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Cancer Immunology Miniature

Cancer Immunology Research

Monocyte-Derived Dendritic Cells with Silenced PD-1 Ligands and Transpresenting Interleukin-15 Stimulate Strong Tumor-Reactive T-cell Expansion

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Abstract

Although allogeneic stem cell transplantation (allo-SCT) can elicit graft-versus-tumor (GVT) immunity, patients often relapse due to residual tumor cells. As essential orchestrators of the immune system, vaccination with dendritic cells (DC) is an appealing strategy to boost the GVT response. Nevertheless, durable clinical responses after DC vaccination are still limited, stressing the need to improve current DC vaccines. Aiming to empower DC potency, we engineered monocyte-derived DCs to deprive them of ligands for the immune checkpoint regulated by programmed death 1 (PD-1). We also equipped them with interleukin (IL)-15 "transpresentation" skills. Transfection with short interfering (si)RNA targeting the PD-1 ligands PD-L1 and PD-L2, in combination with *IL15* and *IL15Rα* mRNA, preserved their mature DC profile and rendered the DCs superior in inducing T-cell proliferation and IFNγ and TNFα production. Translated into an *ex vivo* hematological disease setting, DCs deprived of PD-1 ligands (PD-L), equipped with IL15/IL15Rα expression, or most effectively, both, induced superior expansion of minor histocompatibility antigen–specific $CD8^+$ T cells from transplanted cancer patients. These data support the combinatorial approach of *in situ* suppression of the PD-L inhibitory checkpoints with DC-mediated IL15 transpresentation to promote antigen-specific T-cell responses and, ultimately, contribute to GVT immunity. *Cancer Immunol Res; 5(8); 710–5. ©2017 AACR.*

Introduction

Therapies designed to boost the body's immune system (termed immunotherapies) are currently considered some of the most promising cancer therapies, with the recent approval of several biologicals for clinical use, such as an autologous cellular-based immunotherapy vaccine (Sipuleucel T) and the immune checkpoint inhibitors atezolizumab [programmed death-ligand 1 (PD-L1) inhibitor], nivolumab, and pembrolizumab [both programmed death (PD)-1 inhibitors] (1). In immunotherapy, DC vaccination is a highly studied approach whereby *ex vivo* generated DCs, as the main orchestrators of the immune system, are administered to induce T-cell immunity in cancer patients. Although this has already led to observable clinical responses, the majority of clinical studies report limited efficacy with conventional DC vaccine preparations, indicating the need to design immunostimulatory DC vaccines with fresh approaches, in order to generate superior antitumor immune responses (2, 3).

Incorporating interleukin (IL)15 in the manufacturing process of DC vaccines can turn DCs into potent immune stimulators (4). Because IL15 can stimulate both the innate and the adaptive arms of the immune system, it has become one of the most promising molecules for antitumor immunotherapy (5). To transfer its signal, IL15 uses the α-moiety of its receptor (IL15Rα) to be transpresented to neighboring cells that express the IL15Rβ and -γ subunits, such as natural killer (NK) cells and $CD8^+$ cytotoxic T cells (6, 7). We have shown that transfection of DCs with mRNA encoding both IL15 and its α-receptor renders them able to stimulating innate immunity, with strong NK-cell phenotypic activation profiles, higher NK-cell IFNγ production, and improved cytotoxic properties, compared with DCs lacking transpresented IL15 or soluble IL15 secretion (8). In the context of cancer immunotherapy, the next step is to investigate whether IL15 transpresenting DCs have similar effects on adaptive antigen-specific $CD8^+$ T cells.

In addition to costimulatory interactions, the strength of an immune response is also determined by coinhibitory signals, like those promulgated by PD-1 interactions with its ligands PD-L1 and PD-L2 (9). Because PD-L1 and PD-L2 are highly expressed on mature monocyte-derived DCs, the potency of DC vaccines can be dampened by these dominant coinhibitory immune checkpoints (10). Like IL4-silenced DCs, reducing DC surface expression of inhibitory PD-L1 and PD-L2 by transfection of targeted short interfering RNA (siRNA) resulted in DCs with significantly greater T cell–stimulating properties (11).

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To acquire a tumor-specific T cell–mediated immune response, DCs can be loaded with specific antigens. In the setting of allogeneic stem cell transplantation (allo-SCT), hematopoietic-restricted minor histocompatibility antigens (MiHA) play a key role in the induction of graft-versus-tumor (GVT) effects *in vivo* (12, 13). In addition, MiHA-loaded DCs have proven to boost *in vitro* antitumor $CD8^+$ T-cell immunity, which favors the GVT response (11, 14).

