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Cutting Edge: Hypoxia-Induced Nanog Favors the Intratumoral Infiltration of Regulatory T Cells and Macrophages via Direct Regulation of TGF-β1

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Emerging evidence suggests a link between tumor hypoxia and immune suppression. In this study, we investigated the role of hypoxia-induced Nanog, a stemness-associated transcription factor, in immune suppression. We observed that hypoxia-induced Nanog correlated with the acquisition of stem cell–like properties in B16-F10 cells. We further show that Nanog was selectively induced in hypoxic areas of B16-F10 tumors. Stable short hairpin RNA–mediated depletion of Nanog, combined with melanocyte differentiation Ag tyrosinase-related protein-2 peptide-based vaccination, resulted in complete inhibition of B16-F10 tumor growth. Nanog targeting significantly reduced immunosuppressive cells (regulatory T cells and macrophages) and increased CD8+ T effector cells in tumor bed in part by modulating TGF-β1 production. Additionally, Nanog regulated TGF-β1 under hypoxia by directly binding the TGF-β1 proximal promoter. Collectively, our data establish a novel functional link between hypoxia-induced Nanog and TGF-β1 regulation and point to a major role of Nanog in hypoxia-driven immunosuppression. The Journal of Immunology, 2013, 191: 5802–5806.

Hypoxia is a major component of the tumor microenvironment (1). A relationship between hypoxia and a stem cell–like phenotype has been shown to promote tumor progression (2). We have previously demonstrated that hypoxia induces impairment of immune effector cytotoxicity by several mechanisms (3, 4), including upregulation of NANOG (5). Nanog is a homeodomain transcription factor involved in the maintenance of self-renewal and pluripotency of embryonic stem cells. Increased expression of Nanog has been reported in several types of cancers, where it is associated with apoptosis inhibition, increased migration, invasion, and cell proliferation, as well as stem-like properties of tumor cells (6).

Hypoxia also contributes to the shaping of the tumor environment by promoting immune tolerance through regulation of differentiation of regulatory T cells (Tregs), macrophages, and myeloid-derived suppressor cells (7).

The combination of increased tumor resistance with the recruitment of immunosuppressive cells therefore makes hypoxia a potent inhibitor of immune killing functions and promoter of tumor tolerance. Because the tumor immunosuppressive environment is widely considered a major obstacle in immune-based therapies (8), a better understanding of hypoxia-dependent mechanisms involved in the regulation of immune tolerance could lead to new strategies to enhance antitumor immunity.

In this study, we investigated the putative role of Nanog in the regulation of immune suppression in tumors. We demonstrated, by using the B16-F10 model, that hypoxia-induced Nanog was involved in the control of tumor growth following melanocyte differentiation Ag tyrosinase-related protein-2 (TRP-2, 180–188) peptide-based vaccination. We also showed that Nanog regulated Treg and macrophage infiltration in the tumor bed via enhanced TGF-β1 expression under hypoxia.

Materials and Methods

Cell culture

The B16-F10 melanoma cell line was from the American Type Culture Collection and cultured under hypoxic stress (1% partial pressure of O2) in a hypoxia workstation (Invivo2 400; Ruskinn Technology) (3).

Real-time PCR and Western blot

Real-time PCR and Western blotting were performed as previously described (5).

Immunofluorescence

Hypoxic tumor areas were detected as previously described (4).

Flow cytometry

Abs against TGF-β1, IL-6, IL-10, and arginase-1 were from R&D Systems. Data were collected on a FACS LSR II (BD Biosciences) and analyzed using FlowJo or FACSDivia software.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; NBS, Nanog binding site; shRNA, short hairpin RNA; Treg, regulatory T cell; TRP-2, tyrosinase-related protein-2.

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Gene silencing by small interfering RNA and short hairpin RNA

Predesigned small interfering RNAs against mouse Nanog (Sigma-Aldrich) were transfected by electroporation (5). Control and anti-mouse Nanog lentiviral particle-delivered short hairpin RNAs (shRNAs; Santa Cruz Biotechnology) were transduced following the manufacturer’s instructions. For Nanog targeting, two independent transductions were performed (1, 2). Stable transductants were selected using puromycin (2 μg/ml). Experiments were performed on the selected bulk population in order not to select clone-specific effects.

**ELISA for mouse TGF-β1**

Mouse TGF-β1 was measured in cell-free B16-F10 supernatants using the TGF-β1 ELISA kit (eBioscience).

