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CRISPR Activation Screening Identifies VGLL3–TEAD1–RUNX1/3 as a Transcriptional Complex for PD-L1 Expression

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The PD-L1/2–PD-1 immune checkpoint is essential for the proper induction of peripheral tolerance and limits autoimmunity, whereas tumor cells exploit their expression to promote immune evasion. Many different cell types express PD-L1/2, either constitutively or upon stimulation, but the factors driving this expression are often poorly defined. In this study, using genome-wide CRISPR activation screening, we identified three factors that upregulate PD-L1 expression: GATA2, MBD6, and transcription cofactor vestigial-like protein 3 (VGLL3). VGLL3 acts as a transcriptional regulator, and its expression induced PD-L1 in many different cell types. Conversely, loss of VGLL3 impaired IFN-γ–induced PD-L1/2 expression in human keratinocytes. Mechanistically, by performing a second screen to identify proteins acting in concert with VGLL3, we found that VGLL3 forms a complex with TEAD1 and RUNX1/3 to drive expression of PD-L1/2. Collectively, our work identifies a new transcriptional complex controlling PD-L1/2 expression and suggests that VGLL3, in addition to its known role in the expression of proinflammatory genes, can balance inflammation by upregulating the anti-inflammatory factors PD-L1 and PD-L2. The Journal of Immunology, 2022, 209: 907–915.

The engagement of inhibitory checkpoint receptor PD-1 on CD8+ T cells by its ligands PD-L1 and PD-L2 impairs T cell activation and effectively dampens the immune response (1, 2). Many different cell types express PD-L1 and, to a lesser extent, PD-L2, including tumor cells, immune cells, endothelial cells, and epithelial cells (3–5). Tumor cells express PD-L1 to promote immune evasion, and inhibition of the PD-1/PD-L1 checkpoint is now widely used in immunotherapy against many different tumor types (6, 7). However, immune cells and nonimmune cells, such as keratinocytes, express PD-L1 to induce peripheral tolerance, and the absence of PD-L1 on these cells leads to excessive immune activation and autoimmunity (2, 8–11).

Many cell types express PD-L1 constitutively at low levels, and this is boosted by proinflammatory stimuli, such as TLR activators and IFN-γ. Similar to many other immune genes, the PD-L1 promoter contains binding sites for AP-1, NF-κB, Myc, STAT, and IFN regulatory factor 1 (IRF1), the latter being responsible for IFN-γ–induced expression activation of PD-L1 (12–14). Tumor cell–specific expression of PD-L1 can be mediated via these transcription elements but also via copy number alterations, mRNA stabilization, or 3′ UTR shortening (15–18). Alternative mechanisms inducing PD-L1 expression include activation via YAP/TAZ, HIF1α, or ATF3 factors (19–22). However, these factors fail to explain the differential expression in all tumor cells. Furthermore, the mechanisms driving PD-L1 expression in specific subtypes of immune cells and nonimmune cells remain unknown. Recent genome-wide knockout screens for cell surface expression of PD-L1 have identified CMTM4 and CMTM6 as regulators of PD-L1 protein stability (23, 24), but no new transcriptional regulators.

To identify novel regulators of PD-L1 surface expression, we conducted a genome-wide CRISPR activation screen. This screen yielded a novel transcriptional regulator of PD-L1: transcription cofactor vestigial-like protein 3 (VGLL3). VGLL3 forms a complex with TEAD1 and RUNX1/RUNX3 to mediate PD-L1 expression and also functions in cooperation with IFN-γ. Furthermore, we show that VGLL3 is required for proper induction of PD-L1 and PD-L2 by IFN-γ in keratinocytes. Together, our work identifies new regulatory proteins controlling expression of the PD-L1 and PD-L2 immune checkpoint ligands.