Because both IL15 transpresentation and PD-L silencing on their own can enhance T-cell activation, we hypothesized that the combination of both could be additive. We inserted both manipulations into a single DC vaccine, to create a DC vaccine with superior immune-stimulatory potential. We developed a monocyte-derived DC generation protocol combining *in situ* PD-L silencing, by using siRNA lipofection, and IL15/IL15Rα transfection, by using mRNA electroporation, and investigated the T cell–stimulatory properties of these newly designed DCs.

Materials and Methods

Ethics statement and cell material

This study was approved by the Ethics Committee of the University of Antwerp (Antwerp, Belgium) under the reference number 16/10/123. Experiments were performed using blood samples from anonymous donors provided by the Antwerp branch of the Red Cross Blood Transfusion Center (Mechelen, Belgium) or from patients with hematological malignancies (Table 1) obtained at the Department of Hematology of the Radboud University Medical Center (Nijmegen, the Netherlands). Patients received an allo-SCT prior to blood collection. Cellular material was obtained in accordance with the Declaration of Helsinki and institutional guidelines and regulations (CMO 2013/064).

mRNA and siRNA

The *IL15* and *IL15Rα* mRNA constructs were obtained as described previously (8). PD-L1, PD-L2, and luciferase siRNAs were produced as described in ref. 15.

DC culture and RNA transfection

PD-L–silenced DCs were generated as described in ref. 10 with minor adaptations to implement the IL15 transpresentation mechanism. Briefly, positively selected $CD14^+$ monocytes (Miltenyi Biotec) were differentiated into mature DCs according to a 9-day culture protocol in Phenol red-free X-VIVO-15 culture medium (Lonza) supplemented with 2% human serum (HS; PAA Laboratories; ref. 10). After 3 days, intermediate monocyte-derived DCs (moDC) were harvested and reseeded for transfection in serum-free culture medium with siRNA/SAINT-RED lipoplexes (4 μg/40 μL ratio; luciferase or PD-L1/2 siRNAs) or left untreated (no siRNA; ref. 10). After 1 hour of lipofection at 37°C, medium was replenished with GM-CSF, IL4, and 2% HS to allow further differentiation. At day 7, maturation was induced with tumor necrosis factor (TNF)-α (20 ng/mL, Miltenyi Biotec) and prostaglandin E_2 (1 μg/mL, PGE_2; Prostin E2, Pfizer). At day 9, matured moDCs were harvested and electroporated (200V, 150 μF; Gene Pulser Xcell device, Biorad) without mRNA (mock EP DCs) or a combination of 5 μg *OSP-IL15* mRNA and 5 μg *IL15Rα* mRNA (IL15/IL15Rα EP DCs) in 200 μL Opti-MEM reduced-serum medium without phenol red (Life Technologies) in a 4-mm electroporation cuvette (Biorad), according to a previously described protocol (16). PD-L surface expression, IL15 membrane expression, and maturation phenotype of DCs were analyzed 4 hours after electroporation by staining with fluorochrome-labeled CD80, CD83, CD86, IL15, PD-L1, and PD-L2 antibodies (all from BD, except CD83 from Life Technologies and IL15 from R&D), and corresponding isotype controls. To evaluate PD-L knockdown efficiency, relative expression was calculated as (ΔMFI PD-L siRNA-treated DCs/ΔMFI no siRNA-treated DCs) × 100, with ΔMFI (delta mean fluorescence intensity) representing subtraction of the MFI of the isotype control from the PD-L–specific MFI. Cells were analyzed on a FACScan flow cytometer (BD). For antigen-specific assays, DCs were loaded exogenously with 5 μmol/L MiHA peptide (Table 1) by incubation with peptide during 30 minutes at 37°C.

Allogeneic mixed lymphocyte reaction (Allo-MLR)

Allogeneic $CD3^+$ T cells were isolated by negative selection with magnetic Microbeads (Miltenyi Biotec) and subsequently labeled with 10 μmol/L cell proliferation dye eFluor 450 (eBioscience), according to the manufacturer's instructions. Labeled T cells were cocultured with allogeneic mature DC at a 10:1 ratio in 6 replicates in 96-well round-bottom plates (Nunc) for 5 days at 37°C. Cell-free supernatant was collected and frozen at −20°C until further analysis with ELISA (IFNγ; Endogen) or electrochemiluminescent detection (TNFα, IL1β, IL6, IL12/IL23p40, and IL15; Meso Scale Discovery). Cells were harvested and stained with anti-CD3-PECy7 (Biolegend), anti–CD8-FITC (Beckman Coulter) and 1:1,000 diluted Fixable Viability dye eFluor 780 (eBioscience) for 15 to 30 minutes at 4°C for cell-based proliferation analysis on a Gallios flow cytometer (Beckman Coulter).

