TUMOR IMMUNOLOGY

Local delivery of low-dose anti–CTLA-4 to the melanoma lymphatic basin leads to systemic T_{reg} reduction and effector T cell activation

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Preclinical studies show that locoregional CTLA-4 blockade is equally effective in inducing tumor eradication as systemic delivery, without the added risk of immune-related side effects. This efficacy is related to access of the CTLA-4 blocking antibodies to tumor-draining lymph nodes (TDLNs). Local delivery of anti–CTLA-4 after surgical removal of primary melanoma, before sentinel lymph node biopsy (SLNB), provides a unique setting to clinically assess the role of TDLN in the biological efficacy of locoregional CTLA-4 blockade. Here, we have evaluated the safety, tolerability, and immunomodulatory effects in the SLN and peripheral blood of a single dose of tremelimumab [a fully human immunoglobulin gamma-2 (IgG2) mAb directed against CTLA-4] in a dose range of 2 to 20 mg, injected intradermally at the tumor excision site 1 week before SLNB in 13 patients with early-stage melanoma (phase 1 trial; NCT04274816). Intradermal delivery was safe and well tolerated and induced activation of migratory dendritic cell (DC) subsets in the SLN. It also induced profound and durable decreases in regulatory T cell (T_{reg}) frequencies and activation of effector T cells in both SLN and peripheral blood. Moreover, systemic T cell responses against NY-ESO-1 or MART-1 were primed or boosted ($N = 7$), in association with T cell activation and central memory T cell differentiation. These findings indicate that local administration of anti–CTLA-4 may offer a safe and promising adjuvant treatment strategy for patients with early-stage melanoma. Moreover, our data demonstrate a central role for TDLN in the biological efficacy of CTLA-4 blockade and support TDLN-targeted delivery methods.

INTRODUCTION

Systemic immune checkpoint blockade (ICB) induces robust clinically effective T cell immunity in patients with metastatic melanoma, which is associated with better patient survival (*1*–*4*). However, the risk of autoimmune-related side effects so far has precluded its application in patients with early-stage melanoma (*5*). Of all patients with newly diagnosed melanoma, the majority present with localized early-stage melanoma (i.e., clinical stage I/II) (*6*). According to current guidelines, these patients are treated with a curative-intent resection with additional sentinel lymph node biopsy (SLNB), subject to melanoma Breslow thickness ≥ 0.8 mm or < 0.8 mm with ulceration (*7*). In case of sentinel lymph node (SLN)–positive disease, adjuvant systemic treatment with a programmed cell death-1 (PD-1) monoclonal antibody (mAb) is now approved by the U.S. Food and Drug Administration (FDA) for patients with high-risk stage III melanoma and for patients with pathology-confirmed high-risk stage IIB or IIC melanoma (*8*, *9*). Recurrence rates in early-stage patients run as high as 16 to 30% (*10*, *11*), and the 10-year melanoma-specific survival can run as low as 75%, depending on risk factors such as Breslow thickness, tumor ulceration, and mitotic rate. Patients with SLN-negative disease and pathology-confirmed stage I-IIA localized melanoma have no standard adjuvant treatment available (*12*). Because of the sheer number of patients first presenting with early-stage thin melanoma (≤1 mm, stage I), more of these patients will eventually die compared with patients initially diagnosed with thick melanoma (>4 mm) (*13*). All this considered, there is a good rationale for adjuvant treatment also in patients with pathologically confirmed early-stage melanoma to prevent spread.

New insights into the mechanism of action underlying ICB efficacy point to the importance of immunomodulation in tumor-draining lymph nodes (TDLN) rather than the tumor microenvironment (TME) (*14*–*17*). Effective PD-1 blockade involves the CD28-mediated expansion of tumor-reactive T cell clones by antigen-presenting dendritic cells (DCs) in TDLN, rather than just the reversal of T cell exhaustion in the TME as originally thought (*18*–*20*). In line with this, cytotoxic T lymphocyte–associated protein-4 (CTLA-4) blocking antibodies expand the diversity of tumor-reactive T cell clones through their induction in TDLN (*21*, *22*). This may entail increased antitumor effector T cell priming in the TDLN through facilitated CD28-mediated co-stimulation as well as regulatory T cell (T_{reg}) depletion or impairment of suppressive T_{reg} function (*23*–*28*). As the first-line TDLN, the SLN represents not only the site where naïve T cells are first primed but also the site where even in early-stage melanoma immunosuppression is found and shapes the pre-metastatic niche (*29*). Consequently, the SLN is an attractive target

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for local immune therapeutic intervention in patients with early-stage melanoma, aiming to counteract tumor-induced immunosuppression, boost an antitumor immune response, and prevent disease recurrence. Moreover, the SLN provides a clinical platform to assess the selective effects of immunomodulatory agents on TDLN in the absence of tumor (*30*, *31*).

Preclinical studies indicate that intratumoral administration of CTLA-4 blocking antibody in a low dose is as effective in inducing tumor eradication as systemic delivery, without the risk of immune-related adverse events (irAEs) (*32*, *33*). We hypothesized that, in melanoma patients with clinically node-negative disease, local administration of anti–CTLA-4 would be able to reshape the immune landscape of tumor-conditioned SLN, leading to the release of CTLA-4–mediated inhibitory effects on DC–T cell interactions and ultimately to increased systemic anti-melanoma T cell reactivity. Here, we present the clinical tolerability data of a phase 1 dose escalation study of a single intradermal injection of tremelimumab, a fully human immunoglobulin gamma-2 (IgG2) mAb directed against CTLA-4, at the primary tumor excision site of patients with clinical stage I/II melanoma. We also report its immunological effects as evaluated in the SLN and peripheral blood collected from these patients. Our results showed that local anti–CTLA-4 administration was well tolerated and did not coincide with any serious irAEs. In terms of immunological activity, we demonstrated that locally administered tremelimumab selectively activated migratory DC subsets in the SLN and induced full-range T cell activation while attenuating activated T_{reg} levels both locally and in peripheral blood. In addition, we showed that local and low-dose tremelimumab was able to boost systemic anti-melanoma T cell responses and increase central memory T cell (T_{CM}) formation by specifically conditioning the TDLN. Hence, local administration of tremelimumab aimed at optimal access to and conditioning of the TDLN may serve as a safe and effective adjuvant treatment option for patients with early-stage melanoma at risk for tumor recurrence.

RESULTS

Patient characteristics, safety, and toxicity

Patients with clinical early-stage melanoma were enrolled in this phase 1 study between July 2012 and December 2014. In four dose-escalating cohorts of three patients each, anti–CTLA-4 (tremelimumab) was injected around the scar of the primary tumor excision site, 1 week before the SLNB at doses of 2, 5, 10, and 20 mg. The dose range was based on extrapolation from dose levels of locally applied anti–CTLA-4 in preclinical studies and the highest doses with acceptable low-grade side effects in a clinical study of a single-dose intravenous administration of tremelimumab (*32*), as outlined in detail in the "Study design and objectives" section of Materials and Methods. The average interval between primary tumor excision and tremelimumab administration was 40.2 days (range, 23 to 72 days), with no significant differences between dose level cohorts (see Table 1). Because of the unforeseen transition of the global development rights of tremelimumab as monotherapy during accrual of the highest dose level cohort, only four patients were enrolled in this cohort rather than the originally planned eight patients. As a result, data of a total of 13 patients are described in this report.

Clinicopathological characteristics of each individual study patient are shown in Table 1 (left column). Of the 13 patients, 9 were male (69%) and the average age was 55 years (range, 25 to 76 years). Primary tumor size according to the American Joint Committee on Cancer (AJCC) seventh edition (*34*), in force at time of this study, was ≤1.0 mm (pT1, 3 of 13; 23%), >1.0 to 2.0 mm (pT2, 7 of 13; 54%), >2.0 to 4.0 mm (pT3, 2 of 13; 15%), or >4.0 mm (pT4, 1 of 13; 8%). Pathologic examination revealed 5 of 13 (38%) patients with stage III melanoma based on the presence of tumor cells in the SLN. On the basis of the Melanoma Risk Assessment Tool (www.melanomarisk.org.au/SNLForm), the anticipated risk of metastatic involvement of the SLN showed a trend to be higher for the patients with pathologically proven metastases in the posttreatment SLN biopsy (fig. S1), suggesting that there was no obvious treatment-related interference with staging. All patients with metastasis-positive SLN underwent a subsequent completion lymph node dissection. Median follow-up was 44 months (range, 8 to 79 months). Two patients experienced disease recurrence (both local and distant, unrelated to disease stage or dose level) and received adjuvant treatment with a combination of dabrafenib and trametinib (Table 1). A schematic study outline with treatment schedule and timing of blood and SLN sampling for immune monitoring is shown in Fig. 1A.

Intradermal administration of anti–CTLA-4 was well tolerated. No inadmissible toxicities were observed; all AEs were mild to moderate, were unrelated to disease stage, and were most frequent in the 10- and 20-mg cohort (Table 1). Grade 1 hepatotoxicity and grade 1 anemia developed in three and two patients, respectively. In one patient with pretreatment low white blood cell count, grade 2 leukopenia occurred. Other grade 1 AEs included wound dehiscence and nausea, each in one patient. Furthermore, one patient developed grade 1 local depigmentation of the right nipple after intradermal injection of tremelimumab around the excision site of the primary melanoma located at the upper right arm (fig. S2). None of the patients with reported AEs required supportive treatment. To ascertain whether higher AE rates in the higher dose level cohorts could be due to systemic spread of tremelimumab, we determined tremelimumab titers in posttreatment plasma samples over time (fig. S3). Low dose-dependent concentrations ranging between 0.15 and 0.65 μg/ml were detectable at 1 week after injection, which declined to undetectable levels by 3 months after injection. This contrasted sharply with reported plasma concentrations of between about 10 and 200 μg/ml at 1 week after infusion of a single dose of intravenous tremelimumab in a range that was associated with grade 1 to 3 irAEs (1 to 15 mg/kg); in all these patients, plasma concentrations exceeding 1 μg/ml are still detectable at 3 months after infusion (*35*). Patients receiving the lowest systemic dose level, which resulted in detectable tremelimumab plasma levels in that study (0.1 mg/kg), had plasma levels that still exceeded those measured in the highest dose level in this study. Among those patients, only one case of grade 1 weight loss was reported (*35*). Thus, the exceedingly low but nevertheless slightly elevated plasma levels at the highest dose levels in this study might have accounted for some of the observed side effects, but these were all mild and deemed acceptable.

Immune monitoring

The effect of intradermally administered tremelimumab on various immune cell populations was assessed by means of fluorescence-activated cell sorting (FACS) analysis of freshly isolated SLN single-cell and cryopreserved peripheral blood mononuclear cell (PBMC)

Table 1. Baseline characteristics, adverse events, follow-up, and available samples for immune monitoring of individual study patients. ALT, alanine transaminase; AST, aspartate transaminase; F, female; LRR, locoregional recurrence; DR, distant recurrence; FU, follow-up; M, male; mo, months; TNM, tumor node metastasis status.