**Immune infiltration and tumor growth**

C57BL/6 mice (Charles River) were housed at the Institut Gustave Roussy animal facility and treated in accordance with institutional animal guidelines. TRP-2180–188 vaccination and tumor monitoring were performed as previously described (4). For the analysis of intratumor immune infiltrate, engrafted tumors were explanted and dissociated into single-cell suspensions (mouse tumor dissociation kit; Miltenyi Biotec). All Abs were from eBioscience.

**CD4+ differentiation and arginase-1 production by macrophages**

CD4+ T or F4/80+ cells were sorted from freshly isolated splenocytes by using cell sorter MoFlo (BD Biosciences). Sorted naïve CD4+ T cells (supplemented with 500 IU/ml IL-2) and F4/80+ cells were cultured for 72 h in the presence of the indicated conditioned media. CD4+ T and F4/80+ cells were further analyzed, respectively, for CD25/Foxp3 and arginase-1 expression by flow cytometry.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) was performed after 48 h of hypoxia using anti-Nanog Ab (Cell Signaling Technology) as previously described (9). Primer sequences used were TGF-β1/Nanog-binding site (NBS) 2, forward, 5'-GAGTCATGAGGCTTTGGA-3', reverse, 5'-TGCTGAATTTCCTCTCTGG-3'.

**Statistical analyses**

Data were analyzed with GraphPad Prism. A two-tailed Student t test was used for single comparisons. A p value <0.05 was considered statistically significant.

**Results and Discussion**

**Nanog is induced under hypoxic stress and contributes to the acquisition of cancer stem cell–like features in B16-F10 melanoma cells**

We and others have previously reported that hypoxic stress regulates Nanog expression in cancer cells (2, 5). Here, we showed in B16-F10 cells that hypoxia significantly increased Nanog mRNA and protein levels (Supplemental Fig. 1A, 1B) in an HIF-1α–dependent manner (Supplemental Fig. 1C, 1D).

Nanog endows tumor cells with cancer stem cell–like features such as increased aldehyde dehydrogenase activity, spheroid formation, and tumorigenic potential (10). Supplemental Fig. 1E clearly shows that the proportion of B16-F10 cells with high aldehyde dehydrogenase activity rose significantly after 72 h of hypoxia. This increase was dependent on HIF-1α but not HIF-2α or Nanog (Supplemental Fig. 1F). Moreover, hypoxia-induced increase in spheroid formation was significantly reduced in Nanog-depleted B16-F10 cells (Supplemental Fig. 1G). Finally, when hypoxic B16-F10 cells were s.c. engrafted in immunodeficient mice, tumor initiation was significantly facilitated as compared with engraftment of normoxic cells (Supplemental Fig. 1H). Hypoxic Nanog-depleted B16-F10 cells showed significantly decreased tumor initiation (Supplemental Fig. 1I). Our data suggest that hypoxic conditioning of tumor cells is sufficient to increase their tumorigenic potential, and that hypoxia-induced Nanog plays an important role in driving this tumorigenic potential. This may be associated with Nanog-induced enrichment of cancer stem cell–like populations under hypoxia.

**Nanog is selectively induced in hypoxic zones of B16-F10 tumors, and Nanog targeting improves the efficacy of TRP-2–based vaccination in B16 xenografts**

Nanog was found to be selectively induced in hypoxic zones as shown by the localization of Nanog in pimonidazole-stained hypoxic zones of B16-F10 tumors (Fig. 1A). Similar overlap between HIF-1α and NANOG staining in prostate tumors was previously reported (11). B16-F10 cell lines were stably transfected with shRNA control and shRNA Nanog (Supplemental Fig. 1J, 1K). Nanog-depleted B16-F10 tumors showed a dramatic decrease in tumor growth particularly at days 13, 15, and 17 (Fig. 1B). These results further support the role of Nanog in controlling tumor progression as described earlier (10). Peptide vaccination using TRP-2180–188 peptide in combination with oligodeoxynucleotides containing unmethylated CpG motifs as an adjuvant has been reported to efficiently induce tumor cell–specific CTLs in the B16 melanoma model (12). We asked, therefore, whether Nanog inhibition would potentiate the antitumor effect of TRP-2180–188 peptide vaccination. Strikingly, the combination of TRP-2180–188

![FIGURE 1. Nanog is selectively induced in hypoxic zones of B16-F10 xenografts and regulates tumor progression. (A) Confocal microscopy analysis of Nanog expression in hypoxic zones (pimonidazole [PIMO]) in B16-F10 tumor sections. Blood vessels were stained with anti-CD31Ab (red, I), III is higher magnification (×400) of II. (B) Growth of shRNA-transduced B16-F10 xenografts in immunocompetent C57BL/6 mice (n = 10) without and with TRP-2180–188 vaccination. Error bars indicate ±SEM. Data represents three independent experiments. **p < 0.005, ***p < 0.0005.](http://www.jimmunol.org/content/180/11/5803.full)
vaccination and Nanog depletion resulted in a more pronounced inhibition of tumor growth (Fig. 1B), pointing out that tumor Nanog targeting proved useful in immune-based therapies.