Table 1. Details of the MiHA profile of hematologic cancer patients after allogeneic stem cell transplantation

					% expanded MiHA-specific $CD8^+$ T cells			
Patient	Disease	MiHA type	Peptide	% circulating MiHA-specific $CD8^+$ T cells	Luci/mock DC	Luci/IL15 DC	PDL/mock DC	PDL/IL15 DC
1	MDS-RAEB	HLA-B7 LRH1	TPNQRQNVC	0.003	0.09	0.21	0.26	0.66
2	AML	HLA-B7 LRH1	TPNQRQNVC	0.012	0.60	0.68	0.68	0.76
3	ALL	HLA-A2 HA2	YIGEVLVSV	0.011	0.40	0.62	0.53	0.99
4	AML	HLA-B7 ARGHDIB	LPRACWREA	0.026	0.84	0.82	1.09	1.10
5	AML	HLA-B7 ARGHDIB	LPRACWREA	<0.001	0.02	0.05	0.05	0.14
6	CLL	HLA-A2 HA1	VLHDDLLEA	0.053	2.69	3.89	4.31	8.43

NOTE: Percentages are given of tetramer-positive MiHA-specific $CD8^+$ T cells in peripheral blood (% circulating) and upon expansion in 7-day *ex vivo* cocultures with allogeneic designer DC (% expanded).

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CLL, chronic lymphoid leukemia; DC, dendritic cell; HLA, Human Leukocyte antigen; IL15, *interleukin 15* mRNA electroporated; Luci, *Luciferase* siRNA lipofected; MDS-RAEB, Myelodysplastic Syndrome—Refractory Anemia with Excess of Blasts; mock, electroporated without mRNA; PDL, lipofected with siRNA silencing programmed death ligands.

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MiHA-specific T-cell stimulation assay

MiHA-specific T-cell expansion was evaluated with PBL of hematologic cancer patients stimulated for 7 days with peptide-loaded HLA-A2$^+$ or HLA-B7$^+$ MiHA$^-$ allogeneic moDC of healthy donors at a 10:1 ratio in IMDM + 10% HS in 24-well plates (Elscolab). At day 5 of the coculture, rhIL2 (50 U/mL) was added. Two days later, cells were harvested and stained for 15 minutes at 37°C with MiHA-specific tetramers [0.2 μg PE-labeled and 0.2 μg APC-labeled; kindly provided by Prof. Dr. J.H.F. Falkenburg (Leiden University Medical Center, Leiden, the Netherlands)]. Subsequently, cells were labeled with anti–CD3-PECy7 (Biolegend), anti–CD8-FITC (Beckman Coulter) and 1:1,000 diluted Fixable Viability dye eFluor 780 (eBioscience) for 15 to 30 minutes at 4°C and analyzed using a Gallios flow cytometer (Beckman Coulter).

Statistical analysis

Flow cytometry data were analyzed using FlowJo version 10.0.6 (Treestar). GraphPad Prism 5 software was used for graphing and statistical calculations. Statistical analysis was performed using the repeated-measures one-way analysis of variance with Bonferroni *post hoc* test. Results were considered statistically significant when $P < 0.05$.

Results

PD-L siRNA lipofection retained maturation and IL15 transpresentation and reduced PD-L

We previously designed a DC manufacturing protocol in which DCs were modified to present IL15 on their membrane (8). Now, we further modified our IL15-transpresenting DCs by implementing *PD-L* siRNA lipofection to reduce the expression of both PD-L1 and PD-L2. Therefore, "designer" DCs treated with PD-L or Luci siRNA and subjected to mock or *IL15/IL15Rα* mRNA electroporation were checked for expression of PD-L1 and PD-L2. For both molecules, membrane expression, shown as relative expression, based on delta mean fluorescence intensity (ΔMFI), was significantly reduced on PD-L siRNA DC compared with No siRNA DC ($P < 0.001$) and Luci siRNA DC ($P < 0.001$), regardless of their IL15-transpresenting capacity. Because no difference in PD-L-silencing could be detected between No siRNA and Luci siRNA DCs, results are shown for Luci siRNA DCs only (Fig. 1A). SiRNA lipofection of immature DCs did not affect subsequent mRNA electroporation efficiency of mature DCs, as evidenced by equally high IL15 surface expression (Fig. 1B). Combined lipofection with siRNA and mRNA electroporation preserved the maturation profile of the DCs, as the expression of the most common DC maturation markers (CD80, CD83, and CD86) remained unchanged (Fig. 1C).