Clinicopathological characteristics											Adverse events		Follow-up				Available samples for immune monitoring	
Patient no.	Dose* (mg)	Sex	Age (years)	Site of primary tumor	Breslow (mm)	Ulceration	SLN metastasis	No. of LN metastases/ no. of all dissected LN	TNM*	Stage*	Time between tumor excision and tremelimumab injection (days)	AEs	Grade AEs†	LRR	DR	Adjuvant therapy	Time to last known FU‡ (mo)	PBMC (all time points)	SLN
1	2	M	46	Trunk	1.6	Yes	No		pT2bN0	IIA	31	Elevated AST/ ALT	1	Yes	Yes	Yes	27	Yes	Partial§
2	2	M	53	Trunk	0.9	Yes	No		pT1bN0	IB	34			No	No	No	50	Yes	Yes
3	2	F	51	Trunk	1.0	No	No		pT1aN0	IA	49			No	No	No	70	Yes	Yes
4	5	M	45	Extremities	1.7	No	No		pT2aN0	IB	39			No	No	No	79	Yes	Yes
5	5	M	72	Trunk	1.3	No	No		pT2aN0	IB	29			No	No	No	18	Yes	Yes
6	5	F	25	Extremities	1.9	No	Yes	2/11	pT2aN2a	IIIA	64			No	No	No	8	Yes	Partial§
7	10	M	64	Extremities	2.6	No	No		pT3aN0	IIA	23	Wound dehiscence	1	No	No	No	54	Yes	Partial§
8	10	M	43	Extremities	6.0	Yes	Yes	1/17	pT4bN1a	IIIB	33	Anemia	1	No	No	No	60	Yes	Yes
9	10	M	67	Trunk	3.9	No	Yes	1/14	pT3aN1a	IIIA	30	Elevated AST/ ALT leukopenia	12	Yes	Yes	No	22	Yes	No
10	20	M	66	Extremities	1.4	No	Yes	1/14	pT2aN1a	IIIA	72	Elevated AST/ ALT nausea	11	No	No	No	50	Yes	Yes
11	20	F	57	Extremities	1.0	No	Yes	1/15	pT1aN1a	IIIA	41	Anemia skin depigmentation	11	No	No	No	41	Yes	Yes
12	20	F	76	Trunk	1.1	No	No		pT2aN0	IB	48			No	No	No	50	Yes	Yes
13	20	M	54	Trunk	1.4	No	No		pT2aN0	IB	29			No	No	No	54	Yes	Yes

*According to American Joint Committee on Cancer (AJCC) seventh edition. †According to Common Terminology Criteria for Adverse Events (CTCAE) v3.0. ‡Time from injection of tremelimumab to last known follow-up. §Because of limited SLN cell yield, FACS analysis was limited to phenotypic characterization of the T cell compartment.

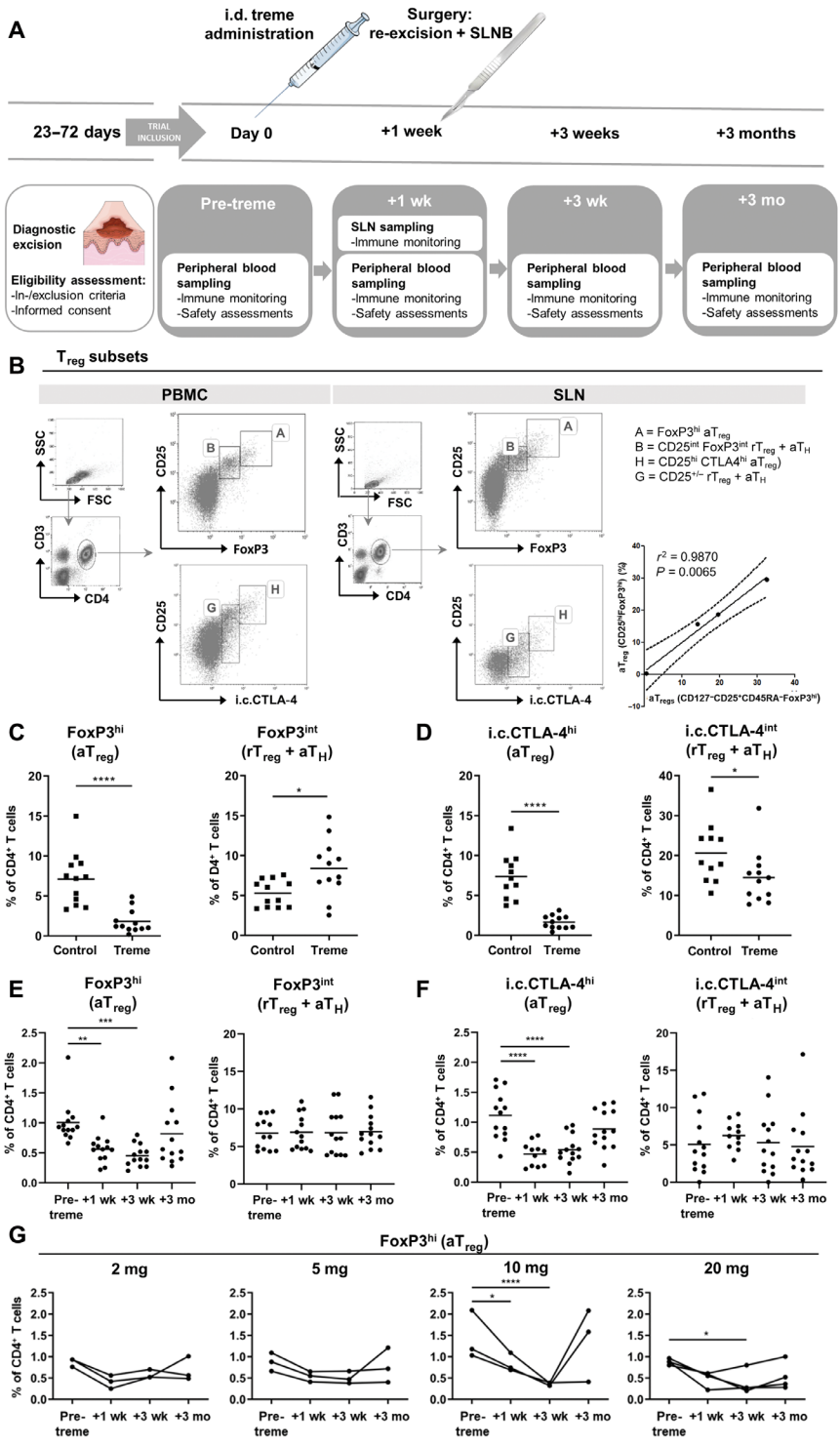

Fig. 1. Study flowchart and T_{reg} frequencies in SLN and PBMC. (**A**) Study design and flowchart. Patients eligible for trial inclusion were scheduled for an intradermal (i.d.) tremelimumab (treme) injection within 4 to 10 weeks after diagnostic excision of the primary melanoma. Subsequently, 1 week after the intradermal tremelimumab administration, patients underwent re-excision and SLNB. Sampling for immune monitoring: gray text boxes. Peripheral blood sampling also included hematology and chemistry testing for safety assessments. Peripheral blood sampling at time point +1 week (day of surgery) was carried out before the start of surgery. (**B**) Gating strategies for T_{reg} subsets in PBMC and SLN cell samples. T_{reg} subsets were all pregated by forward scatter/side scatter (FSC/SSC) lymphocyte live and $CD3^+CD4^+$ gating. Both CTLA-4 and FoxP3 were detected as intracellular (i.c.) antigens; i.c. indicated for CTLA-4. Bottom right: Correlation in four untreated SLN samples of aT_{reg} frequency according to gating as shown in (A) or as proposed by Miyara *et al*. (*37*) with additional $CD127^-CD45RA^-$ pregating. (**C**) Frequencies of $CD25^{hi}FoxP3^{hi}$ aT_{regs} and $CD25^{int}FoxP3^{int}$ rT_{regs} and aT_H in tremelimumab-treated versus untreated SLN. (**D**) Frequencies of $CD25^{hi}CTLA\text{-}4^{hi}$ aT_{regs} and $CD25^{+/-}CTLA\text{-}4^{int}$ rT_{regs} and aT_H in tremelimumab-treated versus untreated SLN. Tremelimumab-treated SLN, $n = 12$; matched control SLN, $N = 12$. aT_{reg} and $rT_{reg} + aT_H$ frequencies according to (**E**) FoxP3 and (**F**) intracellular CTLA-4 expression in PBMC samples of patients ($N = 13$) before tremelimumab and 1 week, 3 weeks, and 3 months after intradermal tremelimumab administration. (**G**) $CD25^{hi}FoxP3^{hi}$ (aT_{reg}) frequencies in PBMC samples of individual patients at tremelimumab doses of 2, 5, 10, and 20 mg, measured before tremelimumab and 1 week, 3 weeks, and 3 months after intradermal tremelimumab administration. Frequencies are all expressed as percentage of total $CD3^+CD4^+$ T cells, and means are shown. $^*P = 0.01$ to 0.05, $^{**}P = 0.001$ to 0.01, $^{***}P = 0.0001$ to 0.001, and $^{****}P < 0.0001$, unpaired *t* test or Mann-Whitney *U* test (C and D); repeated-measures one-way ANOVA or Friedman, with post hoc Dunnett's or Dunn's multiple comparison test (E and F); two-way ANOVA (within dose level cohort), with post hoc Dunnett's multiple comparison test (G). Images from Servier Medical Art and iStock.

samples. Of all 13 patients, PBMC samples from all time points were available [i.e., day 0 (before tremelimumab administration), +1 week (before SLNB), +3 weeks, and +3 months; Fig. 1A]. In three patients, the SLN cell yield was limited and only partial FACS panels were applied, prioritizing T_{reg} and T cell analyses. In one additional case (patient 9), the SLN was smaller than 0.5 cm and found to be unsuitable for SLN cell harvesting by the pathologist (Table 1). A full overview of frequency and activation state of all analyzed immune cell subsets is shown in tables S1 (SLN) and S2 (PBMC). For the SLN analyses, we matched the tremelimumab-treated SLN with a historical control SLN group (i.e., untreated or placebo treated) available from previous studies conducted by our research group (*36*), using the same panels and the same FACS machine; for statistical justification of the selected patients and matching, we refer to the "Matching with historical control SLN" section of Materials and Methods.

Systemic decreases in activated T_{reg} frequencies

Effector or activated aT_{regs} were previously defined as $CD25^{hi}FoxP3^{hi}$ cells ($FoxP3^{hi}$ aT_{reg}) or $CD25^{hi}$ $CTLA\text{-}4^{hi}$ ($CTLA\text{-}4^{hi}$ aT_{reg}) within the $CD3^+CD4^+$ T cell population (*36*, *37*). The $FoxP3^{int}$ and $CTLA\text{-}4^{int}$ populations described in this study likely comprised both resting T_{regs} (rT_{regs}) and activated T helper cells (aT_H) (*37*). For the applied gating strategy, see Fig. 1B. In four untreated and off-study SLN

samples, we applied a post hoc aT_{reg} subset gating procedure according to Miyara *et al.* (*37*), including pregating of $CD4^+$ T cells on $CD25^+$ and $CD127^-$ status and excluding $CD45RA^+$ cells from the $FoxP3^{hi}$ population. A near-perfect correlation with the classical $CD25^{hi}FoxP3^{hi}$ gating applied in the study samples confirmed the aT_{reg} status of the $CD25^{hi}FoxP3^{hi}$ population (see Fig. 1B, bottom right).

