Supplementary Online Content


eMethods

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This supplementary material has been provided by the authors to give readers additional information about their work.
eMethods

Association between proportion of autoimmune adverse events and tissue similarity in anti-PD-1 treated NSCLC patients

Checkpoint inhibitor–induced autoimmune toxic effects can affect virtually any organ of the body. Given that commonly affected organs are of epithelial type (eg, skin), we hypothesized that the development of autoimmune toxic effects may be associated with the degree of tissue similarity between NSCLC and the affected organ. To investigate this association, we used the US Food and Drug Administration Adverse Events Reporting System to analyze organ-specific immune-related adverse events (irAEs) in patients with NSCLC treated with anti–PD-1 (nivolumab or pembrolizumab). irAE frequencies were retrieved for years 2004–2016 for 1,304 patients. Furthermore, tissue similarity between NSCLCs and various affected organs was calculated by retrieving organ-specific gene signatures from the Genotype-Tissue Expression (GTEx) database¹ and RNA-Seq data from 1,145 NSCLC libraries from The Cancer Genome Atlas (TCGA)². Normalized similarity scores were calculated for each organ, and ranged from 0.32 to 0.62. The algorithm used was the ‘single sample’ extension of Gene Set Enrichment Analysis (ssGSEA), which allows to calculate an enrichment score that represents the degree of absolute enrichment of each organ gene set in the NSCLC expression profile. The ssGSEA was implemented using the GSVA package in R.

Bioinformatics analysis for the prediction of shared skin and NSCLC antigens

A pipeline to identify proteins that have a high potential of being NSCLC and skin self-antigens was established. First, RNA sequencing (RNA-Seq) libraries of 551 squamous cell carcinoma (SCC) and 594 adenocarcinoma (AD) tumor samples were obtained from The Cancer Genome Atlas (TCGA)². The count data were normalized using Trimmed Mean of M-values (TMM) method in edge R package, and filtered for highly-expressed genes (>90th percentile in each sample). Second, expression profiles of whole organs and skin cell subtypes were inferred using RNA-Seq samples from the Genotype-Tissue Expression (GTEx)¹ and ENCODE databases³, respectively. Shannon entropy was used to detect genes that are lung- or skin-specific, and further classified into histological skin sites, such as the epidermal compartment. Last, the NCBI Gene Reference Into Function (GeneRIF) database⁴ was mined to identify genes previously described in the literature as self-antigens in autoimmune skin disorders, using keywords such as ‘autoimmunity’, ‘autoantigens’, ‘autoantibodies’, ‘psoriasis’, etc. Overall, this strategy identified genes with the following properties: (1) evidence of self-immunogenicity; (2) high expression in lung tumors; (3) skin-specificity and (4) keratinocyte-specificity. Genes that fulfilled two or more of these criteria were selected, resulting in a list of nine candidate tumor-derived, skin autoantigens (see Table 1). All analyses were conducted in R.

Preparation of peptide pools

Peptide pools of the nine predicted shared skin and NSCLC antigens were ordered from GenScript in the form of lyophilized 15-mers with 5 amino acid overlap. Peptide purity was >85 %. Peptide pools were dissolved in DMSO, diluted in RPMI, aliquoted and stored at -20 °C.

