Novel strategies for inhibiting PD-1 pathway-mediated immune suppression while simultaneously delivering activating signals to tumor-reactive T cells

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Abstract

We previously developed cell-based vaccines as therapeutics for metastatic cancers. The vaccines were aimed at activating type I CD4$^+$T cells and consisted of tumor cells transfected with genes encoding syngeneic MHC class II and CD80 costimulatory molecules, and lacking the MHC II-associated invariant chain. The vaccines showed some efficacy in mice with sarcoma, melanoma, and breast cancer and activated MHC class II syngeneic T cells from breast, lung, and melanoma patients. During the course of the vaccine studies, we observed that CD80 not only costimulated naïve T cells, but also bound to PD-L1 and prevented tumor cell-expressed PD-L1 from binding to its receptor PD-1 on activated T cells. A soluble form of CD80 (CD80-Fc) had the same effect and sustained IFNγ production by both human and murine PD-1$^+$ activated T cells in the presence of PD-L1$^+$ human or mouse tumor cells, respectively. In vitro studies with human tumor cells indicated that CD80-Fc was more effective than antibodies to either PD-1 or PD-L1 in sustaining T cell production of IFNγ. Additionally, in vivo studies with a murine tumor demonstrated that CD80-Fc was more effective than antibodies to PD-L1 in extending survival time. Studies with human T cells blocked for CD28 and with T cells from CD28 knockout mice demonstrated that CD80-Fc simultaneously inhibited PD-L1/PD-1-mediated immune suppression and delivered costimulatory signals to activated T cells, thereby amplifying T cell activation. These results suggest that CD80-Fc may be a useful monotherapy that minimizes PD-1 pathway immune suppression while simultaneously activating tumor-reactive T cells.

Keywords

Introduction

Cancer immunotherapy was recognized by Science Magazine in 2013 as the "Breakthrough of the Year" [1], and monoclonal antibodies (mAbs) to PD-L1 (Programmed Death Ligand 1; also known as B7 homolog 1 (B7-H1) or CD274) and PD-1 (Programmed Death 1 or CD279) were described as "Drugs of the Year" because of their ability to neutralize PD-L1/ PD-1-mediated immune suppression [2].

PD-L1 is a major obstacle to antitumor immunity because it (i) tolerizes/anergizes tumor-reactive T cells by binding to its receptor PD-1 [3, 4]; (ii) renders tumor cells resistant to CD8^{+} T cell and FasL-mediated lysis [5]; and (iii) tolerizes T cells by reverse signalling through T cell-expressed CD80 [6]. Many malignant cells express PD-L1, either constitutively or after induction by IFNγ [3, 5, 7, 8]. The role of PD-1 as a significant obstacle to antitumor immunity is supported by studies demonstrating that antibody blocking of PD-1 improves T cell activation and reduces tumor progression [7, 9–11] and that antibody blocking of PD-L1 reverse signalling through CD80 prevents T cell anergy [6]. These effects are at least partially due to blocking of PD-L1 on dendritic cells (DC) since the expression of PD-L1 by activated DC limits effector cell differentiation and the generation of CD8^{+} T cell memory [12]. The critical role of PD-L1 in human cancer was unequivocally established by recent clinical trials in which 19–30 % of patients with certain advanced cancers treated with antibodies to PD-L1 or PD-1 had partial or complete remissions [13, 14]. On-going studies combining mAb therapy to PD-L1 or PD-1 with immune activating strategies have demonstrated that optimal efficacy is achieved if PD-L1/PD-1 suppression is blocked while concurrently providing immune activation signals such as with cancer vaccines [15, 16]. Based on these findings, we are developing a therapeutic strategy that combines in a single reagent the ability to inhibit PD-1 pathway immune suppression while concomitantly delivering activating signals to tumor-reactive T cells. Our approach makes use of the fact that in addition to binding to the receptor PD-1, PD-L1 also binds to the costimulatory molecule CD80 (also known as B7.1) [17, 18]. This unique binding capacity has led us to hypothesize that a soluble version of CD80 has the potential to facilitate antitumor immunity via three independent mechanisms: (i) CD80 could inhibit the PD-1 suppressive pathway and maintain the activity of activated PD-1^{+} T cells by binding to PD-L1 and thereby preventing the binding of PD-L1^{+} tumor cells to PD-1^{+} T cells; (ii) by binding to PD-L1^{+} cells, CD80 would also prevent the suppression caused by PD-L1–CD80 reverse signalling into CD80^{+} T cells; and (iii) as a dimer, CD80 could simultaneously bind to PD-L1 and CD28. This dual binding could bridge PD-L1^{+} target tumor cells and tumor-reactive CD4^{+} and CD8^{+} T cells and concomitantly prevent PD-1 pathway immune suppression while delivering activating costimulatory signals through CD28. These mechanisms are shown schematically in Fig. 1. This article will review the in vitro and in vivo data from murine and human systems that support our hypothesis and identify a soluble form of CD80 as a potential novel cancer immunotherapeutic reagent.