Both $FoxP3^{hi}$ and $CTLA\text{-}4^{hi}$ aT_{reg} frequencies were significantly lower in the tremelimumab-treated SLN compared with the matched control SLN (Fig. 1, C and D, left). Consistent with this, $FoxP3^{hi}$ and $CTLA\text{-}4^{hi}$ aT_{reg} frequencies in PBMC profoundly decreased after local tremelimumab administration, with significant decreases, pooled across all dose levels, lasting as long as 3 weeks after treatment (Fig. 1, E and F, left). Although not significant, even after 3 months aT_{reg} frequencies in peripheral blood did not appear to have returned to pretreatment levels. As tremelimumab did not change (relative) $CD4^+$ or $CD8^+$ T cell frequencies, the observed decreased aT_{reg} frequencies led to significantly increased $CD8^+$ T cell/ aT_{reg} ratios in both SLN and PBMC after tremelimumab treatment (fig. S4). Although in all dose level cohorts decreased levels of $FoxP3^{hi}$ aT_{regs} in posttreatment PBMC samples were observed (Fig. 1G), these only reached statistical significance in the highest two dose levels (i.e., 10 and 20 mg). In the SLN, no such dose level effects were observed (table S1). In contrast, higher $FoxP3^{int}$ (i.e., rT_{reg} and aT_H) frequencies were observed in SLN after tremelimumab administration, suggesting that tremelimumab induced a relative shift from a suppressive to a nonsuppressive T_{reg} phenotype in the SLN (Fig. 1C, right). $CTLA\text{-}4^{int}$ frequencies in tremelimumab-treated SLN were lower than in control SLN (Fig. 1D, right). Of note though, $CTLA\text{-}4^{int}$ rates in control SLN were twice as high as $FoxP3^{int}$ rates, suggesting that they comprised additional CTLA-4–expressing T_H without FoxP3 expression, which might have decreased upon local tremelimumab delivery. In PBMC, posttreatment levels of both $FoxP3^{int}$ and $CTLA\text{-}4^{int}$ (nonsuppressive) $CD4^+$ T cell subsets remained similar to pretreatment levels, consistent with selective targeting of aT_{regs}. To ascertain whether this selective targeting could be due to increased membrane expression of CTLA-4 on aT_{regs}, we compared intracellular with membrane staining of CTLA-4 within T_H, rT_{reg}, and aT_{reg} subsets, based on CD4, CD25, CD127, and FoxP3 staining, according to Miyara *et al.* (*37*), in four off-study SLN samples. As shown in fig. S5A, intracellular levels of CTLA-4 were significantly higher in aT_{regs} than in rT_{regs} and in rT_{regs} than in T_H, whereas membranous extracellular levels were only significantly higher in aT_{regs} over rT_{regs} or T_H, consistent with the selective posttreatment loss of aT_{regs}. Higher extracellular expression levels of CTLA-4 on intracellular $CTLA\text{-}4^{hi}$ aT_{regs} are also demonstrated in fig. S5B, showing representative extracellular and intracellular double stains in aT_{regs} versus rT_{regs}.

Also, significantly lower frequencies of both circulating monocytic myeloid-derived suppressor cells (mMDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) were observed 1 week after tremelimumab administration, and these rates remained significantly decreased up to 3 months after treatment (Fig. 2, A and B). These systemic MDSC decreases appeared dose dependent (Fig. 2C), but variability in baseline frequencies between the 20-mg cohort and the other cohorts (particularly for PMN-MDSC), and the low patient numbers call for caution in drawing too firm conclusions. This variability in baseline frequencies was not significantly related to time since primary tumor resection or age/sex distribution (see fig. S6 and Table 1). Again, no dose-dependent effect was observed in MDSC subset levels measured in tremelimumab-treated SLN ($N = 9$; table S1). Data from untreated SLN in our historical control group did not include MDSC analyses, precluding comparative analyses.

Local and systemic T cell activation and systemic memory T cell differentiation

To evaluate the influence of intradermal tremelimumab administration on lymphocyte activation state, we analyzed expression of the activation markers human leukocyte antigen–DR (HLA-DR) and inducible T cell costimulator (ICOS) and the checkpoint molecules CTLA-4 and PD-1 on $CD4^+$ and $CD8^+$ T cells (see Fig. 3A for gating). In SLN, tremelimumab induced a dose-dependent increase in HLA-DR and ICOS levels on $CD4^+$ and $CD8^+$ T cells, respectively (Fig. 3B). Corresponding data from historical control SLN were not available. In PBMC, pooled analyses of all study patients revealed significantly increased HLA-DR and ICOS expression levels in both the $CD4^+$ and $CD8^+$ T cell compartment, with highest T cell activation 3 weeks after tremelimumab administration, whereas posttreatment checkpoint molecule expression levels were not increased compared with pretreatment levels, with the exception of a slight increase in PD-1 levels in $CD4^+$ T cells at 3 months (Fig. 3C). Systemic up-regulation of T cell activation and checkpoint molecules was dose dependent, with significant increases observed at the highest dose of 20 mg (shown for ICOS in Fig. 3D). Similar trends were found for HLA-DR and PD-1, but not for CTLA-4 (fig. S7).

Next, we assessed the effects of intradermally administered tremelimumab on T cell differentiation. Memory T cells were identified on the basis of CD45RO expression. In addition, three memory subsets were characterized on the basis of CD45RA and CD27 expression, including $CD27^+CD45RA^-$ T_{CM}, $CD27^-CD45RA^-$ effector memory (T_{EM}), and $CD27^-CD45RA^+$ effector memory expressing CD45RA (T_{EMRA}) T cells (*38*, *39*). See Fig. 4A for gating procedures. Because analysis of T cell effector/memory marker subsets was not carried out in our historical control SLN samples, a direct comparison between untreated and tremelimumab-treated SLN could not be made. No clear dose-dependent effects for T cell differentiation states were found in tremelimumab-treated SLN (Fig. 4B). Systemically, increased posttreatment levels of $CD45RO^+$ memory T cells were observed across all dose levels at 1 and 3 weeks, whereas no shifts in T_{EM} and T_{CM} subset frequencies were observed and only CD4 T_{EMRA} levels significantly increased at 3 months (Fig. 4C). Thus, locally administered tremelimumab was able to induce memory $CD4^+$ and $CD8^+$ T cell differentiation, leading to increased $CD45RO^+$ T cell rates in peripheral blood.

Selective activation of migratory DC subsets in tremelimumab-treated SLN

In the SLN, we identified four conventional DC (cDC) subsets (*40*). These include two migratory $CD1a^+$ cDC subsets, i.e., $CD11c^{int}CD1a^{hi}$ Langerhans cells (LCs) and $CD11c^{hi}CD1a^{int}$ dermal-like DCs (dDCs), and two lymph node–resident (LNR) $CD1a^-$ cDC subsets, i.e., $CD11c^{hi}CD14^-$ LNR-cDC and $CD11c^+CD14^+$ LNR-cDC (for gating, see Fig. 5A). The $CD11c^{hi}CD14^-$ LNR-cDC population consists mostly of a $CD1c^+CD141^+$ cDC2 subset (about 75%) and of a minor $CD1c^-CD141^{hi}$ cDC1 subset (about 25%) (*41*).

In tremelimumab-treated SLN, LCs were detected at significantly lower frequencies and $CD11c^{hi}CD14^-$ LNR-cDCs were detected at

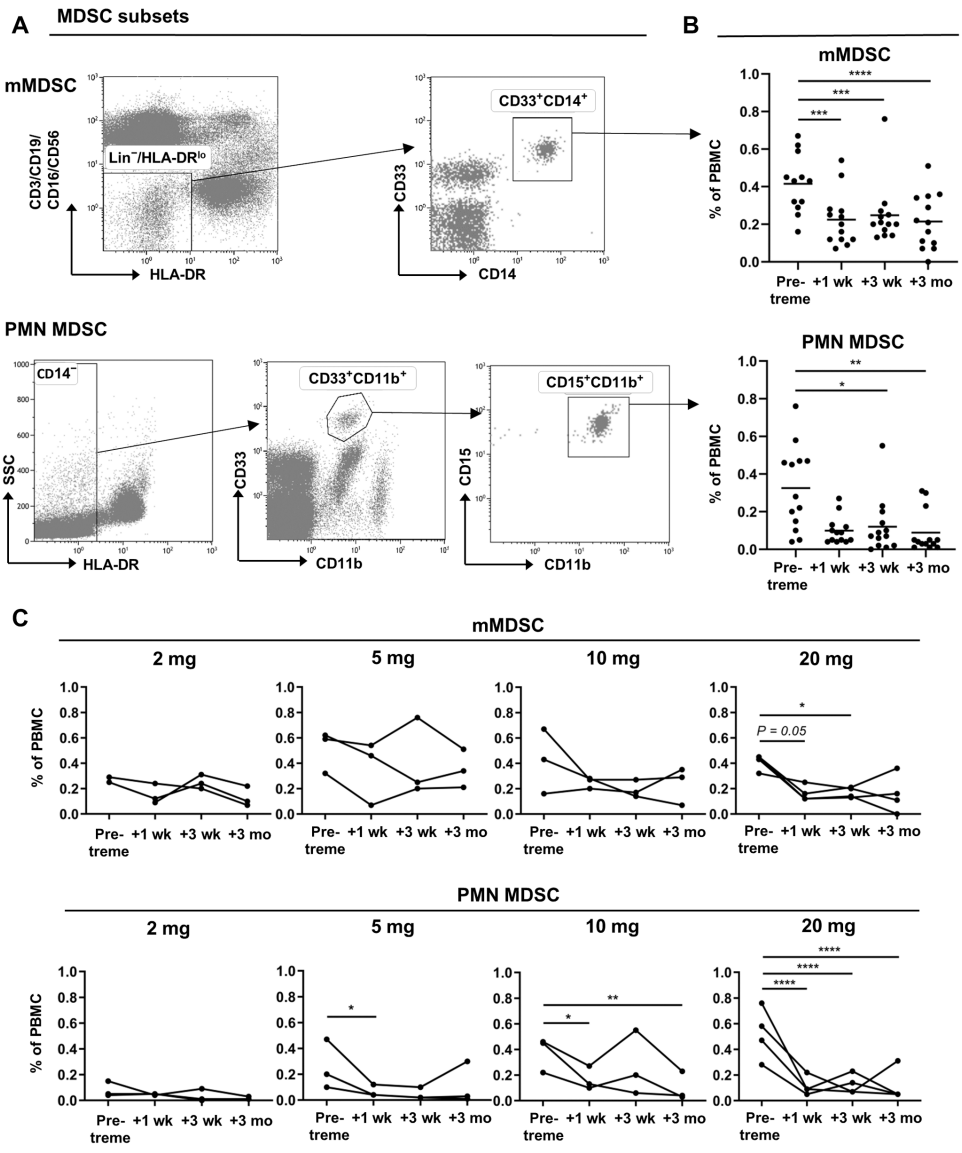

Fig. 2. Decreased systemic MDSC frequencies after intradermal tremelimumab. (**A**) Gating strategy for MDSC subsets: (top row) mMDSC defined as HLA-DR^{lo}Lin^{-}CD33^{+}CD14^{+} and (bottom row) PMN MDSC defined as CD33^{+}CD11b^{+}CD14^{-}CD15^{+}. Frequencies of mMDSC and PMN MDSC in PBMC cell samples before tremelimumab and 1 week, 3 weeks, and 3 months after intradermal tremelimumab administration (**B**) of patients pooled across all dose levels ($N = 13$) and (**C**) of individual patients at dose levels 1 to 4 (i.e., 2, 5, 10, and 20 mg). Frequencies are all expressed as percentage of total PBMC, and means of pooled data are shown. *$P = 0.01$ to 0.05, **$P = 0.001$ to 0.01, ***$P = 0.0001$ to 0.001, and ****$P < 0.0001$, repeated-measures one-way ANOVA or Friedman, with post hoc Dunnett's or Dunn's multiple comparison test (B); two-way ANOVA (within dose cohorts), with post hoc Dunnett's multiple comparison test (C).

higher frequencies compared with control SLN, whereas tremelimumab did not induce changes in frequency of the other two cDC subsets (Fig. 5B). Tremelimumab significantly augmented maturation and activation of both migratory cDC subsets, but not of the LNR-cDC subsets (Fig. 5C). The CD11c^{hi}CD14^{-} LNR-cDC displayed significantly decreased levels of CD83 and CD86 in tremelimumab-treated SLN compared with control SLN, which may be related to suppressive cross-talk with tremelimumab-induced ICOS^{+} T cells (*42*–*44*), borne out by an observed negative correlation between T cell ICOS up-regulation and LNR-cDC activation (fig. S8). The CD11c^{hi}CD14^{+} LNR-cDC subset remained unaffected (Fig. 5C, right column). In conclusion, intradermally administered tremelimumab induced up-regulation of the costimulatory CTLA-4 ligands CD80 and CD86, as well as the activation markers CD83 and CD40, selectively on migratory cDC subsets in the SLN.