**Nanog targeting decreased Tregs and macrophages in B16-F10 tumor bed**

To delineate the role of Nanog in immunosuppression within the hypoxic microenvironment, we examined immune cell subpopulations in Nanog-depleted B16-F10 tumors for the presence of CD4+CD25+Foxp3+ Tregs, F4/80+ macrophages, CD3+CD8+ T cells, CD49b+ NK cells, Gr1+CD11b+ myeloid-derived suppressor cells, and CD11c+ dendritic cells. Nanog-depleted tumors showed a dramatic reduction in the percentage of intratumoral Tregs and macrophages at days 13 and 16 (Fig. 2A, 2B). Interestingly, the decrease in immunosuppressive cells correlated with a doubling of CD3+CD8+ T cells in Nanog-depleted tumors at days 13 and 16 (Fig. 2C). Furthermore, the total number of intratumoral Tregs, macrophages, and CD8+ T cells was evaluated and similar results were obtained (Supplemental Fig. 2A–C). Macrophages from Nanog-depleted tumors contained the same levels of IL-10 but significantly less arginase-1, suggesting their altered immunosuppressive phenotype (Fig. 2D, 2E).

Although hypoxia can promote immune tolerance (13), to the best of our knowledge, this is the first demonstration that Nanog, a transcription factor known to be involved in the maintenance of stem cell–like properties, regulates the recruitment of Tregs, macrophages, and CD8+ T lymphocytes in the tumor bed.

**Hypoxia-induced Nanog directly regulates TGF-β1 and promotes the differentiation of CD4+ T naive cells into Tregs and the macrophage immunosuppressive phenotype**

Among several immunosuppressive cytokines and chemokines (8, 13), we found that TGF-β1 was substantially upregulated under hypoxic stress in B16-F10 cells at mRNA (Supplemental Fig. 2D) and protein levels (Supplemental Fig. 2E). Furthermore, a significant increase in TGF-β1 protein secretion was found in cell-free supernatants of hypoxic B16-F10 cells (Supplemental Fig. 2F). The role of Nanog in regulating TGF-β1 gene expression under hypoxia has not been studied. Nanog targeting in B16-F10 cells significantly decreased hypoxia-induced TGF-β1 mRNA (Fig. 3A) and intracytoplasmic protein levels (Fig. 3B). TGF-β1 protein secretion was also markedly decreased in supernatants from hypoxic Nanog-targeted B16-F10 cells (Fig. 3C). We next analyzed the presence of Nanog binding consensus sequences TAAT(G/T)(G/T) (14) and (C/G)(G/A)(C/G)(G/C)ATTAN(G/C) (15) in the promoter region of TGF-β1 using fuzznuc software. Fourteen putative NBS containing the consensus sequence TAAT(G/T)(G/T) were found. ChIP assay demonstrated significant hypoxia-inducible binding of Nanog to NBS2 (Fig. 3D). This demonstrates that Nanog regulates TGF-β1 expression by directly interacting with NBS in TGF-β1 proximal
promoter under hypoxic stress. We further observed that both mRNA (Supplemental Fig. 2G) and intracellular TGF-β1 expression (Supplemental Fig. 2H) were significantly decreased in B16-F10 Nanog-depleted tumors.

To study the functional consequences of Nanog-mediated TGF-β1 regulation under hypoxia, we analyzed the differentiation of naive CD4+ T cells into CD4+CD25+Foxp3+ Tregs in the presence of cell-free supernatants. Supernatants from hypoxic B16-F10 cells induced a significant larger expansion of CD4+CD25+Foxp3+ Tregs than normoxic ones (Fig. 3E). Moreover, supernatants from Nanog-depleted hypoxic B16-F10 cells significantly failed to induce the expansion of Tregs (Fig. 3E). Similarly, hypoxic supernatants significantly increased arginase-1 production in F4/80+ macrophages (Fig. 3F). There was a significant decrease in percentage of arginase-1+ cells in macrophages incubated with media from Nanog-depleted hypoxic B16-F10 cells (Fig. 3F). Supplementation of supernatants from Nanog-depleted hypoxic B16-F10 cells with rTGF-β1 restored Treg expansion and arginase-1 production in macrophages (Fig. 3E, 3F). These data clearly indicate that hypoxia-induced Nanog mediates Treg expansion and macrophage arginase-1 production by regulating TGF-β1. Furthermore, we did not observe any effect of Nanog targeting on hypoxia-induced chemokines CCL22 (16, 17) and CCL28 (13) shown to regulate Treg infiltration (data not shown). We therefore concluded that the hypoxia-induced Nanog/TGF-β1 axis is a major mediator of Treg expansion and increased arginase-1 production by macrophages.