PD-L–silenced and IL15-transpresenting DCs stimulate allogeneic T cells

To investigate the effects of PD-L–silenced IL15-transpresenting DCs on allogeneic CD3$^+$ T cells, we measured T-cell proliferation and the presence of the proinflammatory cytokine IFNγ in the supernatant of DC–T cell cocultures. T cells cultured without DCs for 5 days did not proliferate, whereas addition of DCs resulted in proliferation of 50% to 60% and 60% to 70% of the CD4$^+$ and CD8$^+$ T cells, respectively (Fig. 2A). PD-L silencing of DCs resulted in a significantly increased T-cell proliferation (PD-L siRNA versus Luci siRNA, $P < 0.001$ for CD4$^+$ T cells and CD8$^+$ T cells) regardless of the presence of IL15 on the membrane of the DCs (Fig. 2A).

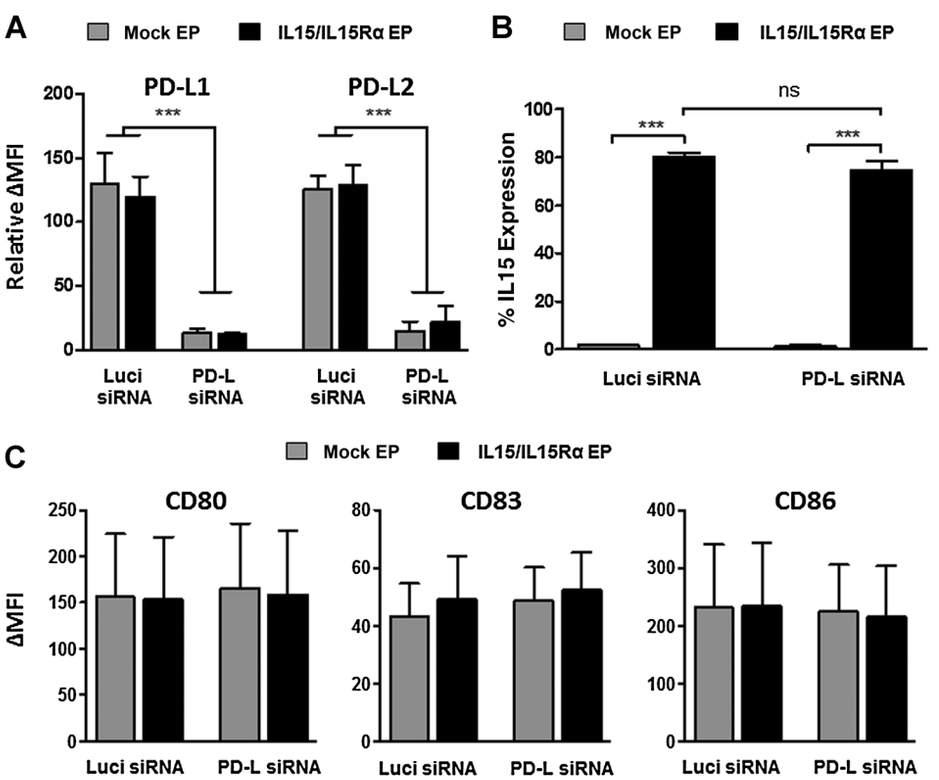

Figure 1.
Phenotypic characterization of PD-L–silenced and IL15-transpresenting DC. Surface expression of PD-L1, PD-L2, IL15, and maturation markers was determined by flow cytometric analysis 4 hours after mock (gray bars) or *IL15/IL15Rα* mRNA (black bars) electroporation of mature Luci siRNA and PD-L siRNA-lipofected DC. **A,** Results are depicted as PD-L surface expression relative to non-lipofected DC based on ΔMFI (see Materials and Methods) for 4 independent donors performed in 2 separate experiments. **B,** IL15 expression is depicted as mean percentage (±SEM, $n = 11$ from 6 independent experiments). **C,** Expression of maturation markers (CD80, CD83, and CD86) is represented as ΔMFI (± SEM) for 4 independent donors from 2 individual experiments. ns, not significant; ***, $P < 0.001$, repeated-measures one-way ANOVA with Bonferroni *post hoc* test. Abbreviations: ΔMFI, delta mean fluorescence intensity; Luci, Luciferase; PD-L, programmed death-ligand; SEM, standard error of the mean.