Locally administered tremelimumab induces systemic melanoma-specific T cell reactivity

To assess the effect of intradermal injection of tremelimumab on systemic melanoma-specific T cell reactivity, we assessed antigen-specific T cell responses directed against the melanoma-associated antigens (MAAs) NY-ESO-1 and MART-1 by means of in vitro restimulation and an interferon-γ (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay in pre- and posttreatment PBMC samples; reactivity against a pool of recall antigen-derived peptides [CEFT (CMV, EBV, Flu and Tetanus)] was taken to assess general immune competence (see Fig. 6A for a schematic overview of the methodology and representative raw ELISpot data—from patient 13). On the basis of the NY-ESO-1/MART-1 ELISpot data, among the 13 patients enrolled in this study, we distinguished tremelimumab immune responders (i.e., patients with a tremelimumab-induced or tremelimumab-enhanced T cell response) and non-responders (i.e., patients with no detectable or an unaltered T cell response after tremelimumab). Tremelimumab immune responders were considered to have (i) a de novo response, when a positive response to either NY-ESO-1 or MART-1 could be detected after treatment only, or (ii) a preexistent, augmented response, when a positive NY-ESO-1– or MART-1–specific T cell response was measurable before treatment, but further significantly enhanced after treatment (see Fig. 6B for representative examples). In Fig. 6C, pretreatment MAA-specific T cell frequencies, as measured by IFN-γ ELISpot readout, of each patient were plotted against the highest measured posttreatment MAA-specific T cell frequencies. In total, 7 of 13 patients (54%) were identified as tremelimumab immune responders and the remaining 6 as nonresponders. In table S3, a complete overview of the pre- and posttreatment T cell responses against NY-ESO-1 and MART-1 per patient, as well as reactivity against the CEFT recall peptide pool, is shown. A CEFT-specific T cell response was found in all patients and served as a measure of general immune competence. In one patient, posttreatment CEFT-specific spots per 100,000 T cells

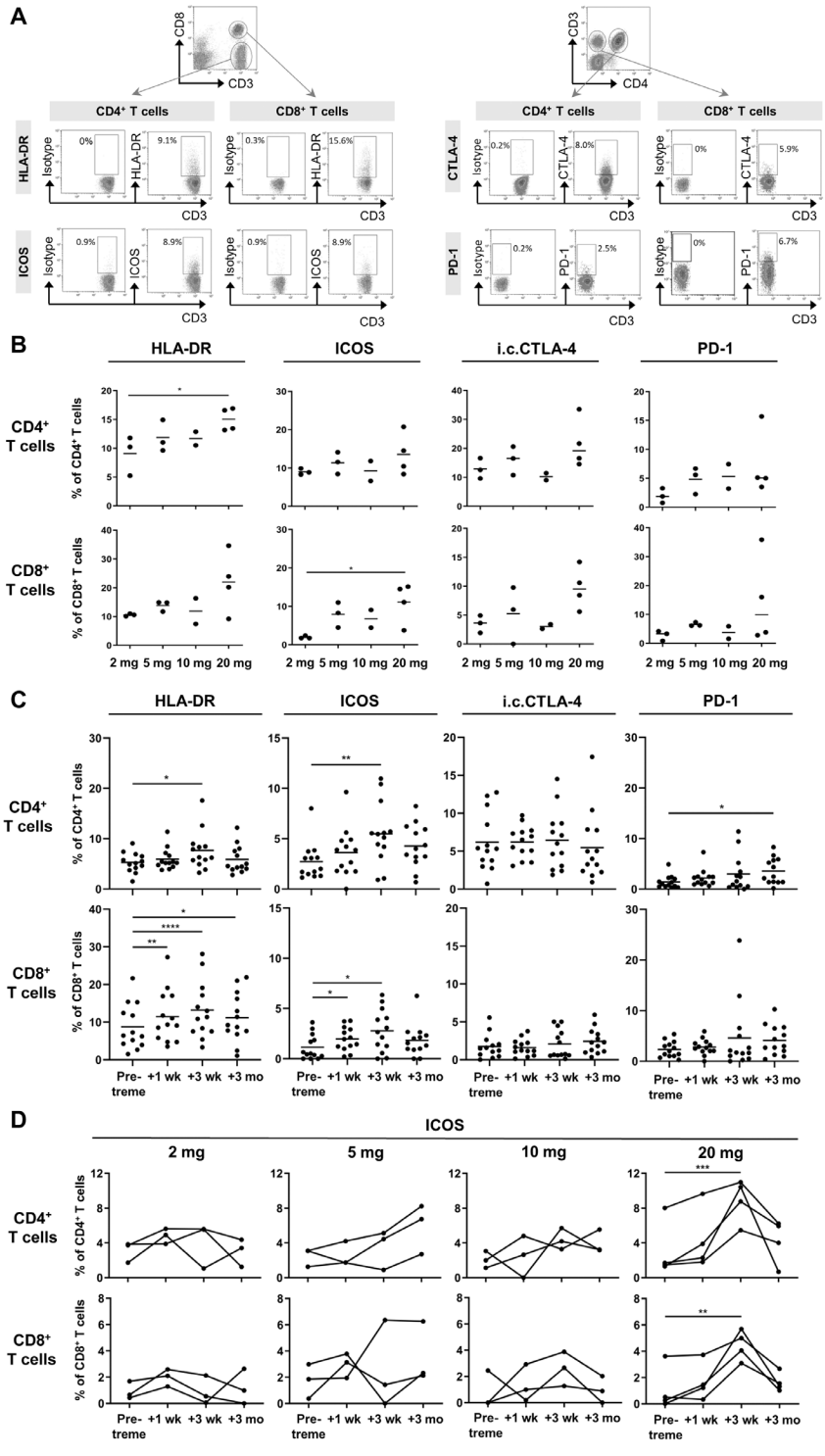

Fig. 3. T cell activation and checkpoint expression levels after intradermal tremelimumab. (**A**) Gating strategy for HLA-DR, ICOS, CTLA-4 (intracellular), and PD-1 expression on $CD4^+$ and $CD8^+$ T cells. FSC/SSC gating was performed to determine viable lymphocytes as shown in Fig. 1B. Because of four-color staining, for HLA-DR and ICOS expression, $CD8^-$ T cells were designated "$CD4^+$ T cells" and, for intracellular CTLA-4 and PD-1, $CD4^-$ T cells were designated "$CD8^+$ T cells." Frequencies of $CD4^+$ and $CD8^+$ T cells expressing activation molecules (HLA-DR and ICOS) and checkpoint point molecules (CTLA-4 and PD-1) in (**B**) tremelimumab-treated SLN were displayed according to dose cohort and (**C**) PBMC samples of patients ($n = 13$) before tremelimumab and 1 week, 3 weeks, and 3 months after intradermal tremelimumab administration. (**D**) Frequency of ICOS-expressing $CD4^+$ and $CD8^+$ T cells in PBMC samples of individual patients at doses of 2, 5, 10, and 20 mg, measured before tremelimumab and 1 week, 3 weeks, and 3 months after intradermal tremelimumab administration. Frequencies are expressed as percentage of total $CD4^+$ T cells or $CD8^+$ T cells, and means are shown. $^*P = 0.01$ to 0.05, $^{**}P = 0.001$ to 0.01, $^{***}P = 0.0001$ to 0.001, and $^{****}P < 0.0001$, one-way ANOVA with post hoc Dunnett's multiple comparison test (B); repeated-measures one-way ANOVA or Friedman, with post hoc Dunnett's or Dunn's multiple comparison test (C); two-way ANOVA (within dose level cohort), with post hoc Dunnett's multiple comparison test (D).

were <100; corresponding MAA T cell reactivity in this patient revealed a nonresponder profile (patient 7; see table S3). Both patients who experienced recurrences (patients 1 and 9) were nonresponders (see Table 1 and table S3).

As a measure of humoral immunity, we also assessed IgG responses to NY-ESO-1 in an enzyme-linked immunosorbent assay (ELISA) with full-length NY-ESO-1 (see fig. S9). In general, titers were low and declining over time, likely reflecting elimination of the antigen source by primary tumor resection. Only two patients showed a marginally positive response: one T cell tremelimumab responder (patient 4) and one nonresponder (patient 9) (fig. S9).