T cell stimulation assays and FACS

Cryopreserved PBMCs were plated at a concentration of 1x10⁶ cells per well in low-adherence 24-well plates (Sarstedt, ref. 83.3922.500) and kept at 37 °C. Cells were resuspended in 2 mL RPMI per well containing 8 % human serum (Biowest, ref. S4190-100), 1 % penicillin-streptomycin, 1 % L-glutamine, 1 % non-essential amino acids, 1 % sodium pyruvate, 0.1 mg/mL kanamycin and 0.1 % 2β-mercaptopethanol. The following day, cells were stimulated with each peptide pool individually at a concentration of 2 µg/mL/peptide. After 48 hours, 1 mL medium was replaced with fresh RPMI (as above) containing IL-2 (final concentration of IL-2 was 150 U/mL). Cells were kept in culture for 10 days and 1 mL medium was replaced with fresh IL-2 containing medium every second day. On day 10, cells were washed and transferred to 96-well plates and re-stimulated with each peptide pool individually for 6 hours in the absence of IL-2 at a concentration of 2 µg/mL/peptide. Brefeldin A was added at the same time of stimulation at a concentration of 10 µg/mL. After 6 hours cells were washed and stained for viability, CD3, CD4, CD8, CD45RA and CD14. Cells were then fixed and permeabilized and stained for IFN-γ and TNF-α. Samples were acquired using BD LSRFortessa and data was analyzed using FlowJo software. IFN-γ and TNF-α production were analyzed to identify antigen-specific T cells. For two patients, the experiment was repeated and CD4⁺/CD8⁺ IFN-γ⁺ T cells were sorted using BD FACSARia III.

TCR analysis

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DNA was extracted from NSCLC and skin lesion samples (formalin-fixed paraffin-embedded samples or fresh biopsy samples) and from sorted PBMCs. DNA was used to carry out T cell receptor beta (TCRβ) sequencing (ImmunoSEQ “Survey” resolution, Adaptive Biotechnologies). Clonotypes represent unique genomic nucleotide sequence resulting from V, D, and J gene rearrangement. Processed data were downloaded from Adaptive Biotechnologies website and analyzed in R. Abundance of clonotypes was estimated using the number of productive templates.

References
Out of a total of 73 patients analyzed, 25 patients (34.2%, 95% CI 24.4%-45.7%) developed autoimmune skin toxic effects. More specifically, 15 out of 22 patients with PR (68.2%, 95% CI 47.3%-83.6%), 4 out of 14 patients with SD (28.6%, 95% CI 11.7%-54.6%) and 6 out of 37 patients with PD (16.2%, 95% CI 7.7%-31.1%). The distribution of patients with autoimmune skin toxic effects was significantly different between the three different groups (p-value<0.001, Chi-square test), with the highest proportion in PR and the lowest in PD.
eFigure 2. T Cell Infiltration in Lung Tumors and Autoimmune Skin Lesions

Skin autoimmune lesions were characterized by an inflammatory infiltrate shown by CD4 (A) and CD8 (B) IHC, as were lung tumors (C-D) (images of a representative patient shown, pre-treatment). T cell counts in skin for this patient were 520 CD4+ T cells/mm² and 320 CD8+ T cells/mm², in the NSCLC 750 CD4+ T cells/mm² and 825 CD8+ T cells/mm².
**eFigure 3. CT Scans and Autoimmune Skin Toxic Effects of NSCLC Patients Under Treatment With Anti-PD-1**

CT scans performed before (A, D, G, J, M, P) and after (B, E, H, K, N, Q) treatment with nivolumab or pembrolizumab showing patients with PR. (C, F, I, L, O, R) Grade 1-2 autoimmune skin toxic effects of anti-PD-1 treated NSCLC patients with PR.
eFigure 4. Comparison of Progression-free Survival in NSCLC Patients With and Without Skin Toxic Effects

Kaplan-Meier analysis of NSCLC patients treated with PD-1 inhibitors reveals better outcome for 25 patients who developed autoimmune skin toxic effects (green line) compared with 48 other patients who did not develop skin toxic effects (red line). One-year progression-free survival rate was 68% in the group with toxic skin effects and 16% in group without toxic skin effects; hazard ratio = 0.22 (95% CI, 0.09-0.49), log-rank p-value=0.001.
**eFigure 5.** Shared TCRs in Autoimmune Skin Lesions and Lung Tumors

