Nenutil R. Colorectal carcinoma is infiltrated by FOXP3-positive lymphocytes (abstr.). *International Meeting ''Molecular targets in cancer therapy: mechanism and therapeutic reversal of immune suppression in cancer''*, Clearwater Beach, FL, USA, January 25–28, 2007)), the patient group with a high prevalence of FOXP3^{+} T_{REG} infiltrating hepatocellular carcinoma showed a significantly lower survival rate (Kobayashi *et al.*, 2007). To this respect, in the present series of 14 PCMs, no conclusions are possible, because only one patient died up to date (15.04% CD3^{+}FOXP3^{+} cells in CD3^{+} lymphocytes infiltrating PCM: case number 12); nevertheless, studies are in progress in our laboratory to determine whether the proportions of FOXP3-bearing lymphocytes infiltrating PCM might even be used as a prognostic marker of reduced survival for melanoma patients.

In conclusion, the present results, showing that phase- and stage-related proportions of TIL bearing the transcription factor FOXP3 occur within the PCM microenvironment (Figure 3c), may favor the hypothesis, as above anticipated, that such infiltrating putative T_{REG} cells can suppress ''*in vivo*'' the local effector antimelanoma immune response. To this respect, immunotherapeutic strategies aimed to counteract ''*in vivo*'' the action of T_{REG} cells, might provide important answers to this hypothesis, and, as already envisioned (Viguier *et al.*, 2004), could have crucial impacts on the design of efficient vaccination protocols for the treatment of melanoma patients. This in fact seems to be the case (Lüpez *et al.*, 2006), as suggested not only in experimental murine melanoma (Shimizu *et al.*, 1999; Steitz et al., 2001; Sutmuller *et al.*, 2001, Nagai *et al.*, 2004; Antony *et al.*, 2005; Li *et al.*, 2006; Quezada *et al.*, 2006; Nair *et al.*, 2007), but also in human melanoma patients (Chakraborty *et al.*, 2004; Dudley *et al.*, 2005; Cesana *et al.*, 2006; Geng *et al.*, 2006).

MATERIALS AND METHODS

Patients

We studied 14 patients affected with PCM. All of them were previously untreated. Experiments had institutional approval and patient consent. The study was conducted in adherence to Helsinki Principles. Excision of PCM was made at the Section of Dermatology, Department of Surgical Sciences, Parma University.

Samples

Skin specimens were collected at the time of surgery and treated as described (Ferrari *et al.*, 2007) at the Pathology Section of the Department of Pathology and Laboratory Medicine, Parma University. Diagnosis was assessed by histopathological examination of formalin-fixed, paraffin-embedded, hematoxylin and eosin-stained tissue samples.

Melanoma staging

Details of the melanoma staging, gathered according to the Melanoma Staging Committee of the American Joint Committee on Cancer (Balch *et al.*, 2004), are given in Table 1. Nine out of the 14 PCMs were stage I melanoma, four of these having ''RGP'' and five having ''VGP'' (Clark *et al.*, 1989), while the remaining five cases were stage III–IV melanoma, according to Balch *et al.* (2004). On the other hand, the five VGP stage I and the five stage III–IV melanomas histopathologically showed not only VGP but also ''brisk'' infiltrate (Elder *et al.*, 1985; Clark *et al.*, 1989; Clemente *et al.*, 1996).

Dermato- and clinico-pathological characterizations of PCMs

Histopathological assessments concerning the investigated PCMs are summarized in Table 1. Specifically, the characterization concerned growth phase, Breslow thickness, ulceration, Clark's level, mitoses/mm^2, regression, cellular type, pigmentation, and TILs.

Antibodies

The following monoclonal antibodies (mAbs), able to identify different lymphocyte subpopulations, were used: anti-CD3 mAb (Neomarkers, Fremont, CA); anti-CD4 mAb (Dako, Glostrup, Denmark); anti-CD8 mAb (Neomarkers); anti-CD25 (IL-2 receptor) mAb (Novocastra, Newcastle, UK); anti-FOXP3 mAb (Novus Biologicals, Littleton, CO); anti-CD20 mAb (Dako).