Tremelimumab-induced melanoma-specific T cell responses are associated with increased T cell activation and memory T cell differentiation

To identify a possible immune signature in the SLN associated with tremelimumab-induced or tremelimumab-augmented systemic MAA-specific T cell reactivity, phenotypic immune profiles in tremelimumab-treated SLN of tremelimumab immune responders were compared with those of nonresponders. T_{reg} frequencies, $CD8^+$ T cell/aT_{reg} ratios, and immune checkpoint expression levels on T_{regs} were similar in tremelimumab responders and nonresponders (fig. S10). However, hierarchical cluster analysis based on treatment-related increases in SLN T cell activation, checkpoint and memory markers, generated two main clusters: patients with both high T cell activation and highly increased memory T cell rates ("high" group) and patients with both low T cell activation and less pronounced T cell memory increases ("low" group) (Fig. 7A). All four patients in the high group were tremelimumab immune responders (all with de novo detectable responses), whereas only three of eight patients in the low group were responders. Two of these patients (patients 2 and 3) had a preexisting MAA-specific T cell response that was augmented after tremelimumab treatment (Fig. 7A and table S3). Although the designation of "de novo response" is arbitrary to a certain extent, as it is defined by a preset cutoff and statistical considerations, these data do support previous evidence that T cell

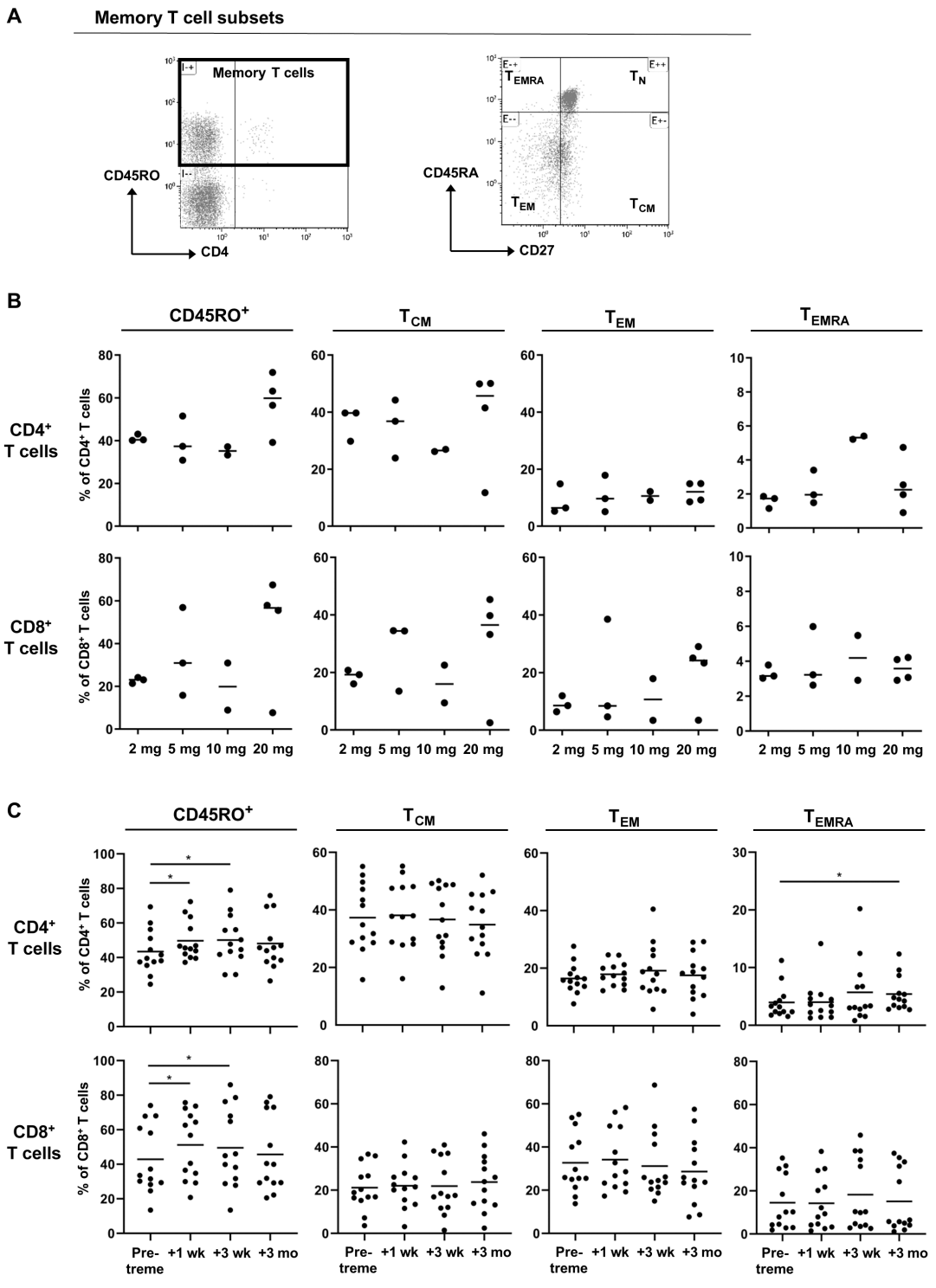

Fig. 4. Intradermal tremelimumab induces a systemic memory T cell response. (**A**) Gating strategy of memory T cell subsets in PBMC cell samples, with examples representing $CD8^+$ memory subsets (i.e., pregated as $CD3^+CD8^+$); for gating of $CD4^+$ memory subsets, cells were pregated as $CD3^+CD8^-$. FSC/SSC gating was performed to determine viable lymphocytes before subsequent subset gating, as shown in Fig. 1B. (**B** and **C**) Columns represent memory T cells and subsets: $CD45RO^+$ memory T cells, the $CD27^+CD45RA^-$ T_{CM} cell subset, the $CD27^-CD45RA^-$ T_{EM} cell subset, and the $CD27^-CD45RA^+$ terminally differentiated effector T cell subset (T_{EMRA}). Frequencies (with bars representing means) of $CD4^+$ and $CD8^+$ memory T cell subsets are shown in (B) SLN of patients 1 week after delivery of different cohort assigned dose levels of intradermal tremelimumab (i.e., 2, 5, 10, and 20 mg) and (C) in PBMC of patients before tremelimumab and 1 week, 3 weeks, and 3 months after intradermal tremelimumab administration (all dose cohorts combined). *$P = 0.01$ to 0.05, repeated-measures ANOVA or Friedman, with post hoc Dunnett's or Dunn's multiple comparison test (C).

activation upon CTLA-4 blockade preferentially leads to the induction of de novo T cell responses and thus broadens the antitumor T cell repertoire (*45*). Because of limited SLN cell yield and prioritizing of T cell subset analyses, natural killer (NK), MDSC, and DC FACS data were available for only nine patients (two nonresponders and seven tremelimumab immune responders), thereby precluding reliable subgroup or hierarchical cluster analyses for these subsets.

Next, to assess whether phenotypic PBMC immune profiles could also serve as a potential biomarker for tremelimumab efficacy, baseline rates and posttreatment changes in PBMC immune subsets were compared between tremelimumab immune responders and nonresponders. No significant association with tremelimumab response was found for any of the baseline PBMC parameters. The pattern of posttreatment decreases in T_{reg} and MDSC frequencies did not differ between tremelimumab immune responders and nonresponders, nor were any changes in NK cell or DC subsets, or in PD-1 or CTLA-4 expression levels associated with either group (fig. S11). In accordance with findings in the SLN, tremelimumab immune responders showed marked elevation of posttreatment $CD4^+$ and $CD8^+$ T cell activation in the peripheral blood, evidenced by significantly increased HLA-DR and ICOS expression levels, whereas nonresponders did not (Fig. 7B). Likewise, there was only significant augmentation of

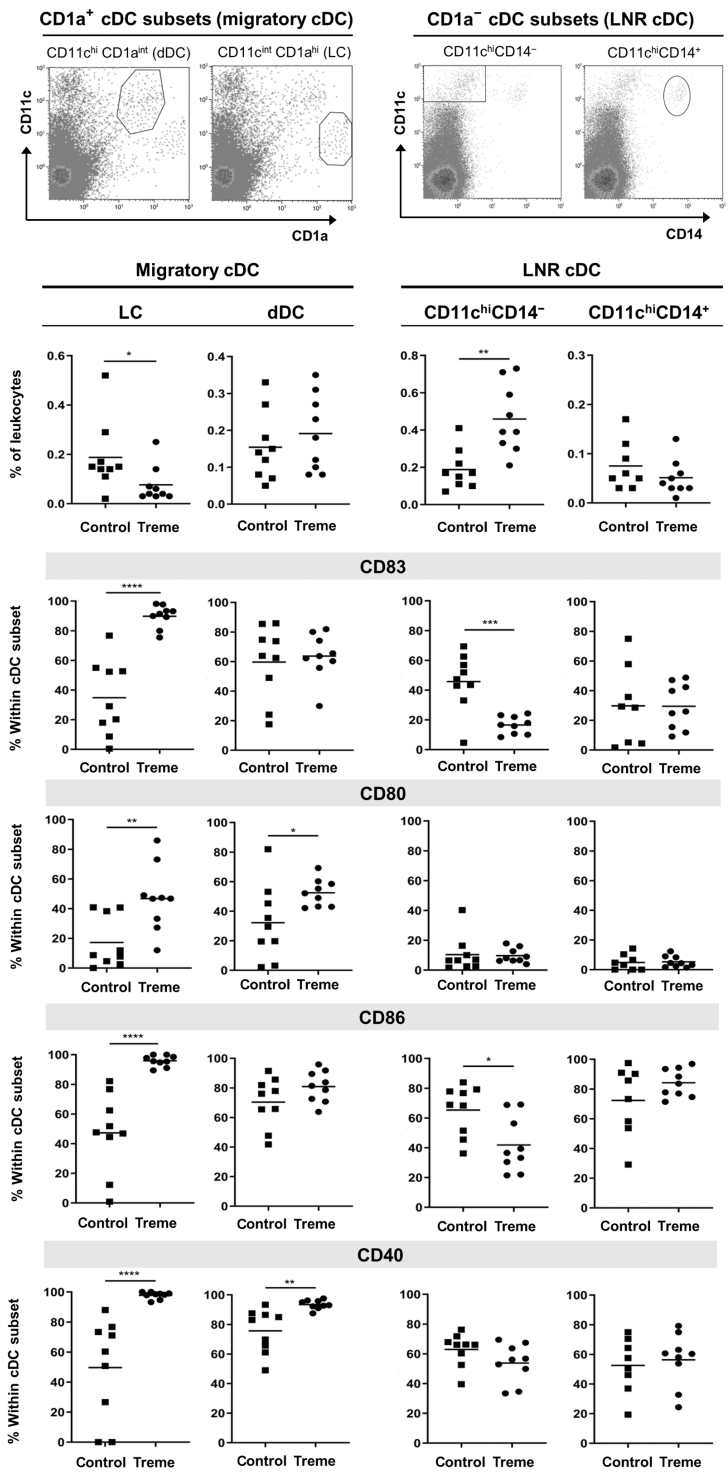

Fig. 5. Intradermal tremelimumab selectively enhances activation and maturation of migratory cDC subsets in the SLN. (**A**) Gating strategies for the four cDC subsets in the SLN, i.e., two CD1a$^+$ migratory cDC subsets [CD11c^{hi}CD1a^{int} (dDC) and CD11c^{int}CD1a^{hi} (LC)] (left plots) and two CD1a$^-$ LNR DC subsets (CD11c^{hi}CD14$^-$ and CD11c^{hi}CD14$^-$) (right plots). Viable cells were gated on the basis of forward scatter and side scatter, and LNR and cDC were pregated on CD1a$^-$. (**B**) cDC subset frequency (top row) and expression levels of maturation markers (rows 2 to 5) of tremelimumab-treated SLN ($N = 9$) compared with data from matched control SLN ($N = 9$). Columns represent the different DC subsets. Left: Two CD1a$^+$ migratory cDC subsets (LC and dDC). Right: Two CD1a$^-$ LNR-cDC subsets (CD11c^{hi}CD14$^-$ and CD11c^{hi}CD14$^+$ LNR-cDC). Frequencies of DC subsets are expressed as percentage of total LN cells; expression levels of maturation (CD83) and costimulatory (CD80 and CD86) and activation (CD40) surface receptors on different DC subsets are expressed as percentage of positive cells within each subset. Bars represent means. *$P = 0.01$ to 0.05, **$P = 0.001$ to 0.01, ***$P = 0.0001$ to 0.001, and ****$P < 0.0001$, unpaired *t* test or Mann-Whitney *U* test.

the CD4$^+$ and CD8$^+$ T cell CD45RO$^+$ and central memory compartment in responders; in nonresponders, posttreatment frequencies of T_{CM} cells were decreased and coincided with increased T_{EMRA} rates, indicative of a more senescent T cell state (Fig. 7C). Thus, local tremelimumab administration resulted in a coordinated systemic T cell memory shift and (MAA-specific) T cell activation.