TCR clonotype analyses performed with patient-matched skin toxicity and NSCLC biopsies from four patients. Shared T cell clones in NSCLC and skin are colored purple in the dot plots. The total number of TCR templates for the four patients analyzed are as follows: 
(A) 3,319 in lung, 270 in skin, 230 shared (6.4%), originating from 38 unique clones; 6,481 in lung, 1,008 in skin, 1,998 shared (26.6%), originating from 164 unique clones (B); 4,851 in lung, 15,474 in skin, 6093 shared (30%), originating from 560 unique clones (C); 326 in lung, 62,081 in skin, 1,174 shared (1.9%), originating from 59 unique clones (D).
We have established a novel strategy to identify proteins that have a high potential of being NSCLC and skin antigens, and to narrow down the number of possibilities. RNA sequencing (RNA-Seq) libraries of 1,145 NSCLC samples were obtained from The Cancer Genome Atlas (TCGA) database, and filtered for highly-expressed genes (>90th percentile in each sample). Expression profiles of whole organs and skin cell subtypes were inferred using RNA-Seq samples from the Genotype-Tissue Expression (GTEx) and ENCODE databases, respectively. An entropy-based algorithm was used to detect genes that are lung- or skin-specific, and further classify them into histological skin sites, such as the epidermal compartment. The NCBI Gene Reference Into-Function (GeneRIF) database was mined to identify genes previously described in the literature as self-antigens in autoimmune skin disorders. This strategy predicted potential shared lung tumor and skin antigens with the following criteria: (1) evidence of self-immunogenicity; (2) high expression in NSCLCs; and (3) skin or lung specificity. Genes that fulfilled two or more of these criteria were selected, resulting in a list of nine candidate genes (see eTable 1).
eFigure 7. Stimulations of PBMCs With Shared NSCLC and Skin Antigens

Stimulations of PBMCs from NSCLC patients with and without skin toxic effects during anti-PD-1 therapy with nine predicted antigens (in the form of peptide pools). Production of IFN-γ by CD8+ (A) and CD4+ (B) T cells was determined for the identification of antigen-specific T cells. Regression analysis revealed an association between development of skin toxic effects and high frequencies of IFNγ+ T-cell response, which was significant for CD8+IFNγ+ T-cell response (odds ratio, 1.99 [95% CI, 1.01-3.90]; P = .046), but was not for CD4+IFNγ+ T-cell response (odds ratio, 1.05 [95% CI, 0.98-1.13]; P = .146).
A bioinformatics analysis based on publicly available RNA-Seq databases was carried out to identify genes highly expressed in NSCLC and skin and that may represent NSCLC antigens (see eFigure 6). 9 candidate genes were selected and are shown in the table.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Name</th>
<th>Protein Function</th>
<th>Evidence of Self-immunogenicity</th>
<th>Tissue Specificity</th>
</tr>
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<tbody>
<tr>
<td><strong>EZR</strong></td>
<td>Ezrin</td>
<td>cell surface structure adhesion, migration and organization</td>
<td>suggested autoantigens in streptococcal-induced autoimmune response in psoriasis</td>
<td>keratinocytes</td>
</tr>
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<td><strong>HSPB1</strong></td>
<td>HSP27</td>
<td>chaperone protein involved in thermotolerance and cell survival under stress conditions</td>
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<td><strong>PRDX2</strong></td>
<td>Peroxiredoxin 2</td>
<td>antioxidant enzyme</td>
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<td><strong>SERPINB5</strong></td>
<td>Maspin</td>
<td>serine protease inhibitor</td>
<td>keratinocytes</td>
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<td>Keratin 6</td>
<td>structural protein</td>
<td>keratinocytes</td>
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<td><strong>KRT14</strong></td>
<td>Keratin 14</td>
<td>structural protein</td>
<td>altered expression in lesional epidermis of patients with autoimmune skin diseases</td>
<td>keratinocytes</td>
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<tr>
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<td>Keratin 17</td>
<td>structural protein</td>
<td>shares epitopes with streptococcal M proteins and may be the target of T cell infiltrating psoriatic lesions</td>
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<td>pathogenic autoantigen in pemphigus vulgaris</td>
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<td><strong>CAMP</strong></td>
<td>LL37</td>
<td>antimicrobial activity</td>
<td>suggested T cell autoantigen in psoriasis</td>
<td>lung</td>
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</tbody>
</table>

**eTable.** Candidate NSCLC Antigens Shared With Skin