Materials and Methods

Cell culture

MelJuSo cells were cultured in IMDM supplemented with 8% FCS, and cell line authentication was performed by Eurofins Genomics (19-ZE-000487). HEK 293T cells were obtained from the American Type Culture Collection (CRL-3216) and cultured in DMEM supplemented with 8% FCS, AKR, A375, FM3, SK-MEL-28, U87, U118, A549, BJeT, HaCaT, and retinal pigment epithelial cells were cultured in DMEM supplemented with 8% serum; DU145, PC3, H460, H1299, PC9, 5637, and BxPC3 cells were cultured in RPMI supplemented with 8% serum. Normal primary human keratinocytes were purchased from Lonza (Morristown, NJ) and cultured in Lonza KGM-gold keratinocyte growth medium according to the manufacturer’s instructions. One day before cytokine stimulation, cells were switched to Lonza KBM medium to remove growth factors. Keratinocytes were used at passage 1 or 2.

Transfections, transductions, and Abs

For the generation of viral particles, HEK 293T cells were transfected using polyethyleneimine (Polysciences) with packaging plasmids pRSRev, pHCMV-G VSV-G, and pMDL/gp80 in combination with the lentiviral vector. The online version of this article contains supplemental material.
construct. Virus was harvested and filtered, and target cells were transduced in the presence of 8 µg/ml polybrene (EMD Millipore). For immunoprecipitation experiments, HEK 293T cells were also transfected using polyethyleneimine.

For small interfering RNA (siRNA)-mediated depletion, cells were reverse transfected with Dharma FECT transfection reagent 1 and 50 nM siRNA (catalog numbers: siCtrl: D0012061-20, siTEAD1: M-012603-01-0005, siTEAD2: M-012611-00-0005, siTEAD3: M-012604-01-0005, siTEAD4: M-019570-03-0005) according to the manufacturer’s protocol. Briefly, siRNAs and Dharma FECT were mixed and incubated for 20 min in a 37°C incubator, and after which cells were added and left to adhere. Cells were analyzed 3 d after siRNA transfections. For gene depletion in keratinocytes, cells were electroporated using the Lonza 4D Nucleofector following the manufacturer’s recommendations.

Abs used in this study were as follows. For flow cytometry, we used PE anti-human CD274 (BioLegend), PE anti-human PD-L2 (BioLegend), and FITC anti-human HLA-ABC (BioLegend). For Western blotting, we used mouse anti–PD-L1 (Cell Signaling Technology, 29122), mouse anti-actin (Sigma-Aldrich, A5441), mouse anti-FLAG (Sigma-Aldrich, M2), rabbit anti-TEAD1 (ProSci, 22-472), mouse anti-TAZ (Santa Cruz Biotechnology, sc-518026), mouse anti-RUNX1 (Santa Cruz Biotechnology, sc-365644), mouse anti-RUNX3 (Santa Cruz Biotechnology, sc-101553), mouse anti-HPA (Covance, 16B12), and rabbit anti-GFP and mouse anti-Myc (9E10) (for the latter two, as described in Ref. 25).

CRISPR activation and knockdown screen

For CRISPR activation, the human CRISPR two-plasmid activation pooled library (SAM) was a gift from Feng Zhang (Addgene, 100000078) and used for CRISPR activation screening. Stable MS2-p65-HSF1 (MPH) expressing MelJuSo cells were generated, and 150 million MelJuSo MPH cells were infected at a multiplicity of infection of 0.3 with the SAM plasmid. The next day, cells were selected with G418 (200 µg/ml) and blastidin (2.5 µg/ml), and, after 5 d, two batches of cells were stained for PD-L1, and the top 10% of PD-L1–expressing cells were sorted out using the FACS. Cells were grown out for another 6 d, and PD-L1high cells were sorted again for both replicates. After the second sort, cells were grown out to reach 10 million, and genomic DNAs (gDNAs) were isolated and amplified using the established protocol (26). gRNAs were sequenced using the Illumina HiSeq 2500, and inserts were mapped to the reference. Statistical analysis was performed using redundant siRNA activity analysis and enrichment >4 was considered a hit (27).