DISCUSSION

The standard of practice is to administer CTLA-4 and PD-1 immune checkpoint inhibitors systemically. This is in keeping with the long-held conventional wisdom that cytostatic treatments in metastatic cancer need to act systemically to eliminate distant metastases. This practice negates the basic principle that adaptive immunity is primed locally (e.g., in response to infection, tissue damage, or a tumor) and can subsequently provide systemic and durable protection through recirculating effector and memory T and B cells. Pivotal in this basic concept is the role of locoregional lymph nodes where T cells are primed by migrating and/or LNR-cDC. By local administration of immune checkpoint inhibitors in or around the primary tumor, high concentrations and bioavailability of these drugs in the tumor and TDLN can be reached, allowing for priming of effector and memory T cells while minimizing actual systemic exposure and subsequent toxicity (*15*, *33*, *46*–*48*). Although the concept of intratumoral immunomodulation and/or ablation to achieve in vivo vaccination has long been proposed and pursued (*46*, *49*–*52*), there are very few clinical studies that have explored the effects of local delivery of immune checkpoint inhibitors (NCT03889912, NCT03316274, NCT04090775, and NCT03755739).

With this phase 1 clinical trial, we demonstrated that intradermal administration of a single low-dose anti–CTLA-4 (tremelimumab) around the scar of the primary tumor excision site in patients with early-stage melanoma was well tolerated and did not coincide with any serious toxicity. Furthermore, local treatment with tremelimumab reshaped the immune microenvironment in the melanoma-draining SLN and also and bolstered the systemic anti-melanoma T cell response. The clinical setting of this study, i.e., subsequent to surgical removal of the primary tumor and prior to the SLNB, allowed for the specific assessment of the role of TDLN in the biological efficacy of CTLA-4 blockade. The mode of action of CTLA-4 blockade is generally believed to involve targeting of the CD28 ligands CD80/CD86, which are mostly restricted to professional antigen-presenting cells (APCs) that typically reside in peripheral lymphoid tissues. The higher avidity of CTLA-4 for these costimulatory ligands would lead to interference with proper costimulation

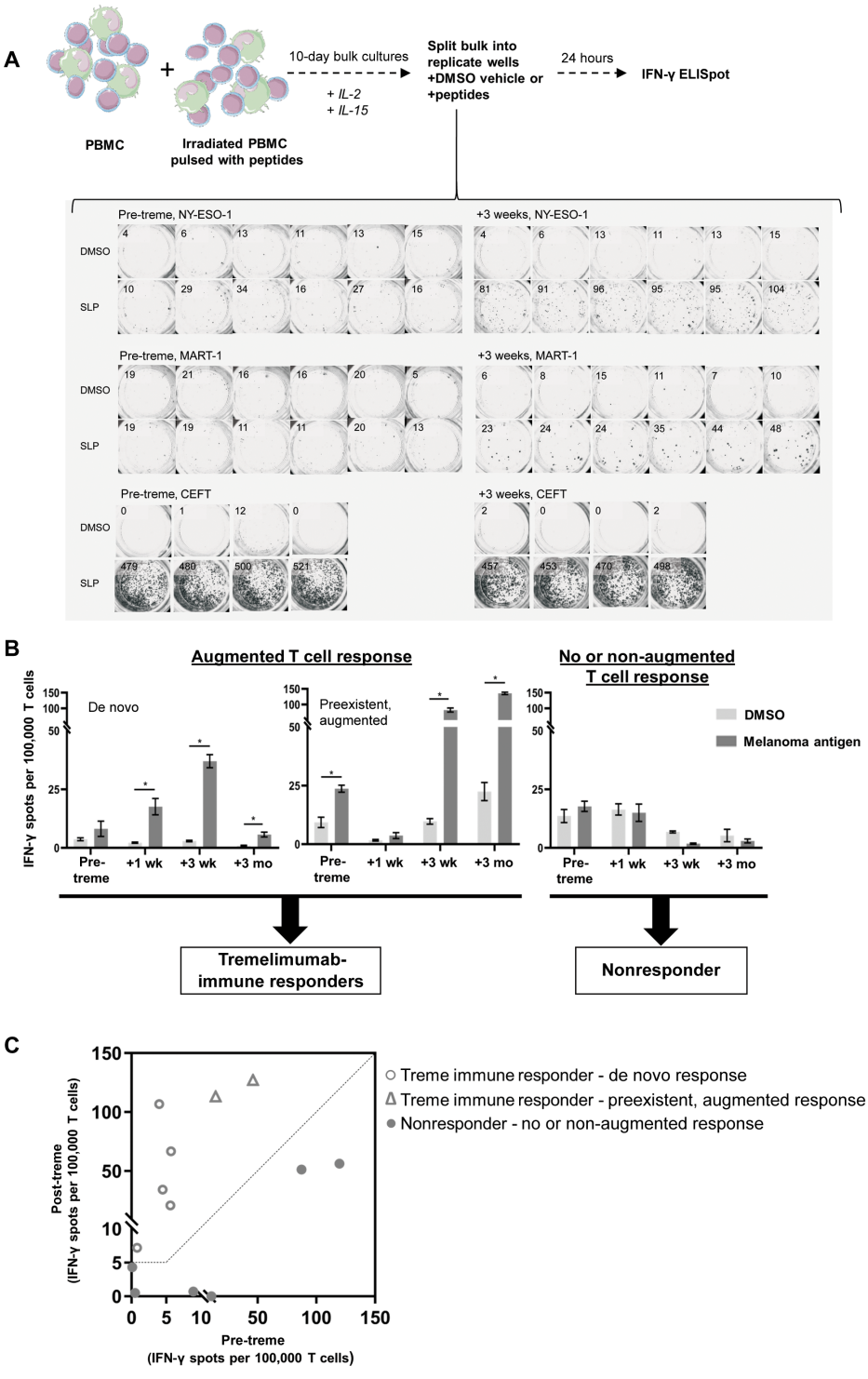

Fig. 6. Systemic MAA-specific T cell responses after intradermal tremelimumab administration. (**A**) Schematic overview of melanoma-specific T cell stimulation to quantify the induction of systemic MAA-specific T cell responses by means of an IFN-γ ELISpot assay. Cryopreserved PBMCs were thawed and incubated at a 1:1 stimulator-to-responder ratio with irradiated autologous PBMC pulsed with MAA-specific peptides (NY-ESO-1, MART-1) or CEFT peptides as positive recall control. Cells were harvested at day 10 and seeded in 2× four to six replicate wells at a density of 1×10^5 to 2.5×10^5 per well for 24-hour restimulation with DMSO vehicle, MAA peptides (NY-ESO-1 and MART-1), or CEFT peptides in an anti–IFN-γ–coated multiscreen 96-well plate followed by ELISpot development. An example of a typical appearance of IFN-γ–positive spots in an ELISpot assay of one tremelimumab immune responding patient before tremelimumab administration (Pre-treme) and after tremelimumab treatment (+3 weeks) with raw counts is shown. (**B**) Representative data of three individual patients each with a different pattern of T cell reactivity against MAA (overlapping peptides covering NY-ESO-1 and/or MART-1 sequence) measured by IFN-γ ELISpot readout in PBMC samples before tremelimumab and 1 week, 3 weeks, and 3 months after tremelimumab administration. * indicates positive T cell response according to preset criteria (see Materials and Methods). Patients were considered tremelimumab immune responders when (i) a de novo positive response to either NY-ESO-1 or MART-1 could be detected after treatment or, (ii) in case of a preexisting (i.e., pretreatment) positive response to either NY-ESO-1 or MART-1, posttreatment antigen-specific T cell frequencies were significantly increased compared with pretreatment in a post hoc test of a one-way ANOVA. (**C**) MAA-specific (i.e., either against MART-1 or NY-ESO-1) T cell responses before and after treatment, shown for all individual patients ($N = 13$). Highest antigen-specific spots at any post-treatment time point are shown. Open dots indicate patients with a de novo T cell response, open triangles indicate patients with an augmented T cell response, and closed dots indicate patients with neither de novo nor augmented T cell responses. Images from Servier Medical Art.

and thereby dampen the priming of tumor-specific effector T cells. Thus, CTLA-4 blockade would primarily restore effective T cell priming (*23*). Our data are consistent with this notion and support a model in which interference in CTLA-4–mediated interactions between migratory DC and aT_{regs} in TDLN results in enhanced locoregional and systemic antitumor immunity.

CTLA-4 is able to capture B7 ligands (i.e., CD80 and CD86) from APCs by transendocytosis, followed by degradation of these ligands in the CTLA-4–expressing cell (*27*). This CTLA-4–mediated transendocytosis of CD80 and CD86 primarily targets migratory cDC subsets in peripheral LN (*25*) and is mediated by T_{regs} through their constitutive surface expression of high levels of CTLA-4. Our current findings in patients with early-stage melanoma support the idea that T_{reg}-mediated transendocytosis can be abrogated by CTLA-4 blockade, resulting in increased CD80 and CD86 expression of migratory cDCs in LN. The increased expression of CD83 and CD40 on migratory cDC subsets in tremelimumab-treated SLN in our study was likely an indirect result of enhanced pro-inflammatory cytokine signaling and/or decreased aT_{reg} rates at the dermal injection