Immunohistochemistry

First, paraffin-embedded serial sections were treated for immunohistology to reveal, in single labeling experiments, positive TILs of

Table 1. Clinicopathological parameters of 14 PCMs, including four PCMs of stage I with RGP (numbers 1–4), five PCMs of stage I with VGP (numbers 5–9), and five PCMs of stages III–IV (numbers 10–14)

PCM case number	AJCC staging		Dermatopathological parameters								
	TNM	Stage	Growth phase	Breslow thickness	Ulceration	Clark's level	Mitoses/mm^2	Regression	Cellular type	Pigmentation	TIL
1	T1bN0M0	IB	RGP	0.30	Yes	II	1	No	Epit	Yes	Brisk
2	T1aN0M0	IA	RGP	0.69	No	II	1	No	Epit	Yes	Brisk
3	T1aN0M0	IA	RGP	0.53	No	II	0	No	Spin	Yes	Brisk
4	T1aN0M0	IA	RGP	0.69	No	II	0	No	Epit	Yes	Brisk
5	T1bN0M0	IB	VGP	0.93	Yes	III	13	No	Epit	Yes	Brisk
6	T2aN0M0	IB	VGP	1.25	No	III	1	No	Epit	Yes	Brisk
7	T1aN0M0	IA	VGP	0.68	No	III	1	Yes	Epit	Yes	Brisk
8	T2aN0M0	IB	VGP	1.95	No	V	2	No	Epit	No	Brisk
9	T1aN0M0	IA	VGP	0.90	No	III	2	Yes	Spin	Yes	Brisk
10	T3bNxM1b	IV	VGP	3.60	Yes	IV	5	No	Epit	Yes	Brisk
11	T2bN2cM0	IIIB	VGP	1.30	Yes	III	3	No	Epit	Yes	Brisk
12	T1aNxM1c	IV	VGP	0.90	No	III	1	Yes	Spin	Yes	Brisk
13	T2aN2cM0	IIIB	VGP	1.38	No	III	5	No	Spin	Yes	Brisk
14	T2aN2aM0	IIIA	VGP	1.4	No	II	10	No	Epit	Yes	Brisk

AJCC, American Joint Committee on Cancer; Epit, epithelioid cells; PCM, primary cutaneous melanoma; RGP, radial growth-phase; Spin, spindle cells; TIL, tumor-infiltrating lymphocyte; TNM, tumor node metastasis; VGP, vertical growth phase.

different lymphocyte subsets infiltrating the PCM mass. For such a purpose, a previously described technique (Ferrari *et al.*, 2007) was performed, with minor modifications. Briefly, the deparaffinized sections were first treated with 3% hydrogen peroxide to block endogenous peroxidase, thereafter were incubated with the primary mAbs anti-CD20, -CD3, -CD4, -CD8, -CD25, and -FOXP3. For antigen retrieval, sections were treated with pH 9 Tris-EDTA buffer for 30 minutes in a water bath at 98°C. The sections were immunostained with HRP Polymer (Ultravision LP Large Volume Detection System, Lab Vision, Fremont, CA) in accordance with the manufacturer's specifications. Diaminobenzidine was used for staining development, and part of the sections was counterstained with Harris hematoxylin.

Double labeling experiments

Serial sections were thereafter treated by a double staining technique, in order to establish the percentages of FOXP3-positive cells in the context of the CD3-, CD4-, CD8-, and CD25-positive subpopulations. For such a purpose, after labeling of FOXP3 as mentioned above, and after a blocking step, the second mAb was immunostained with streptavidin–biotin complex (LSAB2 System, AP, Dako), in accordance with the manufacturer's specifications. Permanent red chromogen was used for staining development. A similar double labeling technique was used for CD4/CD25 double labeling.