For knockout screening, we used the human CRISPR Brunello genomewide knockout library, a gift from David Root and John Doench (Addgene, 73178). MelJuSo cells stably expressing FLAG-VGLL3 were generated, and two batches of 100 million cells were infected at a multiplicity of infection of 0.3. Transduced cells were selected using puromycin (1 µg/ml), and, after 5 d, cells were stained for PD-L1, and the lowest 5% of GFP-positive PD-L1low–expressing cells were sorted. Cells were grown out and sorted again using the same gating strategy as for the first sort. After this sort, cells were grown out to reach 10 million, the gDNA was isolated, and gDNAs were amplified using the established protocol (26). gRNAs were sequenced using the Illumina NovaSeq 6000, and inserts were mapped to the reference.

Hit validation and subcloning

For hit validation, two individual guides per gene were cloned into the LentiSAM vector (a gift from Feng Zhang, Addgene plasmid 75112), and MelJuSo MPH cells were stably transduced with these guides or with the empty LentiSAM vector (a gift from Feng Zhang, Addgene plasmid 75112), and the indicated MelJuSo cells were transduced with the Dharma FECT transfection reagent and 100 nM siRNA (VGLL3 IS A NOVEL REGULATOR OF PD-L1 EXPRESSION). For knockout screening, we used the human CRISPR Brunello genomewide knockout library, a gift from David Root and John Doench (Addgene, 73178). MelJuSo cells stably expressing FLAG-VGLL3 were generated, and two batches of 100 million cells were infected at a multiplicity of infection of 0.3. Transduced cells were selected using puromycin (1 µg/ml), and, after 5 d, cells were stained for PD-L1, and the lowest 5% of GFP-positive PD-L1low–expressing cells were sorted. Cells were grown out and sorted again using the same gating strategy as for the first sort. After this sort, cells were grown out to reach 10 million, the gDNA was isolated, and gDNAs were amplified using the established protocol (26). gRNAs were sequenced using the Illumina NovaSeq 6000, and inserts were mapped to the reference.

Hit validation and subcloning

For hit validation, two individual guides per gene were cloned into the LentiSAM vector (a gift from Feng Zhang, Addgene plasmid 75112), and MelJuSo MPH cells were stably transduced with these guides or with the empty LentiSAM vector as a control. Guide sequences used were as follows: GATA2-1: 5'-GGACTCCTGGAGTACC-3', GATA2-2: 5'-GTCCGCAATTCCTCGAACCC-3', MBED-1: 5'-GGTCTCTCAGCGGCCTGGC-3', MBED-2: 5'-TGCGAGTGGTCTCTGGGAA-3', VGLL3-1: 5'-GTCGGCTAAGCCCTCTGCTG-3', VGLL3-2: 5'-CTCTGGTCCCCATGCTG-3', ABCB5-1: 5'-CATCAAACTGTCCGCCTGC-3', ABCB5-2: 5'-TCTAGTATCCTAAGAGTC-3', GPR173-1: 5'-AGTGGCCTAAGAGGGAATG-3', GPR173-2: 5'-GGTCTGCAACTGTAGGCGAG-3', SMARCC1-1: 5'-GATGAGCCACCCAGGATGG-3', SMARCC1-2: 5'-GTGAAGAAAGCAGGCTTCC-3', CD45RA-1: 5'-TACCACTGATGCTGACTG-3', CD45RA-2: 5'-GATGCTCCGTCTGACCTCC-3', CD45RA-3: 5'-CTCCCTTGGCAGCAGGCA-3'.

For whole-cell lysate analyses, cells were lysed directly in SDS sample buffer. Samples were boiled before loading, and proteins were separated by SDS-PAGE and transferred to Western blot filters. Blocking of the filter andAb incubations were performed in PBS supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) milk powder. Blots were imaged using the Odyssey Imaging System (LI-COR Biosciences) or the Amersham Imager. Full, uncropped Western blots are shown in Supplemental Fig. 4.