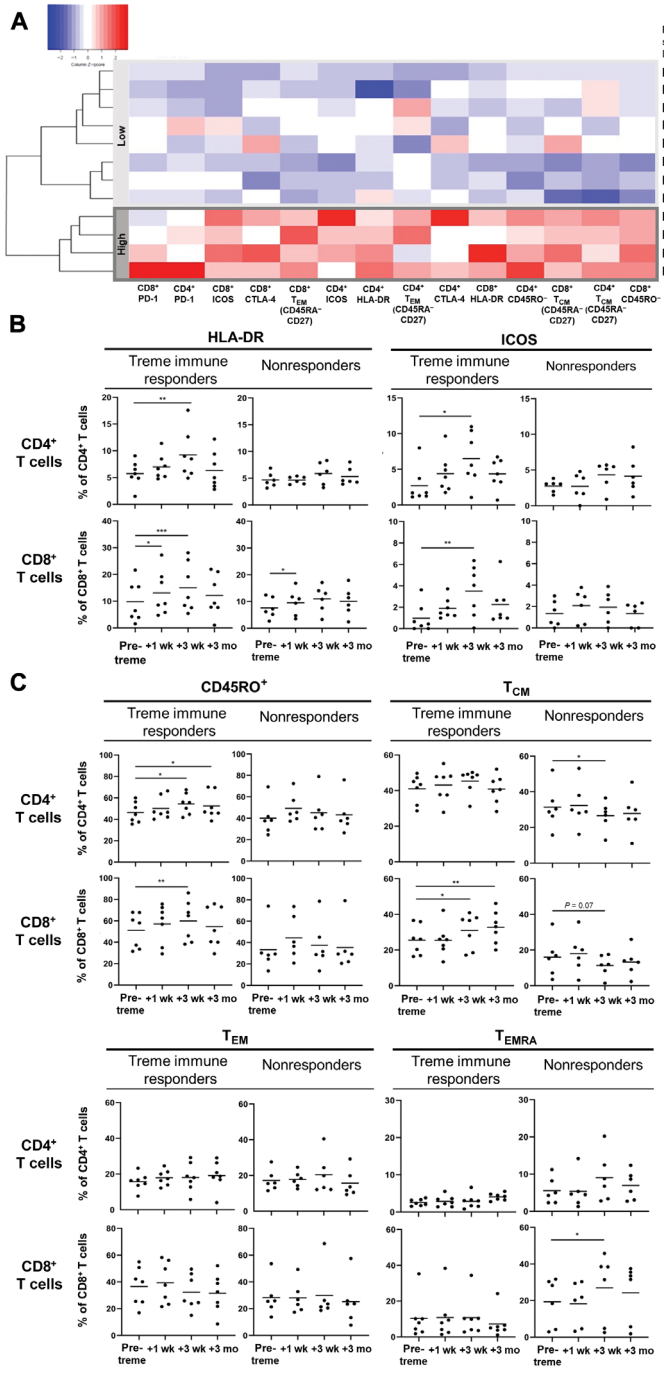

Fig. 7. Tremelimumab-induced melanoma-specific T cell responses are associated with increased T cell activation and augmented memory T cell phenotype. (**A**) Heatmap of hierarchical cluster analysis according to expression of T cell activation, immune checkpoint, and memory markers in tremelimumab-treated SLN. Corresponding patient numbers (according to numbering in Table 1), dose level, and presence or absence of a systemic (i.e., measured in PBMC) tremelimumab immune responder profile are shown. (**B** and **C**) Frequencies of (B) $CD4^+$ and $CD8^+$ T cells expressing activation molecules (HLA-DR and ICOS) and (C) frequencies of ($CD4^+$ and $CD8^+$) $CD45RO^+$ memory T cells, $CD27^+CD45RA^-$ T_{CM} cells, $CD27^-CD45RA^-$ T_{EM} cells, and terminally differentiated effector T cells (T_{EMRA}) in PBMC samples of immune responders ($N = 7$) and nonresponders ($N = 6$) before tremelimumab and 1 week, 3 weeks, and 3 months after intradermal tremelimumab administration. Frequencies are expressed as percentage of total $CD4^+$ T cells or $CD8^+$ T cells, and means are shown. $^*P = 0.01$ to 0.05, $^{**}P = 0.001$ to 0.01, and $^{***}P = 0.0001$ to 0.001, repeated-measures one-way ANOVA or Friedman, with post hoc Dunnett's or Dunn's multiple comparison test.

site. The finding that CTLA-4 blockade preferentially interferes with migratory cDC is in keeping with their higher expression levels of CD80 and CD86 (*40*) and their reported cross-talk with T_{regs} during or right after their migration to skin-draining lymph nodes, which is involved in the maintenance of self-tolerance (*53*–*57*). We thus provide clinical evidence that CTLA-4–mediated interactions between migratory cDC and aT_{reg} in TDLN (with elevated surface levels of CTLA-4) are pivotal in setting the levels of both local and systemic aT_{regs}. High numbers of aT_{regs} in melanoma TDLN may interfere with migratory cDC activation and consequently with proper priming of anti-melanoma effector T cells. CTLA-4 blockade appears to redress this disbalance.

Whereas the above proposed mechanism of action provides a strong rationale to clinically explore local CTLA-4 blockade for optimal access to TDLN, more recent insights hold that CTLA-4 efficacy, at least in part, depends on antibody-dependent cellular cytotoxicity (ADCC)–mediated depletion of T_{regs} (*24*, *26*, *58*) or interference with their suppressive functionality in the TME (*25*, *27*). Others show no decrease in absolute T_{reg} numbers in the TME by quantitative immunohistochemistry (*48*). Differences may in part be explained by assessment of T_{reg} levels at different time points after CTLA-4 blockade (*24*, *53*, *54*, *58*). Previous studies, limited to patients with advanced or metastatic melanoma, have not reported a consistent pattern of change in peripheral blood T_{reg} frequency after systemic anti–CTLA-4 treatment (*59*–*62*). Our study demonstrated consistent decreases in T_{reg} frequencies in both peripheral blood and melanoma-draining LN after local anti–CTLA-4 treatment, specifically aimed at TDLN conditioning. Although we can only speculate as to whether these observations were due to aT_{reg} conversion or depletion, we did show significantly higher CTLA-4 surface expression on aT_{regs} than on either rT_{regs} or conventional T_H. A high turnover rate of CTLA-4 is reported, accounting for its predominant intracellular localization in activated effector T cells (*63*). Higher membrane levels in aT_{regs} may be due to storage of CTLA-4 in recycling submembrane vesicles rather than in the more remote trans-Golgi compartment, which may account for higher surface expression at any given moment (*63*). As a consequence, increased tremelimumab binding as well as a higher likelihood of subsequent ADCC-mediated depletion of aT_{regs} may result.

It is unclear how our observations of the systemic immunomodulating effects of intradermal delivery of anti–CTLA-4 compare with systemic administration. Unfortunately, there are no data available for the systemic use of tremelimumab in patients with early-stage melanoma. However, in keeping with our observations, increased expression levels of activation (i.e., ICOS and HLA-DR) and memory markers (CD45RO) on $CD4^+$ and $CD8^+$ T cells in peripheral

blood of patients with urothelial carcinoma and/or metastatic melanoma are detected upon systemic CTLA-4 blockade (*44*, *58*–*60*, *64*–*67*). These parameters, and increased levels of $CD4^+ICOS^+$ T cells in particular, are also linked to clinical response and overall survival (*58*, *64*, *68*). Although decreased T_{reg} rates are reported in the TME upon systemic CTLA-4 blockade, in peripheral blood no such decreases were observed (*59*–*62*), which contrasts starkly with our findings for local CTLA-4 blockade. As for MDSCs, conflicting findings in terms of on-treatment systemic increases or decreases are reported (*69*), but in general lower baseline or posttreatment frequencies are related to favorable clinical outcome upon systemic CTLA-4 blockade (*70*). mMDSC rates in peripheral blood are unaffected by intradermal injection of a saline placebo (*71*), providing further evidence that local injection of tremelimumab in this study was responsible for the observed decline in systemic mMDSC rates.

One could speculate that the systemic biological efficacy that we observed in our study could have been due to systemic spread of the locally delivered tremelimumab, which would also be in keeping with the dose dependence that we often observed for these effects. However, the exceedingly low levels of tremelimumab that we detected after treatment argue against this. Plasma levels that are equated to efficacy of systemic tremelimumab treatment exceed 30 μg/ml at 4 weeks after infusion (*35*), whereas we found 0.4 μg/ml by 3 weeks after injection—even for the highest dose level. More likely, the observed systemic effects in our study were due to relatively high tremelimumab levels in the TDLN, resulting in decreased aT_{reg} rates and increased frequencies of activated and memory effector T cells. A similar observation was made in a mouse model of pancreatic cancer, where local low-dose anti–CTLA-4 delivery resulted in profoundly lower serum levels of the antibody as compared with systemic high-dose administration, but antitumor efficacy and effector T cell infiltration were comparable between both treatment strategies. Whereas T_{reg} rates in this tumor model were significantly elevated in TDLN and spleen upon systemic delivery, they remained low upon local delivery (*72*).

In conclusion, findings in this report demonstrate that intradermal administration of tremelimumab at the primary tumor excision site of patients with early-stage melanoma is feasible and safe. Moreover, they point to a role for the interference with CTLA-4–mediated cross-talk between migratory cDC and aT_{regs} in TDLN in the systemic biological efficacy of local CTLA-4 blockade, resulting in decreases in aT_{reg} rates and increases in conventional T cell activation and central memory conversion, ultimately leading to the induction of systemic anti-melanoma T cell responses. Although caution is obviously called for in view of the small numbers of patients enrolled in this phase 1 trial and the absence of randomized placebo controls, these observations suggest that local administration of anti–CTLA-4 may offer a safe and promising adjuvant treatment strategy for patients with early-stage melanoma. In addition, this study underscores the importance of immunomodulation of TDLN in generating de novo antitumor T cell responses and supports the concept of local instead of systemic anti–CTLA-4 blockade for future adjuvant immunotherapeutic strategies in patients with early-stage melanoma as well as for neoadjuvant approaches (*73*). This is in keeping with growing evidence from preclinical studies pointing to the key role of TDLN in the efficacy of ICB (*48*) and may allow for combined treatment with other immunomodulators for optimal efficacy without increased risk of unwanted side effects. On the basis of its safety and biological efficacy profile, we would recommend the highest tested dose of 20 mg of tremelimumab for further clinical exploration of locally applied and TDLN-targeted CTLA-4 blockade in either adjuvant or neoadjuvant approaches.

MATERIALS AND METHODS

Study design and objectives

This phase 1, two-center, open-label, dose-escalating clinical trial (NCT04274816) was designed to evaluate the safety, tolerability, and immunological activity of escalating single doses of intradermally administered tremelimumab in patients with clinical stage I/II melanoma 7 days before SLN excision. Patients were treated in four cohort doses of 2, 5, 10, or 20 mg of tremelimumab with three patients per cohort and an intended expansion phase with five additional patients at the highest tolerable dose of 20 mg. Patients were observed for dose-limiting toxicities for 28 days. If no not-acceptable toxicity (NAT) was experienced during this period by any of the patients in a cohort, dose escalation proceeded. NAT was defined as any (drug-related) grade ≥3 AE or autoimmune-related grade ≥2 AE not attributable to another etiology. All AEs were graded according to the National Cancer Institute Common Toxicity Criteria (CTC) grading system version 3.0 (NCI-CTCAE v3.0). We based the selected dose range on extrapolation of findings by Simmons *et al.* (*32*) who compared the effects of systemic high-dose (150 μg) versus local low-dose (10 μg) anti–CTLA-4 in combination with a granulocyte-macrophage colony-stimulating factor (GM-CSF)–secreting cell-based vaccine in a mouse model and demonstrated antitumor effects with as little as 10 μg of local anti–CTLA-4 with reduced serological signs of collateral autoimmunity. At clinically used systemic dose levels of 70 to 350 mg of anti–CTLA-4 (based on an average of 70 kg human body weight and an applied dose range of 1 to 5 mg/kg), this would come to an approximate range of 5 to 23 mg for equivalent local dosing. In addition, we selected the dose range of 2 to 20 mg based on the assumption that no serious toxicities should be expected, which in these patients with early-stage melanoma would have been unacceptable. Intravenous administration of comparable doses (0.01 to 0.1 mg/kg) in a previous phase 1 trial of tremelimumab resulted in one case of grade 1 weight loss and one case of grade 2 nausea (out of six patients with metastasized melanoma tested) (*35*).

Study population

Patients ≥18 years, with clinically stage I/II melanoma (AJCC seventh edition), who were scheduled for an SLNB, were eligible for inclusion. For a detailed overview of all inclusion and exclusion criteria, see table S4. The study was approved by the Institutional Review Boards of the VU University Medical Center and Spaarne Gasthuis, and written informed consent was obtained from all patients. SLN samples were collected and handled according to medical ethical guidelines described in the Code of Conduct for Proper Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies or protocols approved by the Institutional Review Boards of the participating hospitals.