Morphometric cell analysis

Quantitative evaluation of positively stained cells, either single or double labeled, was performed by cell analysis of TIL-infiltrated PCM microenvironment. Specifically, the lymphoid component was morphometrically evaluated on images acquired with a videocamera (JVC, Yokoama, Japan), through a light microscope (Olympus BX 51, Tokyo, Japan) (magnification $\times 25$), using an image analyzer (Image Pro Plus ver. 4.5, Media Cybernetics Inc., Silver Spring, MD).

The number of immunostained cells within a microscopic field (0.04 mm^2) was recorded, and a mean of 13 such fields from each sample was calculated, thus giving an observed total tissue area of 1.38 mm^2. The percentages of the different lymphocyte subsets were calculated by dividing the number of each cell type by the total number of immunostained cells, and multiplying the value by 100.

Statistical analysis

Statistical analysis has been performed with Statgraphics Centurion software (Herndorn, VA).

A preliminary statistical analysis has been carried out on the total FOXP3$^+$CD3$^+$ cell counts and CD3$^+$ cell counts in all the microscopic fields for the three considered melanoma groups, namely, (i) RGP stage I melanoma, (ii) VGP stage I melanoma, (iii) VGP stage III–IV melanoma (Table 2). Data have been treated by the χ^2-test. The three groups resulted in a χ^2 value of 150.3, corresponding to a $P<0.001\%$. The same *P*-value has been obtained by comparing both the frequencies of (i) with (ii) ($\chi^2=20.1$), and pooled frequencies of (i) plus (ii) with (iii) ($\chi^2=133.1$). This demonstrates that in melanoma, the proportions of FOXP3$^+$ cells strongly depend on the phase and on the stage of melanoma.

A further statistical analysis of data has been carried out on the percentages of FOXP3$^+$CD3$^+$ cells in CD3$^+$ cells in single microscopic fields. They were compared by non-parametric tests. At first, a non-parametric analysis of variance (Kruskaal–Wallis) has been accomplished, using the three considered PCM groups, as a

Table 2. Counts of the FOXP3$^+$CD3$^+$ cells and of the CD3$^+$ cells in the three considered melanoma groups, obtained as sum of the counts of positive cells of all the considered microscopic fields

Positive cells	Melanoma groups		
	RGP stage I	VGP stage I	VGP stages III–IV
FOXP3$^+$ CD3$^+$	438	944	1,252
CD3$^+$	4,498	7,384	6,658

FOXP3, forkhead box protein P3; RGP, radial growth-phase; VGP, vertical growth phase.

classification factor. The significance level resulted $P<0.1\%$. Wilcoxon test was employed as ''*post-hoc*'' test. It showed significant difference between (i) and (ii) ($P=0.1\%$), and between (ii) and (iii) ($P<0.1\%$). Analogous result was obtained comparing pooled data of (i) and (ii) *versus* those of (iii) ($P<0.1\%$). This demonstrates that, with a *P*-value of 0.1%, also in single microscopic fields, the proportions of FOXP3$^+$ cells are significantly higher in VGP *versus* RGP, and in late *versus* early melanoma stages.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This study was supported by Grants ''FIL O5'' and ''FIL O6''(to GDP) from the Italian Ministry for the University, Rome, Italy. We thank surgeons operating within the Section of Dermatology of the Department of Surgical Sciences of Parma University (Alfredo Zucchi, Rocco Tortorella), for providing specimens. The expert technical assistance in the morphometric counts of Miss Anna Maria Bonafede, as well as the valuable secretarial work by Mrs Rosetta Gandolfi, are gratefully acknowledged.