Chromatin immunoprecipitation and Western blotting

For chromatin immunoprecipitation experiments, cells were lysed in lysis buffer (0.5% Nonidet P-40, 5% glycerol, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, supplemented with complete EDTA-free protease inhibitor cocktail [Roche]) and cleared by centrifugation. Lysates were incubated with Myc-Trap beads (Chromotek) or protein G-Sepharose 4 FF resin preloaded with the indicated Abs. Following incubations, beads were washed extensively with lysis buffer before addition of SDS sample buffer (2% SDS, 10% glycerol, 5% 2-ME, 60 mM Tris-HCl, pH 6.8, and 0.01% bromophenol blue).

For whole-cell lysate analyses, cells were lysed directly in SDS sample buffer. Samples were boiled before loading, and proteins were separated by SDS-PAGE and transferred to Western blot filters. Blocking of the filter andAb incubations were performed in PBS supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) milk powder. Blots were imaged using the Odyssey Imaging System (LI-COR Biosciences) or the Amersham Imager. Full, uncropped Western blots are shown in Supplemental Fig. 4.

Chromatin immunoprecipitation (ChIp)-qPCR

{}
PD-L2 qPCR forward 5'-CCAGGGGCTCATAACTGCTG-3'
CD274 qPCR reverse 5'-GTCTTGGGAGCCAGGGTGAC-3'
GAPDH qPCR reverse 5'-CTCCACGAGCTACTCAGCG-3'
VGLL3 VHFAAA reverse 5'-GGCTTGGCCCAAAGCTCTTGAGGCGGCTTCATCCGCTACTGACCC-3'
GATA2 C373R forward 5'-ATGGAAAGGATGAGTGACTCTGC-3'
HIS forward 5'-TCCCACATGGTGGATAGATAGC-3'
HIS reverse 5'-CAGGAGGGCGGTGGCGGTGGTGCATGTGG-3'
GATA2 C373R reverse 5'-TGAAAGTGCAAATGGCAAGC-3'
FLAG-VGLL3 at the PD-L1 promoter site (Fig. 2A). To analyze whether VGLL3 directly regulated PD-L1 transcription, we performed ChIP-qPCR experiments using anti-FLAG Abs and were able to detect VGLL3 binding at the PD-L1 promoter region.

To induce PD1 expression on T cells, transduced T cells were activated with αCD3/αCD28 Dynabeads (Thermo Fisher Scientific) at a cell-to-bead ratio of 1:1 in the presence of 150 U/ml IL-2. Every 72 h, beads were magnetically removed, and the T cells were harvested and restimulated with a fresh batch of αCD3/αCD28 Dynabeads and IL-2. After three restimulations, T cells were harvested. Surface PD1 expression was confirmed by flow cytometry analysis, and the T cells were used in cytotoxicity assays.

To measure cytotoxicity, AKR and MelJuSo HLA-A2 YFP cells transduced with FLAG or FLAG-VGLL3 were plated in 96-well plates and cocultured with MART1 TCR T cells at 0:1, 1:1, and 1:5 E:T ratios for 6 h. Dead cells were quantified by flow cytometry with the Zombie NIR viability kit (BioLegend) within GFP+ target cells. To determine cytotoxic production by T cells, culture medium was supplemented with 5 μg/ml brefeldin A (BioLegend), and intracellular cytokine staining was performed after 6 h of coculture using the CytoFix/Cytoperm Kit (BD Biosciences) according to the manufacturer’s recommendations.

**Results**

A CRISPR activation screen identifies GATA2, MB6D, and VGLL3 as regulators of PD-L1 expression