Treatment and safety assessment

One single dose of tremelimumab was administered intradermally around the (diagnostic) excision scar of the primary melanoma and 7 days before scheduled re-excision and SLN procedure. Tremelimumab was provided as an intravenous solution at a 20 mg/ml concentration

(20 ml per vial), allowing for intradermal injection of 20 mg in 1 ml at the highest dose of 20 mg. This volume was kept constant for all dose levels by diluting the needed volume dose from the vials with saline solution. After tremelimumab administration, patients were clinically monitored for 1 hour for early autoimmune or allergic reactions. Additional safety assessments included monitoring of AEs and evaluation of laboratory parameters at baseline and during follow-up after 1 week, 3 weeks, and 3 months of the tremelimumab injection.

Immune monitoring

For immune monitoring purposes, cell samples derived from the surgically removed SLN (1 week after intradermal tremelimumab administration) were collected. In addition, 50 ml of heparinized peripheral blood was collected before tremelimumab administration and 1 week, 3 weeks, and 3 months after tremelimumab injection (see Fig. 1A for a structured study flowchart).

Collection and processing of SLN single-cell and peripheral blood samples

All SLNs were identified and retrieved by the triple technique as described (*74*). In short, a lymphoscintigraphy was performed at least 4 hours before surgery to dynamically determine lymphatic drainage. Immediately before surgery, a blue inert dye was injected adjacent to the scar of the primary melanoma excision. On the basis of blue staining of the draining lymphoid tissues and radiation intensity as monitored by a handheld gamma probe, the SLN was identified during surgery. Immediately after removal, the SLN was collected in a dry sterile container and taken to the pathology department of the participating hospitals for retrieval of viable cells under sterile conditions. Before routine histopathological examination and after confirmation by the pathologist that the SLN was suitable for cell harvesting (i.e., >0.5 cm), viable cells were scraped from the SLN using a previously described method, without interfering with standard diagnostic procedures (*75*, *76*). Collected SLN cells were washed twice in complete medium, comprising Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT, USA), sodium penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml), 2 mM L-glutamine (P/S/G), and 0.01 mM 2-mercaptoethanol. The obtained viable SLN single-cell samples were used directly for flow cytometric analysis. PBMCs were isolated by density gradient centrifugation with Lymphoprep (Axis-Shield, Oslo, Norway) and cryopreserved until further use as previously described (*77*).

Flow cytometric staining and analyses

The effect of intradermally administered tremelimumab on various immune cell populations was assessed by means of four-color FACS analysis of freshly isolated SLN and PBMC single-cell samples. A full overview of the used phenotypic definitions for each of the assessed immune subsets and analyzed activation/checkpoint molecules in SLN and PBMC is presented in tables S1 (SLN) and S2 (PBMC). Gating strategies for T cell and cDC subsets are shown in Figs. 1 to 5. FACS analysis was performed using fluorochrome-labeled mAbs; for an overview of specifications of the used mAbs, see table S5. Stainings were performed in FACS buffer, consisting of phosphate-buffered saline supplemented with 0.1% bovine serum albumin and 0.02% sodium azide, for 30 min. For mAbs labeled with biotin, an additional incubation step with streptavidin-allophycocyanin (eBioscience) was performed. For detection of T_{regs} and activated conventional T cell subsets, additional intracellular stainings with mAbs against FoxP3 and CTLA-4 were performed using the eBioscience anti-human FoxP3 staining set (eBioscience, San Diego, CA, USA), following the manufacturer's instructions. Matching isotype antibodies were used as negative controls. Per measurement, a minimum of 1×10^5 (membrane staining) and 2×10^5 (intracellular staining) cells were required. After incubation, cells were washed in FACS buffer to remove excess antibodies and used for flow cytometric analyses. Flow cytometric analyses were performed on a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest data acquisition and analysis software or (for advanced T_{reg} gating) on an LSR Fortessa X-20 flow cytometer (BD Biosciences). Data were analyzed using Kaluza or FlowJo (v.10.7.1) analysis software. Matching isotype antibodies were used as negative controls. In three cases, SLN cell yield was limited and partial FACS panels were applied, prioritizing T_{reg} and T cell analyses. In one additional case, the SLN was smaller than 0.5 cm and found to be unsuitable for SLN cell harvesting by the pathologist.

Melanoma-specific T cell stimulation and IFN-γ ELISpot assay

We quantified the induction of systemic MAA-specific T cell responses by means of IFN-γ ELISpot assay after a 10-day in vitro stimulation of pre- and posttreatment PBMC samples with a pool of overlapping long peptides spanning the entire sequence of the NY-ESO-1 (pool of 43 peptides, 15-mers with 11–amino acid overlap; PepMix Human NY-ESO-1; JPT) and MART-1 (pool of 27 peptides, 15-mers with 11–amino acid overlap; PepMix Human Melan-A/MART-1; JPT) protein. As a positive recall control, a CEFT pool consisting of 27 peptides selected from defined HLA class I– and II–restricted T cell epitopes from *Clostridium tetani*, Epstein-Barr virus (HHV-4), human cytomegalovirus (HHV-5), and influenza A was used (PepMix CEFT; JPT). Cryopreserved PBMCs were thawed and incubated at a 1:1 stimulator-to-responder ratio with irradiated autologous PBMC pulsed with either NY-ESO-1, MART-1 (10 μg/ml), or CEFT peptides (all 1 μg/ml per peptide) with β_2-microglobulin (3 μg/ml) (30C-cp1022U; Fitzgerald Industries, International). PBMCs were cultured for 10 days at 1×10^6/ml IMDM + 10% HPS (Human Pooled Serum) + P/S/G for 10 days in the presence of interleukin-2 (IL-2) (10 IU/ml; Novartis) and IL-15 (10 ng/ml; eBioscience). Cells were harvested at day 10 and seeded in 2× four to six replicate split wells at a density of 2.5×10^5 per well (NY-ESO-1 and MART-1) or 1.0×10^5 per well (CEFT) in a multiscreen 96-well plate coated with an IFN-γ catch antibody (Mabtech). Cells were either rechallenged overnight with the peptides to which they were initially stimulated or cultured with a dimethyl sulfoxide (DMSO) vehicle control. As a technical control, phytohemagglutinin at a concentration of 5 μg/ml was tested in duplicate (10,000 cells per well) for each plate. The following day, cells were removed and the plates were rinsed and developed according to the manufacturer's instructions [Human IFN-γ ELISpot PLUS kit (ALP), Mabtech, 3420-4APT-2]. Spots were counted by an automated ELISpot reader (AID Diagnostika). Specific spots (i.e., antigen-specific T cell frequencies) were calculated by subtracting the mean number of spots of the DMSO control from the mean number of spots in the peptide-stimulated wells. Antigen-specific T cell frequencies were considered to reflect positive responses when (i) the mean number of spots in the peptide-stimulated wells was significantly higher than the mean number of

spots in the DMSO control wells in an unpaired two-sided Student's *t* test ($P < 0.05$), (ii) the mean number of spots of peptide-stimulated wells exceeded the mean number of spots of the DMSO control wells by at least twofold, and (iii) the absolute difference in mean number of spots between the peptide-stimulated and DMSO control wells was at least 5. Patients were considered tremelimumab-induced MAA-specific immune responders when (i) a de novo detectable positive response to either NY-ESO-1 or MART-1 could be detected after tremelimumab treatment or, (ii) in case of a preexisting (i.e., before tremelimumab treatment) positive response to either NY-ESO-1 or MART-1, posttreatment antigen-specific T cell frequencies were significantly increased compared with pretreatment in a post hoc test of a one-way analysis of variance (ANOVA).

Matching with historical control SLN

As the phase 1 study design did not include a placebo-controlled arm, we matched the tremelimumab-treated SLN with a historical control SLN group (i.e., untreated or placebo-treated melanoma SLN) available from previous studies conducted by our research group (*36*) to enable immunological comparisons between "naïve" and anti–CTLA-4–modulated SLN. Because FACS analyses of these previous studies focused on either T cell or DC subsets, separate pools were formed of unmatched control SLN with available T cell data (T cell pool, $N = 15$) and of SLN with available DC data (DC pool, $N = 22$). Unmatched control SLN in the T cell and DC pool was then matched to tremelimumab-treated SLN, of which DC ($N = 9$) and/or T cell ($N = 12$) FACS analyses were performed. Matching was performed on the basis of SLN status and Breslow thickness. Because it was not possible to construct well-matched groups using propensity-score matching due to small sample sizes of both the tremelimumab and control SLN, we performed exact matching for SLN status followed by nearest neighbor matching for Breslow thickness. We used standardized mean difference (SMD) as a metric to assess covariate balance between treatment and control groups. An SMD of <0.1 was considered to indicate a negligible difference. The matched cohort showed improved covariate balance compared with the unmatched cohort, with SMD equal to zero for SLN status and less than 0.1 for Breslow thickness (table S6).

Statistical analyses

Data were tested for normal distribution using the Shapiro-Wilk normality test. To assess tremelimumab-induced effects on immune cell populations in PBMC and SLN, FACS analysis data of posttreatment samples (for PBMC taken at three different time points, i.e., 1 week, 3 weeks, and 3 months after intradermal tremelimumab injection) were compared with pretreatment samples (PBMC) and with available data of matched historical control samples (SLN). Data of all dose levels were pooled for a general assessment of tremelimumab effect and compared using a one-way repeated-measures ANOVA (normal distribution) or the Friedman test (nonnormally distributed data) with Dunnett's or Dunn's multiple comparison test as posttest, respectively (PBMC), or alternatively using an unpaired *t* test or Mann-Whitney *U* test (SLN). In addition, the dose level effect of tremelimumab was assessed by comparing pre- and posttreatment time points within each dose level cohort (PBMC) in a two-way repeated-measures ANOVA with the post hoc Dunnett's multiple comparison test and also by comparing data between different dose level cohorts (SLN), in an ordinary one-way ANOVA (normal distribution) or Kruskal-Wallis test (nonnormally distributed data) with Dunnett's or Dunn's multiple comparison test as posttest, respectively. In case of missing values, mixed-effects analysis was performed, with post hoc Dunnett's multiple comparison test. Differences were considered statistically significant when $P \leq 0.05$, as indicated with asterisks (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$). Statistical analyses were performed using GraphPad Prism software (version 8). Hierarchical cluster analysis was carried out using Euclidean distance and Ward.D2 clustering methods with the function heatmap.2 in RStudio version 1.1.423 (RStudio, USA).

SUPPLEMENTARY MATERIALS

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Figs. S1 to S11
Tables S1 to S7

View/request a protocol for this paper from *Bio-protocol*.

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Local delivery of low-dose anti–CTLA-4 to the melanoma lymphatic basin leads to systemic Treg reduction and effector T cell activation

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Under the skin

Although systemic immune checkpoint blockade (ICB) displays therapeutic efficacy in cancers, it induces immune-related adverse events (irAEs). Avoiding irAEs is crucial for effective ICB. Van Pul *et al*. performed a phase 1 clinical trial testing the effects of an intradermal injection of anti–CTLA-4 at the site of primary tumor excision in patients with early-stage melanoma. Seven of 13 patients immunologically responded to the treatment, with little to no irAEs. Responders had more tumor antigen–specific T cells in the blood, increased migratory DC activation in the sentinel lymph node (SLN), and decreased T_{reg} in both the SLN and blood. Thus, intradermal anti–CTLA-4 after primary tumor excision in melanoma induced a favorable immune response and has promise as a treatment option for patients with early-stage melanoma.

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