REFERENCES
Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR *et al.* (2005) 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* 174:2591–601

Asano M, Toda M, Sakaguchi N, Sakaguchi S (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184:387–96

Balch CM, Soong SJ, Atkins MB, Buzaid AC, Cascinelli N, Coit DG *et al.* (2004) An evidence-based staging system for cutaneous melanoma. *CA Cancer J Clin* 54:131–49

Banham AH (2006) Cell-surface IL-7 receptor expression facilitates the purification of FOXP3$^+$ regulatory T cells. *Trends Immunol* 27:541–4

Cesana GC, DeRaffaele G, Cohen S, Moroziewicz D, Mitcham J, Stoutenburg J *et al.* (2006) Characterization of CD4$^+$CD25$^+$ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J Clin Oncolol* 24:1169–77

Chakraborty NG, Chattopadhyay S, Mehrotra S, Chhabra A, Mukherji B (2004) Regulatory T-cell response and tumor vaccine-induced cytotoxic T lymphocytes in human melanoma. *Hum Immunol* 65:794–802

Clark WH Jr, Elder DE, Guerry DIV, Braitman LE, Trock BJ, Schultz D *et al.* (1989) Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst* 81:1893–904

Clarke SL, Betts GJ, Plant A, Wright KL, El-Shanawany TM, Harrop R *et al.* (2006) CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells suppress anti-tumor immune responses in patients with colorectal cancer. *PLoS ONE* 1:e129 (doi:10.1371/journal.pone.0000129)

Clemente CG, Mihm MC Jr, Bufalino R, Zurrida S, Collini P, Cascinelli N (1996) Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 77:1303–10

Coulie PG, Connerotte T (2005) Human tumor-specific T lymphocytes: does function matter more than number? *Curr Opin Immunol* 17:320–5

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P *et al.* (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10:942–9

Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP *et al.* (2005) Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 23:2346–57

Elder DE, Guerry DIV, VanHorn M, Hurwitz S, Zehngebot L, Goldman LI *et al.* (1985) The role of lymph node dissection for clinical stage I malignant melanoma of intermediate thickness (1.51–3.99 mm.). *Cancer* 56:413–8

Ferrari D, Lombardi M, Ricci R, Michiara M, Santini M, De Panfilis G (2007) Dermatopathological indicators of poor melanoma prognosis are significantly inversely correlated with the expression of NM23 protein in primary cutaneous melanoma. *J Cutan Pathol.* (in press)

Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat Immunol* 4:330–6

Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY (2005) Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. *Immunity* 22:329–41

Fontenot JD, Rudensky AY (2005) A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6:331–7

Gajewski TF (2006) Identifying and overcoming immune resistance mechanisms in the melanoma tumor microenvironment. *Clin Cancer Res* 12:2326s–30s

Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA *et al.* (2007) FOXP3-dependent programme of regulatory T-cell differentiation. *Nature* 15:771–5

Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A *et al.* (2006) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci USA* 103:6659–64

Geng H, Zhang GM, Xiao H, Yuan Y, Li D, Zhang H *et al.* (2006) HSP70 vaccine in combination with gene therapy with plasmid DNA encoding sPD-1 overcomes immune resistance and suppresses the progression of pulmonary metastatic melanoma. *Int J Cancer* 118:2657–64

Gray CP, Arosio P, Hersey P (2003) Association of increased levels of heavy-chain ferritin with increased CD4$^+$CD25$^+$ regulatory T-cell levels in patients with melanoma. *Clin Cancer Res* 9:2551–9

Guilloux Y, Viret C, Gervois N, Le Drean E, Pandolfino MC, Diez E *et al.* (1994) Defective lymphokine production by most CD8$^+$ and CD4$^+$ tumor-specific T cell clones derived from human melanoma-infiltrating lymphocytes in response to autologous tumor cells *in vitro*. *Eur J Immunol* 24:1966–73

Harlin H, Kuna TV, Peterson AC, Meng Y, Gajewski TF (2006) Tumor progression despite massive influx of activated CD8$^+$ T cells in a patient with malignant melanoma ascites. *Cancer Immunol Immunother* 55:1185–97

Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057–61

Ichihara F, Kono K, Takahaschi A, Kawaida H, Sugai H, Fujii H (2003) Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 9:4404–8

Javia LR, Rosenberg SA (2003) CD4$^+$CD25$^+$ suppressor lymphocytes in the circulation of patients immunized against melanoma antigens. *J Immunother* 26:85–93