Although an indirect regulation of TGF-β1 by NANOG has previously been reported in human mesenchymal stem cells in normoxic conditions (18), we show in this study that in a murine model of melanoma, hypoxic Nanog modulated the expansion of Tregs and arginase-1 production by macrophages in part by directly regulating TGF-β1. TGF-β signaling inhibition suppresses tumor progression by decreasing Tregs and increasing CD8+ T cells in tumors (19). TGF-β also increases macrophage recruitment enabling tumor evasion of the immune system (20). Therefore, it is conceivable that the reversal or switch in the numbers of intratumoral Tregs, macrophages, and CD8+ T cells as a result of Nanog silencing was subsequent to a decrease in TGF-β1. However, we cannot
exclude that other hypoxia-induced factors (including cytokines and chemokines) may cooperate with TGF-β1 to regulate Nanog-mediated immune suppression.

In conclusion, in this study we provide novel mechanistic insight into a dual functional role of Nanog in promoting tumor growth through its capacity to both control self-renewal and favor immunosuppression by directly regulating TGF-β1 and increasing Tregs and macrophages in tumor bed. These findings connect stem cell–associated factors with inhibition of the immune response in the hypoxic tumor environment and open new opportunities for enhancing immune therapies.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Fig. 1: Hypoxia-induced Nanog expression regulates CSC-like characteristics in B16-F10 melanoma cells.

(A) mRNA levels of Nanog and HIF-target genes under hypoxia. (B) Western-blot analysis of Nanog protein levels under hypoxia. (C) mRNA levels of Hif-1α, Hif-2α, and Nanog in B16-F10 cells electroporated with the indicated siRNAs after 48h of hypoxia. (D) Western-blot for Nanog protein following siRNA-targeting of Hif-1α and Hif-2α under hypoxia. (E, F) ALDH activity of viable (not stained by propidium iodide) B16-F10 cells following hypoxic stress (E) and after siRNA-targeting of Hif-1α, Hif-2α, and Nanog (10^4 events) after 48h of hypoxia (F). (G) Spheroid number per 100 B16-F10 cells under normoxia and hypoxia after clonal dilution at d15. (H) B16-F10 cells cultured under normoxia or hypoxia for 48h were sub-cutaneously injected into Nude mice (n=3). (I) B16-F10 transduced with control or Nanog targeting shRNAs were kept under normoxia or hypoxia for 48h, sub-cutaneously injected (3x10^3 cells/mouse) into Nude mice (n=3) and tumor growth was monitored. (J, K) RT-qPCR (J) and western-blot (K) evaluation of shRNA efficiency in transduced B16-F10 cells. For Nanog targeting, two independent transductions were performed (sh-Nanog 1 and sh-Nanog 2). Stable transductants were selected using puromycin (2μg/ml). Error bars indicate mean ± SD (A, C, G and J) or SEM (H, I). Data represents 3 independent experiments.

Supplemental Fig. 2: Nanog-depleted B16-F10 xenografts have decreased number of intra-tumoral CD4+ CD25+ Foxp3+ Treg and macrophages and hypoxia-induced Nanog regulates Tgf-β1 expression in B16-F10 cells.

(A, B, C) Total number of intra-tumoral CD4+ CD25+ Foxp3+ (A), F4/80+ (B) and CD3+ CD8+ T (C) cells in control and Nanog-depleted B16-F10 tumors grown in C57Bl/6 mice (n=3) at indicated days. The percentage of the respective immune population was multiplied by the total number of CD90.2+ cells (CD4+ CD25+ Foxp3+ and CD3+ CD8+ T) and of CD45+ cells (F4/80+) and divided by 100. The obtained number was then divided by the mass (in gram) of the resected tumor. Error bars indicate mean ± SD. Data represents 2 independent experiments. (D) mRNA levels of the indicated genes under hypoxia. (E) Intra-cellular expression of Tgf-β1, IL-6, and IL-10 evaluated by flow cytometry. (F) Tgf-β1 concentration in cell-free supernatants of B16-F10 cells in hypoxia. (G, H) Tgf-β1 mRNA (G) and intra-cellular protein (H) levels in Nanog-depleted B16-F10 xenografts.