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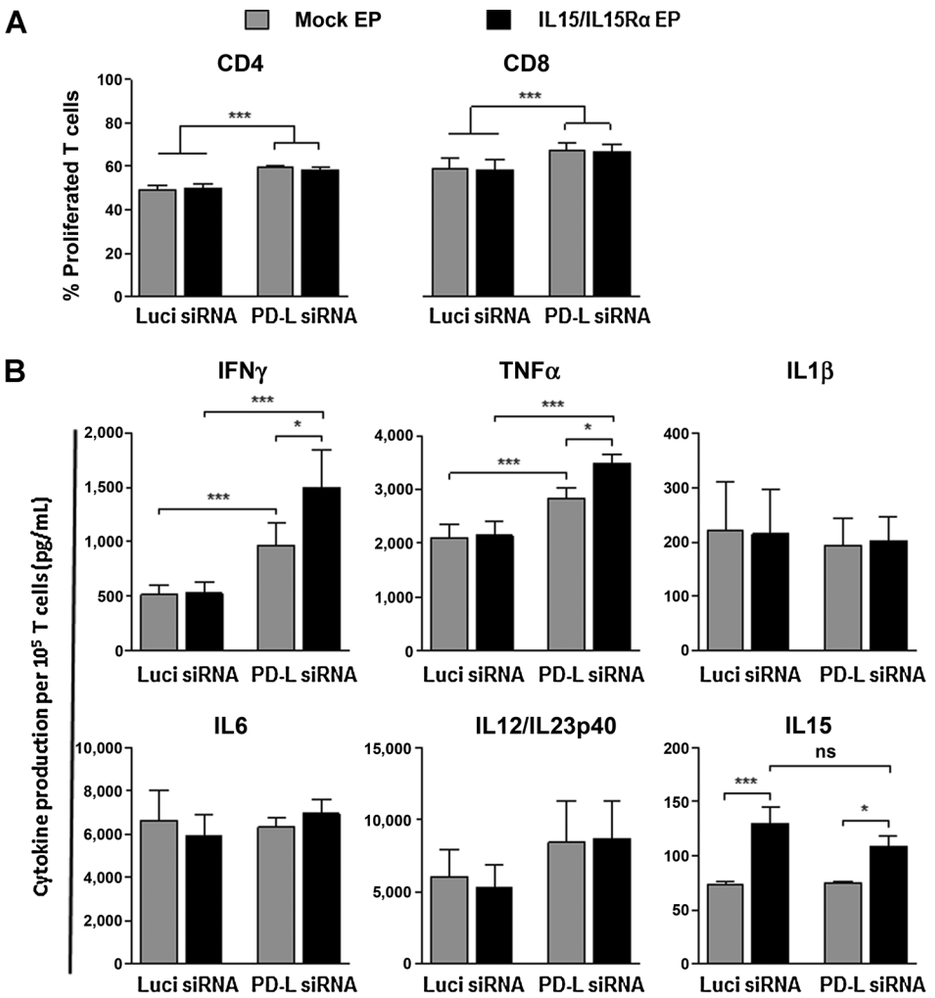

Figure 2.
Allogeneic T cell–stimulating capacity of PD-L–silenced and IL15-transpresenting DC. Bar graphs depict proliferation of allogeneic CD3$^+$ T cells upon 5-day coculture with mock-electroporated (gray bars) or *IL15/IL15Rα* mRNA-electroporated (black bars) mature DCs treated with Luci or PD-L siRNA, as indicated, at a 10:1 T cell/DC ratio. Cocultures were analyzed for CD4$^+$ T-cell and CD8$^+$ T-cell proliferation within the viable CD3$^+$ T-cell population by flow cytometry (**A**), and supernatant was analyzed for IFNγ secretion by ELISA and secretion of TNFα, IL1β, IL6, IL12/IL23p40, and IL15 by electrochemiluminescent detection. **B,** Data are shown as mean (± SEM) for 5 (**A**) and 4 (**B**) independent donors from 3 (**A**) and 2 (**B**) individual experiments, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, repeated-measures one-way ANOVA with Bonferroni *post hoc* test. Abbreviations: IFNγ, interferon gamma; Luci, Luciferase; PD-L, programmed death-ligand; SEM, standard error of the mean; TNFα, tumor necrosis factor alpha.