The immune checkpoint inhibitor PD1-PD-L1 is expressed in many tissues at varying levels, suggesting that several mechanisms are in play controlling its expression. To identify novel regulators promoting PD-L1 cell surface expression, MelJuSo cells were transduced with a genome-wide CRISPR/Cas9-mediated gene activation library targeting 23,000 genes of the human genome (26, 30) (Fig. 1A). This human cutaneous melanoma cell line expresses low levels of PD-L1, which provides a window for detecting increased expression. Following transduction with the activation library, cells with increased PD-L1 surface expression were sorted twice by FACS and analyzed for gRNA enrichment compared with the input (Fig. 1A). Hits were considered on the basis of two criteria: two or more gRNAs per gene showed fourfold enrichment in the sorted population in two independent sorts, and this enrichment had to be statistically significant according to RSA analysis (Fig. 1B and Supplemental Fig. 1A). Based on these criteria, several hits were identified, with the top one being CD274, the gene encoding PD-L1. To validate these results, cell lines stably expressing individual gRNAs were generated. Analysis of PD-L1 surface expression in these cells revealed that guides activating GATA2, MB6D, and VGLL3 upregulated PD-L1 expression (Fig. 1C). Meanwhile, these guides did not affect expression of another cell surface marker, MHC class I. Indeed, these gRNAs upregulated the expression of their respective target genes (Supplemental Fig. 1B), identifying the transcription factor GATA2, transcriptional coactivator VGLL3 and polycomb-binding protein MB6D as regulators of PD-L1 cell surface expression in the melanoma cell line MelJuSo.

GATA2 and VGLL3 regulate PD-L1 transcription

Due to their defined functions as transcriptional regulators, we followed up on GATA2 and VGLL3. To confirm gRNA specificity and eliminate the possibility of off-target effects, PD-L1 expression was assessed after overexpressing GATA2 and VGLL3 by cDNA expression plasmids. Both FLAG-tagged GATA2 and VGLL3 robustly upregulated expression of PD-L1 at the cell surface, protein, and mRNA levels (Fig. 1D–G). Interestingly, IFN-γ stimulation of GATA2- and VGLL3-overexpressing cells further boosted their PD-L1 expression, suggesting that GATA2 and VGLL3 cooperate with IFN-γ in promoting PD-L1 expression (Fig. 1E–G). GATA2 and VGLL3 also upregulated the expression of the PD-L1 homolog PD-L2 (Fig. 1E). Increased PD-L1 mRNA could be the consequence of mRNA stabilization or increased transcription. To test for the first, the cells were cultured with transcriptional inhibitor actinomycin D, showing that GATA2 or VGLL3 did not affect the half-life of PD-L1 mRNA (Supplemental Fig. 1C). Overall, our results demonstrate that GATA2 and VGLL3 promote transcription of PD-L1 and PD-L2. GATA2 regulating PD-L1 expression was recently shown in glioblastoma cells as well (31).

**VGLL3 induces PD-L1 in multiple cell types and impairs T cell–mediated cytotoxicity**

VGLL3 is a transcriptional activator that has recently been shown to drive expression of several proinflammatory genes and is reported to be involved in the induction of autoimmunity in women (32–34). Our data show that VGLL3 also promotes the expression of the anti-inflammatory genes PD-L1 and PD-L2. To test whether VGLL3 directly regulated PD-L1 transcription, we performed ChIP-qPCR experiments using anti-FLAGAbs and were able to detect FLAG-VGLL3 at the PD-L1 promoter site (Fig. 2A). To analyze the breadth of PD-L1 regulation, VGLL3 was introduced in a panel of different cell lines. Many but not all cell types induced PD-L1 upon VGLL3 expression, indicating that upregulation of VGLL3 can induce PD-L1 expression in multiple cell types (Fig. 2B). Increased PD-L1 expression renders cells less sensitive to T cell–mediated cytotoxicity, suggesting that VGLL3 could dampen T cell activation. To test this, MARTI TCR+ T cells were used in a