Membrane-bound human or mouse CD80 prevents PD-L1–PD-1 interactions and maintains IFNγ production by activated PD-1^{+} T cells in the presence of PD-L1[+] human or mouse tumor cells

We initially observed that tumor cells transfected with membrane-bound CD80 plus MHC class II molecules syngeneic to the recipient were therapeutic vaccines for mice with up to 21-day established, large (up to 6 mm diameter) vascularized, subcutaneous tumors with extensive stroma [19], as well as for mice with established, spontaneous metastatic disease [20]. At the time we attributed the efficacy of CD80 to its serving as a costimulatory molecule. In vitro studies confirmed that the genetically modified tumor cells were both antigen-presenting cells (APC) for tumor peptides [21, 22] and a source of tumor peptide/MHC I and II complexes for "cross-dressing" dendritic cells [23, 24]. When we realized that most of the mouse tumors were strongly PD-L1[+], or were induced by IFNγ to express PD-L1, we examined the possibility that CD80 was also maintaining T cell activation by preventing PD-L1–PD-1 interactions. We tested this hypothesis by expressing membrane-bound CD80 on seven different PD-L1[+] human tumor cell lines including lung adenocarcinoma, mammary carcinoma, and cutaneous and uveal melanomas. For all PD-L1[+] tumors, co-expression of membrane-bound CD80 prevented the binding of PD-1 as assessed by flow cytometry [25]. The functional relevance of preventing PD-L1–PD-1 interactions was shown by coculture experiments in which activated human T cells were incubated with human PD-L1^{+} tumor cells. Whereas PD-L1^{+} tumor cells suppressed IFNγ production, CD80^{+}PD-L1^{+} tumor cells prevented the suppression and maintained IFNγ production by activated PD-1^{+} human T cells. IFNγ production by both CD4^{+} and CD8^{+} T cells was sustained. Expression of membrane-bound CD80 on two PD-L1^{+} murine tumor cell lines similarly prevented PD-1 binding and maintained IFNγ production by PD-1^{+} murine T cells [26], further suggesting that CD80 has potential as a therapeutic agent to prevent PD-L1–PD-1-mediated immune suppression [25].

A soluble form of CD80 similarly maintains IFNγ production by activated PD-1^{+} human or mouse T cells in the presence of PD-L1^{+} tumor cells

The studies with membrane-bound CD80 provided proof-of-principle for CD80 as a potential therapeutic; however, therapy with a membrane-bound molecule is not feasible. We therefore set out to determine whether a soluble form of CD80 was similarly effective. Membrane-bound CD80 consists of an amino-terminal Ig-like variable domain (IgV) followed by an Ig-like constant domain (IgC), a transmembrane region, and a cytoplasmic domain. PD-L1 has a similar structure (Fig. 2a). Mutation studies have established that CD80 and PD-1 bind to a similar location within the IgV domain of PD-L1 [17, 18] (Fig. 2b, c). To construct a soluble form of CD80 that binds to PD-L1, we generated recombinant fusion proteins consisting of the IgV plus IgC domains of human or mouse CD80 fused to the Fc domain of either human IgG1 or murine IgG2a, respectively (CD80-Fc). To determine whether CD80-Fc was as effective as membrane-bound CD80, phytohemag-glutinin (PHA)-activated human peripheral blood mononu-clear cells (PBMC) were cocultured with PD-L1[+] human C8161 cells ± human CD80-Fc or irrelevant fusion protein