Kaestner KH, Knochel W, Martinez DE (2000) Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* 14:142–6

Khattri R, Cox T, Yasayko SA, Ramsdell F (2003) An essential role for scurfin in CD4$^+$CD25$^+$ T regulatory cells. *Nat Immunol* 4:337–42

Kobayashi N, Hiraoka N, Yamagami W, Ojima H, Kanai Y, Kosuge T *et al.* (2007) FOXP3$^+$ regulatory T cells affect the development and progression of hepatocarcinogenesis. *Clin Cancer Res* 13:902–11

Lau KM, Cheng SH, Lo KW, Lee SA, Woo JK, van Hasselt CA *et al.* (2007) Increase in circulating FOXP3$^+$CD4$^+$CD25$^{(high)}$ regulatory T cells in nasopharyngeal carcinoma patients. *Br J Cancer* 26:617–22

Li B, Lalani AS, Harding TC, Luan B, Koprivnikar K, Huan Tu G *et al.* (2006) Vascular endothelial growth factor blockade reduces intratumoral regulatory T cells and enhances the efficacy of a GM-CSF-secreting cancer immunotherapy. *Clin Cancer Res* 12:6808–16

Liu W, Putnam Al, Xu-Yu Z, Szot GL, Lee MR, Zhu S *et al.* (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4$^+$ T reg cells. *J Exp Med* 203:1701–11

Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G *et al.* (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 169:2756–61

Loddenkemper C, Schernus M, Noutsias M, Stein H, Thiel E, Nagorsen D (2006) *In situ* analysis of FOXP3$^+$ regulatory T cells in human colorectal cancer. *J Transl Med* 4:52–9

Lopes JE, Torgerson TR, Schubert LA, Anover SD, Ocheltree EL, Ochs HD *et al.* (2006) Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor. *J Immunol* 177:3133–42

López M, Aguilera R, Pérez C, Mendoza-Naranjo A, Pereda C, Ramirez M *et al.* (2006) The role of regulatory T lymphocytes in the induced immune response mediated by biological vaccines. *Immunobiology* 211:127–36

Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN *et al.* (2004) Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 103: 1755–62

Morgan ME, van Bilsen JH, Bakker AM, Heemskerk B, Schilham MW, Hartgers FC *et al.* (2005) Expression of FOXP3 mRNA is not confined to CD4$^+$CD25$^+$ T regulatory cells in humans. *Hum Immunol* 66:13–20

Mortarini R, Piris A, Maurichi A, Molla A, Bersani I, Bono A *et al.* (2003) Lack of terminally differentiated tumor-specific CD8$^+$ T cells at tumor site in spite of anti-tumor immunity to self-antigens in human metastatic melanoma. *Cancer Res* 63:2535–45

Mukherji B, Guha A, Chakraborty NG, Sivanandham M, Nashed AL, Sporn JR *et al.* (1989) Clonal analysis of cytotoxic and regulatory T cell responses against human melanoma. *J Exp Med* 169:1961–76

Mukherji B, Wilhelm SA, Guha A, Ergin MT (1986) Regulation of cellular immune response against autologous human melanoma. I. Evidence for cell-mediated suppression of *in vitro* cytotoxic immune response. *J Immunol* 136:1888–92

Nagai H, Horikawa T, Hara I, Fukunaga A, Oniki S, Oka M *et al.* (2004) *In vivo* elimination of CD25$^+$ regulatory T cells leads to tumor rejection of B16F10 melanoma, when combined with interleukin-12 gene transfer. *Exp Dermatol* 13:613–20

Nair S, Boczkowski D, Fassnacht M, Pisetsky D, Gilboa E (2007) Vaccination against the forkhead family transcription factor Foxp3 enhances tumor immunity. *Cancer Res* 67:371–80

Nakajima T, Ueki-Maruyama K, Oda T, Ohsawa Y, Ito H, Seymour GJ *et al.* (2005) Regulatory T-cells infiltrate periodontal disease tissues. *J Dent Res* 84:639–43

Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E (1999) Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res* 59:3128–33

Owen CJ, Jennings CE, Imrie H, Lachaux A, Bridges NA, Cheetham TD *et al.* (2003) Mutational analysis of the FOXP3 gene and evidence for genetic heterogeneity in the immunodysregulation, polyendocrinopathy, enteropathy syndrome. *J Clin Endocrinol Metab* 88:6034–9

Quezada SA, Peggs KS, Curran MA, Allison JP (2006) CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J Clin Invest* 116:1935–45

Romero P, Dunbar PR, Valmori D, Pittet M, Ogg GS, Rimoldi D *et al.* (1998) *Ex vivo* staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J Exp Med* 188: 1641–50

Roncador G, Brown PJ, Maestre L, Hue S, Martínez-Torrecuadrada JL, Ling KL *et al.* (2005) Analysis of FOXP3 protein expression in human CD4$^+$CD25$^+$ regulatory T cells at the single-cell level. *Eur J Immunol* 35:1681–91

Roncarolo MG, Levings MK (2000) The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol* 12:676–83

Sakaguchi S (2005) Naturally arising Foxp3-expressing CD25$^+$CD4$^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 6:345–52

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151–64

Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M *et al.* (2001) Immunologic tolerance maintained by CD25$^+$CD4$^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 182:18–32

Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A *et al.* (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 203:1693–700

Shevach EM (2000) Regulatory T cells in autoimmunity. *Annu Rev Immunol* 18:423–49

Shevach EM (2002) CD4$^+$CD25$^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2:389–400

Shimizu J, Yamazaki S, Sakaguchi S (1999) Induction of tumor immunity by removing CD25$^+$CD4$^+$ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 163:5211–8

Steitz J, Brüch J, Lenz J, Knop J, Tüting T (2001) Depletion of CD25$^+$CD4$^+$T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon α-induced, CD8$^+$ T cell-dependent immune defense of B16 melanoma. *Cancer Res* 61:8643–6

Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP *et al.* (2001) 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* 194:823–32

Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN (2004) Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. *J Exp Med* 200:771–82

Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L *et al.* (2004) Foxp3 expressing CD4$^+$CD25^{high} regulatory T cells are over-represented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 173:1444–53

Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH *et al.* (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4$^+$CD25$^-$ T cells. *J Clin Invest* 112:1437–43

Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y *et al.* (2004) Tumor-specific human CD4$^+$ regulatory T cells and their ligands: implications for immunotherapy. *Immunity* 20:107–18

Wang HY, Peng G, Guo Z, Shevach EM, Wang RF (2005) Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4$^+$ regulatory T cells. *J Immunol* 174:2661–70

Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE (2007) Transient expression of FOXP3 in human activated nonregulatory CD4$^+$ T cells. *Eur J Immunol* 37:129–38

Weisstein EW (1999) *CRC Concise Encyclopedia of Mathematics*. Boca Raton (FL): CRC Press, 161

Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G *et al.* (2001) Regulatory CD4$^+$CD25$^+$ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 61:4766–72

Yazdi AS, Morstedt K, Puchta U, Ghoreschi K, Flaig MJ, Rocken M *et al.* (2006) Heterogeneity of T-cell clones infiltrating primary malignant melanomas. *J Invest Dermatol* 126:393–8

Yee C, Thompson JA, Roche P, Byrd DR, Lee PP, Piepkorn M *et al.* (2000) Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of T cell-mediated vitiligo. *J Exp Med* 192:1637–44

Ziegler SF (2006) FOXP3: of mice and men. *Annu Rev Immunol* 24:209–26

Ziegler SF (2007) FOXP3: not just for regulatory T cells anymore. *Eur J Immunol* 37:21–3

Zippelius A, Batard P, Rubio-Godoy V, Bioley G, Lienard D, Lejeune F *et al.* (2004) Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. *Cancer Res* 64:2865–73