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<td><strong>Primer Name</strong></td>
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<td>GATA2 C373R forward</td>
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<td>GATA2 C373R reverse</td>
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<td>VGLL3 VHFAAA forward</td>
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**To induce PD1 expression on T cells, transduced T cells were activated with αCD3/αCD28 Dynabeads (Thermo Fisher Scientific) at a cell-to-bead ratio of 1:1 in the presence of 150 U/ml IL-2. Every 72 h, beads were magnetically removed, and the T cells were harvested and restimulated with a fresh batch of αCD3/αCD28 Dynabeads and IL-2. After three restimulations, T cells were harvested. Surface PD1 expression was confirmed by flow cytometry analysis, and the T cells were used in cytotoxicity assays.**
FIGURE 1. CRISPR activation screen identifies novel regulators of PD-L1 expression. (A) Schematic setup of the screen. MelJuSo melanoma cells stably expressing MS2-p65-HSF1 were transduced with a pooled gRNA library containing dCAS9 and sorted by FACS for cells displaying high levels of PD-L1. (B) Genes for which at least two different gRNAs were significantly enriched (greater than fourfold) in the sorted population versus control population in both replicate sorts. Plotted are p values based on RSA analysis. (C) MelJuSo MPH cells stably expressing the SAM vector with or without the indicated activation gRNAs were analyzed for cell surface expression of PD-L1 and MHC class I (HLA-ABC). Data represent three independent experiments (± SD), and statistical significance was determined by paired Student t test (*p < 0.05, **p < 0.01). (D) MelJuSo cells stably expressing FLAG (EV), GATA2-FLAG, or FLAG-VGLL3 were analyzed for cell surface expression of PD-L1 using flow cytometry. (E) MelJuSo cells as in D were either stimulated or not with IFN-γ for 48 h, and cell surface expression of PD-L1 and PD-L2 was measured using flow cytometry. (F) MelJuSo cells as in D were either stimulated or not with IFN-γ for 24 h, and expression of the indicated proteins was determined by Western blot analysis. (G) MelJuSo cells as in D were treated with IFN-γ for 24 h when indicated, and mRNA levels of the indicated genes were analyzed using quantitative real-time PCR and normalized to GAPDH. All data represent three independent experiments (± SD), and statistical significance was determined by ANOVA using Dunnett’s multiple comparison test (*p < 0.05, **p < 0.01).
cytotoxicity experiment with target cells either expressing VGLL3 or not. As target cells, AKR (HLA-A2\(^*\) MART1\(^{+}\)) melanoma cells and MelJuSo cells (MART1\(^{+}\)) stably expressing HLA-A2-YFP were used. In both cases, PD-L1 expression was upregulated by VGLL3 (Fig. 2C). VGLL3 expression impaired killing of both cell types by T cells from two different donors in a manner correlating with the degree of PD-L1 upregulation (Fig. 2D). In agreement with this, T cells exposed to AKR cells expressing VGLL3 expressed less IFN-\(\gamma\) and TNF-\(\alpha\) (Fig. 2E). Thus, VGLL3 can impair T cell–mediated killing.

**VGLL3 regulates IFN-\(\gamma\)–induced PD-L1 and PD-L2 expression in keratinocytes**

Physiologically, VGLL3 is highly expressed in keratinocytes (32), so we tested whether VGLL3 regulates PD-L1 and PD-L2 expression in keratinocytes. Indeed, mice overexpressing VGLL3 specifically in keratinocytes (under the keratin-5 promoter) expressed higher levels of PD-L1 and PD-L2 mRNA (33) (Fig. 3A). To validate that endogenous VGLL3 controls PD-L1/2 expression in these cells, primary keratinocytes were depleted for VGLL3 by RNAi, and expression of PD-L1 was assessed using Western blot and qPCR analyses. Although VGLL3 depletion did not significantly affect the basal levels of PD-L1/2 expression in these cells, IFN-\(\gamma\)–induced expression of PD-L1/2 was hampered when VGLL3 was depleted (Fig. 3B, 3C). Overall, these results show that VGLL3 is a physiological activator of IFN-\(\gamma\)–induced expression of PD-L1 and PD-L2 in keratinocytes.