TROY-Fc (toxicity and JNK inducer or TNFRSF19). PD-L1^{+} human tumor cells suppressed the activated CD4^{+} and CD8^{+} T cells, and inclusion of CD80-Fc reversed the suppression [26]. Experiments with three additional PD-L1^{+} human tumors (MCF10 mammary carcinoma, H292 squamous cell carcinoma, and H358 bronchioloal-veolar adenocarcinoma) showed similar suppression and rescue of activation in the presence of CD80-Fc [25]. The activated human T cells expressed high levels of both PD-1 and PD-L1, resulting in susceptibility to PD-1 suppression. To ascertain that CD80-Fc rescues T cell activation by preventing PD-1 suppression, we compared the ability of human PD-1-Fc vs. CD80-Fc to prevent PD-1 suppression [26]. Mouse PD-1 served as a negative control. Both human CD80 and human PD-1, but not mouse PD-1, restored IFNγ production in the presence of PD-L1[+] human tumor cells, with CD80 being more effective.

Murine CD80 similarly prevented the binding of murine PD-1 to murine PD-L1^{+} tumor cells and maintained T cell production of IFNγ in the presence of PD-L1[+] murine tumor cells. The 4T1 mammary carcinoma constitutively expresses PD-L1, and low levels (100 units) of IFNγ increase PD-L1 expression. PD-L1^{+} 4T1 cells bound soluble PD-1, and inclusion of CD80-Fc prevented the binding of PD-1. Addition of the mAb (43H12), which disrupts murine PD-L1–CD80 interactions [6], confirmed that CD80 prevented PD-1 binding because CD80 is physically associated with PD-L1. Co-expression of membrane-bound CD80 by PD-L1[+] 4T1 or B16 MELF10 cells, or inclusion of CD80-Fc also prevented suppression of antigen-activated transgenic murine T cells [26]. These results demonstrated that CD80 masks PD-L1 and maintains T cell activation in the presence of multiple human and mouse PD-L1[+] tumors.

CD80-Fc is more effective than mAbs to PD-L1 or PD-1 for maintaining activated T cells

MAbs to PD-L1 and PD-1 have significant efficacy in clinical trials as either monotherapies [13, 14, 27, 28], or in combination with other checkpoint blockers or cancer vaccines [29, 30]. To preliminarily determine how soluble CD80 compares to existing mAbs, we tested human CD80-Fc and five antihuman PD-L1 (29E.2A3, MIH1, 5H1, MIH3, and 130021) and five antihuman PD-1 (PD1.3.1.3, EH12.1, EH12.2H7, J116, and MIH4) mAbs in vitro for their ability to sustain T cell production of IFNγ in the presence of six different PD-L1[+] human tumor cell lines (non-small cell lung cancers H292 and H358, mammary carcinoma MCF10, uveal melanoma MEL202, and cutaneous melanoma C8161). Cocultures of activated T cells plus PD-L1^{+} tumor cells containing CD80-Fc or CD80-transfected tumor cells consistently produced more IFNγ than cocultures with mAbs to PD-L1 or PD-1 [25, 26, 31]. These results demonstrated that in vitro, CD80-Fc is more effective in sustaining T cell activation than mAbs to PD-L1 or PD-1.

CD80-Fc enhances T cell activation by simultaneously inhibiting PD-1 suppression and costimulating

CD80 was originally identified as a ligand that delivers a costimulatory signal to T cells via its receptor CD28 [32]. To determine whether CD80 was also functioning as a costimulatory

molecule in our tumor systems, two in vitro approaches were used. (i) Using human tumor cells and PBMC from healthy donors, CD80–CD28 costimulation was prevented by inclusion of mAbs to CD28. (ii) In a murine system, costimulation was eliminated by using T cells from CD28-deficient mice. By blocking or eliminating CD28, costimulation was prevented and CD80 could only facilitate T cell activation by preventing PD-1 suppression. Therefore, IFNγ production in cultures blocked for CD28 represents activity exclusively due to inhibiting PD-1 suppression, while the differential in IFNγ levels between CD28 blocked and unblocked cultures represents activity due to costimulation. In both human and mouse systems, CD80-Fc maintained T cell production of IFNγ in the presence of PD-L1$^+$ tumor cells; however, IFNγ levels were not as high as when costimulation was not inhibited [26, 31]. Therefore, blocking costimulation reduces, but does not eliminate the ability of CD80 to maintain T cell activation, indicating that CD80-Fc acts by both costimulating and blocking the PD-1 suppressive pathway.

