T Cells Dominate the Local Immune Response Induced by Intralesional IL-2 in Combination with Imiquimod and Retinoid for In-Transit Metastatic Melanoma

TO THE EDITOR

The management of in-transit metastatic melanoma remains challenging. Regional metastatic lesions are often treated surgically but recurrence is common (Maverakis et al., 2015). Recent reports demonstrate that intralesional IL-2 has an excellent complete response rate (Radny et al., 2003), especially when combined with topical imiquimod and tretinoin cream (Garcia et al., 2011; Shi et al., 2015). However, the potential of IL-2 to adversely affect antitumor T-cell responses (Scisiel et al., 2015; Sim et al., 2014) remains a concern, as patients often have subclinical systemic disease. Herein, we characterize the immune response induced by an IL-2-based intralesional therapy for patients with in-transit metastatic melanoma to determine the molecular mechanisms leading to complete tumor regression and observe the therapy’s impact on distant immune cells.

Patients (n = 4) received intralesional therapy (Figure 1a and Supplementary Materials and Methods online), which resulted in intense erythema at treatment sites and durable complete responses (Figure 1b) with no evidence of recurrence after a mean follow-up of 15.3 months (range, 5-25 months). Gene expression profiling of inflamed skin revealed widespread increases in genes coding for cytokines/chemokines, cell surface markers, transcription factors, and effector molecules (Figure 1c). Interestingly, T helper type 1 (Th1)-promoting transcription factors TBX21 (T-bet), STAT4, and STAT1 were greatly increased (97.4-, 30.2-, and 8.5-fold, respectively), whereas the gene expression of Th2- and Th17-promoting transcription factors GATA3 and RORC, respectively) was significantly decreased (0.3- and 0.2-fold, respectively). Expression of IFNG was also strongly increased in treated tumors, although it was not graphed because untreated samples did not show an appreciable signal.

Of the cell surface markers analyzed by quantitative real-time PCR, expression of IL2RA (regulatory T cells [Tregs]) and activated lymphocytes), CD69 (activated T cells and natural killer [NK] cells), CDB4 (cytotoxic T cells), CD3E (T cells), and CD52 (mature lymphocytes) increased the most (88-, 73-, 53-, 37-, and 37-fold, respectively) (Figure 1c). Immunohistochemical analysis of the treated tumors also revealed a robust cellular response comprising mainly cells bearing T-cell markers (CD3, CD4, CD8, CD45RO) and to a lesser extent cells expressing CD56 (marker for NK and NK T cells) (Figure 1d and e).

Examination of the peripheral immune response by flow cytometry (Figure 2a) revealed a therapy-induced dramatic increase in the CD4+/CD8+ T-cell ratio (Figure 2b), a twofold increase in the percentage of B cells (Figure 2c), and a shift in NK cells toward a CD56+CD16+ cell surface phenotype (Figure 2d). To further dissect the nature of the peripheral T-cell response, we also characterized effector and memory T-cell subpopulations (i.e., T cells not within the CD3+, CD4+, CD25high, CD127low Treg fraction) by flow cytometry. Activated T cells, as identified by their expression of FAS receptor (CD95), were expanded after initiation of therapy (Figure 2e). We

**Abbreviation:** Th1, T helper type 1; Treg, regulatory T cell

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also observed that central (CCR7+ and effector (CCR7-) memory (CD45RO+) CD8+ T cells (CD3+) proportionally increased during and after completion of therapy (Figure 2f). Responsiveness of the peripheral T-cell repertoire was then assessed by in vitro stimulation of peripheral blood mononuclear cell cultures with anti-CD3/CD28, revealing that intralesional therapy was associated with an increased capacity of T cells to produce IFN-γ, tumor necrosis factor, GM-CSF, and IL-5 (Figure 2g).

Finally, to characterize the effect of IL-2 on Treg cells, CD3+CD4+CD25high CD127low cells were monitored for their expression of Foxp3 by flow cytometry. Within this well-defined population, the percentage of Treg cells (Foxp3-expressing) increased from 19% before treatment to 71% (3.73-fold) during therapy (Figure 2h), a finding consistent with that seen in patients receiving systemic low-dose IL-2 as a cancer immunotherapy regimen (Zorn et al., 2006).