**VGLL3 controls PD-L1 transcription via TEAD1**

Although VGLL3 upregulated PD-L1 in many cell types, the fact that not all cell types responded equally suggests that VGLL3 alone is not sufficient to promote PD-L1 expression. VGLL3 may then require additional (transcriptional) factors. To identify factors cooperating with VGLL3 in driving PD-L1 transcription, we performed a genome-wide CRISPR knockout screen in the MelJuSo FLAG-VGLL3–expressing cells, sorting for cells with reduced PD-L1 expression (Fig. 4A).

gRNA enrichment analysis of the sorted cells identified CD274 as the top hit, substantiating the screen (Fig. 4B). In addition to CD274, gRNAs targeting the NFs RUNX1, TAZ (WWTR1), and TEAD1 were significantly enriched (Supplemental Fig. 2A). It is reported that TAZ regulates PD-L1 expression in many tumor cells in conjunction with the TEAD transcription factors (21, 22). Moreover, VGLL3 interacts with TEAD transcription factors via its TDO domain to act as a transcriptional coactivator (35). This suggests that VGLL3 may interact with TEAD1 to drive PD-L1 expression in a manner similar to TAZ. To test this, we generated TAZ, TEAD1, and RUNX1 knockout cells using the top two gRNAs.
from the screen in both MelJuSo FLAG and MelJuSo FLAG-VGLL3 cells. gRNA-mediated knockout of TEAD1 in FLAG-VGLL3 cells almost completely abrogated VGLL3-mediated PD-L1 expression (Fig. 4C, 4D), whereas the effect on constitutive PD-L1 expression in MelJuSo cells was less prominent (Fig. 4D, right panel). However, the effect of TAZ knockout on PD-L1 surface expression was not affected by VGLL3 overexpression (Fig. 4D). This argues that TAZ is involved in canonical PD-L1 expression in MelJuSo cells and acts independently from VGLL3. Last, we found that the knockout of RUNX1 did not affect the existing PD-L1 expression but reduced VGLL3-mediated expression by ∼50%.

To validate that the interaction between VGLL3 and TEAD1 is critical to drive PD-L1 expression, we generated a TDU domain mutant that in other VGLL family members abolishes TEAD binding (V166A, H167A, F170A). FLAG-tagged WT but not the vhfaa-VGLL3 mutant was able to communoprecipitate with MYC-tagged TEAD1 from transfected HEK293 cells, indicating that these mutations indeed abolished the interaction between VGLL3 and TEAD1. Indeed, the VGLL3(vhfaa) mutant also failed to upregulate PD-L1 expression in MelJuSo cells (Fig. 4E). Of note, mutating a histidine stretch that is required for VGLL3 to control upregulated RUNX1 as a candidate involved in PD-L1 expression in keratinocytes. (A) Upregulation of VGLL3, PD-L1, and PD-L2 transcripts in the skin of mice overexpressing VGLL3 from the keratin 5 promoter. Data are from a transcriptome dataset (33). (B) Keratinocytes were transfected with siCtrl or siVGLL3 and treated or not with IFN-γ for 48 h, after which cells were lysed and PD-L1 expression was analyzed by Western blotting. n = 2. (C) Keratinocytes were transfected with siCtrl or siVGLL3, and mRNA for the indicated genes was analyzed from cells either treated or not with IFN-γ for 48 h. Shown are data from three independent experiments (+ SD); statistical significance was determined by ANOVA using Dunnett’s multiple comparison test (*p < 0.05).

**FIGURE 3.** VGLL3 regulates IFN-γ-induced PD-L1 expression in keratinocytes. (A) Upregulation of VGLL3, PD-L1, and PD-L2 transcripts in the skin of mice overexpressing VGLL3 from the keratin 5 promoter. Data are from a transcriptome dataset (33). (B) Keratinocytes were transfected with siCtrl or siVGLL3 and treated or not with IFN-γ for 48 h, after which cells were lysed and PD-L1 expression was analyzed by Western blotting. n = 2. (C) Keratinocytes were transfected with siCtrl or siVGLL3, and mRNA for the indicated genes was analyzed from cells either treated or not with IFN-γ for 48 h. Shown are data from three independent experiments (+ SD); statistical significance was determined by ANOVA using Dunnett’s multiple comparison test (*p < 0.05).
which both have significant PD-L1 expression. Furthermore, GATA2 is highly expressed in hematopoietic stem cells, such as PD-L1/2, and their expression correlates during the different phases of stem cell development (38). It will be interesting to test whether GATA2 is indeed responsible for PD-L1/2 expression during hematopoiesis and what the physiological function is for this expression.