CD80-Fc delays murine tumor progression and increases tumor-infiltrating T cells (TIL) more effectively than mAbs to PD-L1

In mouse models, mAbs to PD-L1 or PD-1 augment immunotherapy approaches such as adoptive T cell therapy and cancer vaccines [11, 15, 16, 33–37], and in some cases, antibody monotherapy reduces tumor progression and increases survival [9, 38–40]. However, some tumors are refractory or minimally responsive to PD-L1 or PD-1 monotherapy [34, 37, 41, 42]. To determine whether CD80-Fc is more effective in vivo than mAb monotherapy, we compared the efficacy of mAbs to PD-L1 vs. CD80-Fc for impacting tumor progression in mice with CT26 tumors. BALB/c mice were inoculated in the flank on day 0 with 5×10^5 PD-L1$^+$ CT26 colon carcinoma cells and subsequently untreated or treated intraperitoneally with CD80-Fc, PD-L1 mAb 10F.9G2, or rat IgG2b isotype control (200 μg/mouse) on days 3, 6, 9, and 22. Tumor diameters were measured twice a week. Surviving mice were killed on day 42, and tumors were excised and weighed, and then analyzed by immunohistochemistry for TIL following staining with mAbs to CD3. All of the CD80-Fc-treated mice were alive at day 42, whereas only 67, 0, and 50 % of the PD-L1 mAb-treated mice, isotype control mice, and untreated mice, respectively, were alive. At day 42, the tumors in the CD80-Fc-treated group were approximately half the volume of the tumors in the surviving mice of the other groups. Immunohistochemistry revealed that the tumors from the CD80-Fc-treated group were highly infiltrated with TIL and contained many more TIL than tumors from the other groups (Fig. 3).

Conclusions

Since many human tumors, as well as activated T cells and DC, express PD-L1, the PD-1 suppressive pathway is a significant obstacle to both natural antitumor immunity and immune therapies aimed at activating a patient's immune system. Inhibitors of the p38 MAP kinase pathway reduce transcription of PD-L1 by DC and may thereby enhance DC-based cancer vaccines [43]. However, these drugs cannot be used in vivo, so other therapy strategies are needed. Binding of CD80 to PD-L1 in both murine and human systems has been known since 2007 and 2008, respectively [17, 18]. However, prior to the studies

reviewed here, it was not appreciated that CD80 binding to PD-L1 prevents PD-L1/ PD-1 interactions and therefore neutralizes PD-1-mediated immune suppression. Because CD80 is also a potent costimulatory molecule that facilitates T cell activation when bound to its receptor CD28, CD80 serves the dual function of activating T cells while simultaneously inhibiting PD-1 pathway immune suppression (Fig. 1). This combination of effects by a single reagent may explain the heightened efficacy of soluble CD80 as compared to anti-PD-L1 or PD-1 monotherapy.

PD-L1 also tolerizes T cells by reverse signalling through T cell-expressed CD80, so soluble CD80 has the potential to also neutralize this additional suppressive mechanism. Whether or not soluble CD80 prevents suppression by reverse signalling remains to be tested.

A potential limitation of soluble CD80 as a therapeutic agent is its binding to CTLA-4, another molecule capable of suppressing activated T cells [44] (Figs. 1, 2c). However, the enhancement of T cell activation that is routinely observed with soluble CD80, plus our preliminary data using antibodies to block CTLA-4 (Horn and Ostrand-Rosenberg, unpublished), indicates that CD80-Fc does not cause immune suppression by binding to CTLA-4. Given that the predicted binding sites for PD-L1 and CTLA-4 on CD80 are in the same region, and that the dissociation constant for CTLA-4–CD80 interactions is smaller than that for PD-L1–CD80 binding, it is surprising that CD80-Fc does not appear to suppress T cell activation through CTLA-4. The apparent lack of suppression through CTLA-4 could be due to the inability of CD80-Fc to cross-link CTLA-4, or because binding of CD80 to PD-L1 perturbs the CTLA-4 binding site. Additional studies are clearly needed to resolve this issue.