The role of systemic therapy for in-transit only metastatic melanoma remains controversial. In this setting, intralesional immunotherapy has emerged as a promising treatment option. Our data demonstrate that intralesional IL-2 combined with topical imiquimod and retinoid...
completely eradicates in-transit metastatic melanoma by inducing a strong local T-cell response (CD4+ and CD8+). Gene expression profiling of infiltrated tissue revealed increased expression of Th1-associated genes (IFNG, TBX21, STAT4, and STAT1) and genes associated with a cytotoxic T-cell responses (e.g., CD8A, PRF1 [perforin], and GZMB [granzyme B]). Increased expression of CXCL9, CXCL10, and CXCL11, which encode chemokines with known anticancer properties, was also detected.

In contrast, therapy resulted in a decrease in Th2- and Th17-associated transcription factors (GATA3 and RORC, respectively), which is likely beneficial because these immune responses are not effective at eradicating tumors. Other upregulated cytokines have both pro- and anticancer properties. Thus, their role in the eradication of the melanomas remains questionable.

There was also a treatment-induced regulatory response, as is evident by the observed increase in preregulatory genes (e.g. FOXP3, IL10, IDO1, and TGFBI) and in circulating Tregs, a concerning finding as IL-2-induced highly active or persistently elevated Tregs have been shown to correlate with cancer progression (Cesana et al., 2006; Sim et al., 2014). However, there was no evidence to support an overly detrimental Treg response in our patients, as activated T cells were expanded not only at the site of treatment but also within the periphery. Central memory CD8+ T cells, known to be important in anticancer immune responses (Klebanoff et al., 2005), were also expanded despite the increase in Tregs.

T cells require antigen recognition (signal 1) in the setting of appropriate costimulation (signal 2) for optimal activation, differentiation, and expansion—processes that are aided by cytokine stimulation (signal 3). T-cell paralysis can occur when antigen naive T cells encounter cytokines, such as IL-2, in the absence of signals 1 and 2 (i.e., out of sequence) (Skicsel et al., 2015). Although paralysis remains a concern with intralesional therapy, we did not find any evidence for this. In contrast, therapy was associated with increased activity of peripheral T cells, as evident by their expression of activation markers and increased capacity to produce cytokines, which may be a result of a preexisting rather than primary anticancer immune response being present when the patients received the IL-2. Also, IL-2 is a known mitogen for both CD4+ and CD8+ T cells; however, because only relative counts of circulating T cells were measured, we cannot rule out the possibility that the dramatic increase in the ratio of CD4+ to CD8+ T cells was due to a selective loss of CD8+ T cells. However, the clear increase in CD8+ T cells within the treatment sites, as demonstrated by gene expression profiling and immunohistochemistry (Figure 1c–e), would argue against CD8+ T-cell loss as a mechanism. Also, in the setting of HIV, low-dose IL-2 is known to dramatically increase CD4+ T cells whereas CD8+ T cells remain stable, which accounts for the ability of IL-2 to dramatically increase the CD4 to CD8 T-cell ratio in these patients (Kovacs et al., 1995, 1996).
Cancer-Associated Long Noncoding RNA
SMRT-2 Controls Epidermal Differentiation


TO THE EDITOR

Long noncoding RNAs (lncRNAs) are increasingly appreciated to play functional roles in homeostasis and cancer, yet their presence in cutaneous squamous cell carcinoma (SCC), the second most common cancer in the United States, is still being defined. Within the skin, anti-differentiation noncoding RNA (ANCR) and terminal differentiation-induced noncoding RNA (TINCR) are required to maintain the undifferentiated state in tissue progenitor cells and induce the normal terminal differentiation program in epidermis (Kretz et al., 2012, 2013). Aberrant expression of TINCR and the recently identified PICSAR lncRNA occur in SCC (Kretz et al., 2013; Piipponen et al., 2016). Thus, while efforts to characterize key genetic anomalies in this malignancy have traditionally focused on changes that affect protein-coding genes, altered expression of lncRNAs may also play a role in SCC development.

To identify previously unrecognized lncRNAs that are dysregulated in SCC, RNA sequencing with an average of 111 million reads per sample was performed on three primary cutaneous SCC and patient-matched normal skin following institutional approval and written, informed patient consent (Supplementary Table S1 online). Differential expression analysis identified...