We also identified VGLL3 as a novel regulator of both PD-L1 and PD-L2 expression. VGLL3 is a transcriptional coactivator that can induce transcription via the TEAD transcription factors, as well as ETS1 (35, 36). We define a tripartite transcription complex consisting of VGLL3, TEAD1, and RUNX1/3 in the control of PD-L1 expression. The TEAD transcription factors contain four members, and although in our system TEAD1 appears to be dominant, it could be that in different cell types other TEAD family members act in concert with VGLL3. Similarly, RUNX1 and RUNX3 are very homologous and act redundantly to afford PD-L1 induction. The prerequisite for RUNX1/3 sets VGLL3 apart from YAP/TAZ, which also drive PD-L1 expression via the TEAD family and bind to the PD-L1 promoter (21, 22, 39, 40). However, RUNX1 and RUNX3 also interact with these factors, suggesting that VGLL3 and TAZ potentially act mechanistically similarly to drive PD-L1 expression. VGLL1, however, did not induce PD-L1 expression, and VGLL4 has been reported to induce PD-L1 expression via the TEAD family and bind to the PD-L1 promoter (21, 22, 39, 40). However, RUNX1 and RUNX3 also interact with these factors, suggesting that VGLL3 and TAZ potentially act mechanistically similarly to drive PD-L1 expression. VGLL1, however, did not induce PD-L1 expression, and VGLL4 has been reported to induce PD-L1 expression via the TEAD family and bind to the PD-L1 promoter (21, 22, 39, 40).

VGLL3 is a transcriptional activator that displays a female-dominant expression and is linked to several autoimmune diseases that are more prevalent in women (32). It regulates the expression of many
autoimmune-associated proinflammatory genes, and overexpression in keratinocytes leads to the development of a lupus-like rash and systemic autoimmunity, classified by enhanced B cell reactivity (32–34). Our data demonstrate that complementary to promoting expression of proinflammatory genes, VGLL3 induces expression of the anti-inflammatory factors PD-L1 and PD-L2, leading to evasion of T cell–mediated killing. Using the same models as before, mice expressing VGLL3 in keratinocytes were found to upregulate PD-L1/2, and keratinocytes were shown to require VGLL3 for full induction of PD-L1/2 by IFN-γ. Thus, VGLL3 promotes expression of genes from both sides of the pro- and anti-inflammatory equation. This resembles IFN-γ, which also promotes proinflammatory cytokines such as CXCL9 and CXCL10, as well as anti-inflammatory factors such as PD-L1 and PD-L2. However, expression of VGLL3 is limited to specific tissues. VGLL3 is not expressed in immune cells, which express high levels of PD-L1/2, but rather has its highest expression in trophoblasts, especially extravillous trophoblasts and syncytiotrophoblasts (Supplemental Fig. 3) (42, 43), which form the outer layer and protrusions of the placenta. These are also among the highest PD-L1- and PD-L2–expressing cells and secrete proinflammatory factors to recruit and shape an immune cell repertoire that maximally protects the fetus (44). We propose that these tissues use VGLL3 to create a well-balanced but active immune environment.

In summary, using a genome-wide CRISPR activation screen in combination with a dedicated genome-wide CRISPR knockout screen, we identified novel regulators of PD-L1 expression, including GATA2 and MBD6. VGLL3, a female-biased transcriptional activator linked to autoimmune disease, in complex with TEAD1 and RUNX1/3 can regulate transcription of PD-L1 and PD-L2. VGLL3 is required for full induction of PD-L1/2 expression by IFN-γ in keratinocytes and thus regulates not only expression of proinflammatory genes but also expression of the anti-inflammatory immune checkpoint molecules PD-L1 and PD-L2.

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