A phase I trial of superagonist mAb TGN1412 to CD28 created a cytokine storm (cytokine release syndrome) in all six volunteers [45]. Recent studies demonstrated that this effect involved TGN1412 binding to the Fc gamma receptor IIb (FcγRIIb) on monocytes, which is up-regulated by high-density culture [46]. Our in vitro studies have not produced elevated levels of cytokines characteristic of a cytokine storm. Likewise, tumor-bearing mice treated with CD80-Fc did not display symptoms of cytokine release syndrome. The absence of these symptoms is most likely because the binding constant of CD80-Fc for CD28 is approximately five orders of magnitude lower than mAb binding to CD28 [47]. However, use of an Fc domain that does not bind to the FcγRIIb could overcome the potential for cytokine release syndrome.

Clearly, more extensive studies in experimental animal models and clinical trials are needed to establish whether a soluble form of CD80 will be more effective than current mono or combination antibody therapies for patients with cancer. However, our initial studies indicate that soluble CD80 has efficacy and supports the continued testing of soluble CD80 as a novel cancer immunotherapy.

Acknowledgments

Abbreviations

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APC	Antigen-presenting cell
CD80-Fc	Soluble CD80 (two extracellular domains of CD80 fused to the Fc domain of Ig)
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cells
IgC	Immunoglobulin-like constant region
IgV	Immunoglobulin-like variable region
mAbs	Monoclonal antibodies
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
TcR	T cell receptor for antigen
TIL	Tumor-infiltrating T cells
TROY-Fc	Toxicity and JNK inducer or TNFRSF19 protein fused to the Fc domain of Ig

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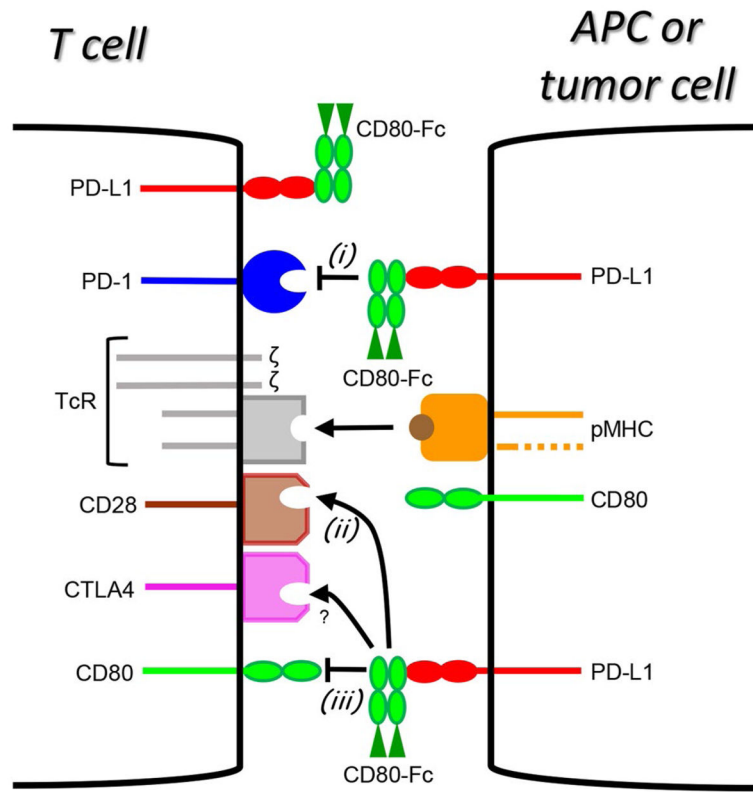
Fig. 1.
A soluble recombinant molecule consisting of the extracellular domains of CD80 linked to the Fc domain of Ig (soluble CD80 or CD80-Fc) has the potential to maintain and facilitate T cell activation through three independent mechanisms. (i) CD80-Fc could block the PD-1 suppressive pathway by binding to PD-L1 on tumor cells or activated T cells and preventing binding to PD-1 on activated T cells; (ii) CD80-Fc could activate tumor-reactive T cells by binding to PD-L1 on tumor cells and simultaneously costimulating through CD28 on T cells; (iii) CD80-Fc could prevent the tolerance induced by reverse signalling of PD-L1 through CD80 on T cells by binding to PD-L1 on tumor cells. TcR = T cell receptor; pMHC = peptide-MHC complex; APC = antigen-presenting cell; ? = CD80-Fc probably does not bind to CTLA4 although theoretically it should bind
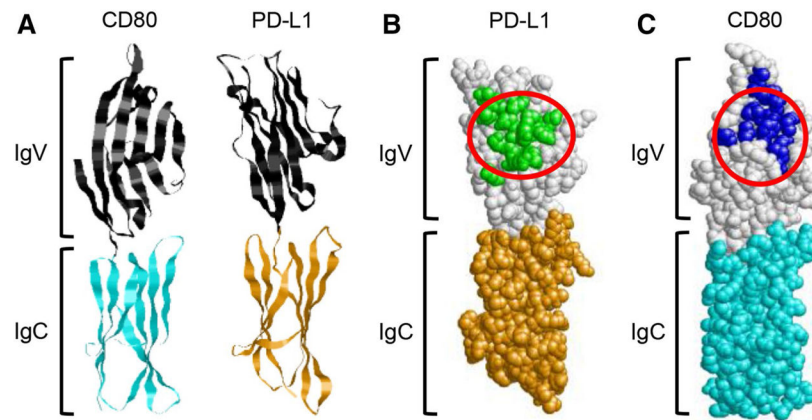
Fig. 2.

CD80, PD-1, PD-L1, and CTLA-4 share common binding sites. **a** Ribbon structures showing the amino-proximal IgV-like and the amino-distal IgC-like domains of CD80 and PD-L1 (structures are from RCSB Protein Databank (http://www.rcsb.org/pdb/home/home.do); accession numbers 1I8L and 3BIK, respectively). **b** PD-L1 space-fill structure showing the predicted binding sites for PD-1 (*green residues*) and CD80 (*area circled in red*). **c** CD80 space-fill structure showing the predicted binding sites for CTLA-4 (*blue residues*) and PD-L1 (*area circled in red*). Predicted binding sites are based on [17, 48, 49], and identification of amino acid residues that are within 3.9Å of the binding partner
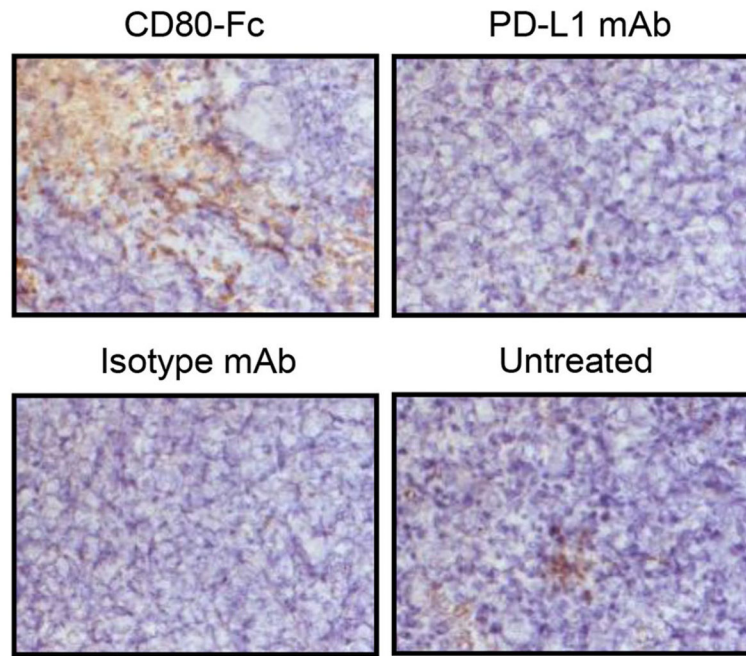
Fig. 3.
Tumors of mice treated with soluble CD80 (CD80-Fc) contain large areas of tumor-infiltrating CD3$^+$ T cells. Tumors were excised on day 40 (Isotype control group) or 42 (all other groups) post-CT26 inoculation, embedded and frozen in O.C.T, and subsequently sectioned and stained for immunohistochemistry with mAbs to CD3. Magnification is ×200