In accordance with the enhanced proliferation of both CD4$^+$ and CD8$^+$ T cells, cocultures of PD-L siRNA DCs and allogeneic T cells resulted in significantly increased IFNγ and TNFα levels compared with Luci siRNA DCs ($P < 0.001$; Fig. 2B). When the PD-L siRNA DCs also transpresented IL15, a synergistic effect on IFNγ and TNFα production was observed, resulting in significantly enhanced levels compared with PD-L siRNA DCs without IL15 transpresentation ($P < 0.01$) and IL15-transpresenting Luci siRNA DCs ($P < 0.001$; Fig. 2B). Expression of ILβ, IL6, and IL12/IL23p40 was comparable in all conditions where designer DCs were cocultured with allogeneic T cells. Secretion of IL15 was elevated in cocultures in which IL15-transpresenting DCs were included, compared with DCs without IL15 conditioning (Luci siRNA DC, $P < 0.001$; PD-L siRNA DC, $P < 0.05$; Fig. 2B). These increased IL15 concentrations likely originate from the IL15-transpresenting DCs rather than the T cells in the coculture.

Robust DC-mediated expansion of MiHA-specific T cells from hematologic cancer patients

Next, we investigated whether low frequencies of MiHA-specific memory T cells present in the peripheral blood of hematological cancer patients after allo-SCT could be efficiently boosted with the different MiHA-loaded DC vaccines (characteristics of patient samples in Table 1). In this context, integrating the PD-L–silencing strategy into the DCs increased the expansion of MiHA-specific T cells compared with Luci siRNA DCs (Fig. 3A and Table 1). In contrast, exploiting the IL15-transpresentation mechanism also resulted in a detectable increase of MiHA-specific T cells compared with the mock electroporated DC condition. Notably, combining PD-L silencing and IL15 transpresentation showed the highest levels, reaching 2-fold higher percentages of MiHA-specific T cells versus the IL15-transpresenting Luci siRNA DCs or the mock EP PD-L siRNA DCs (Fig. 3A and Table 1). Comparing absolute counts of MiHA-specific T cells, the effect of the combination treatment on expansion of MiHA-specific T cells is significantly better compared with mock EP PD-L siRNA DC lacking IL15 expression ($P < 0.01$; Fig. 3B).

Discussion

Current therapies to treat cancer patients with hematologic malignancies, such as allo-SCT, are often insufficient in clearing all tumor cells, due to inadequate amounts of functional immune killer cells (17). Therefore, adjuvant therapies are needed to boost GVT immunity, without inducing graft-versus-host disease

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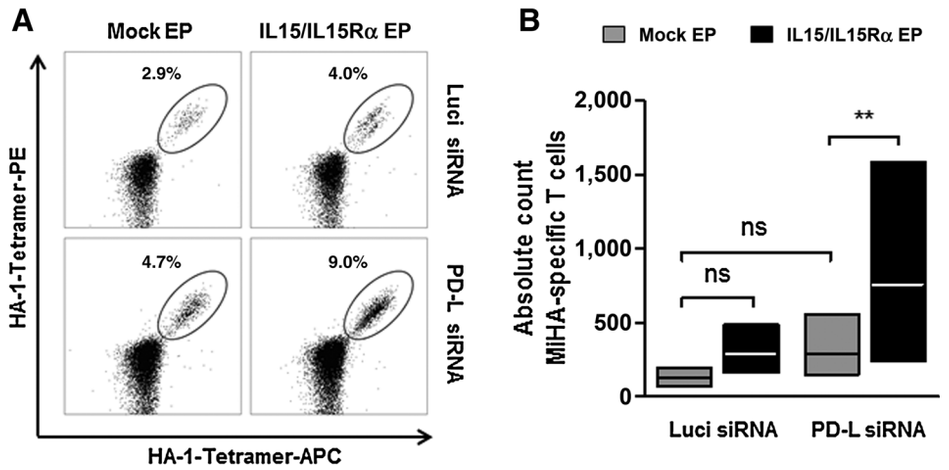

Figure 3.

PD-L-silenced and IL15-transpresenting DC induce strong expansion of MiHA-specific $CD8^+$ T cells from hematological cancer patients. Flow cytometry plots (one representative patient out of 6 patients; **A**) or floating bar graphs ($n = 6$ from 4 independent experiments; **B**) illustrate percentage and absolute counts, respectively, of expanded MiHA-specific $CD8^+$ T cells from allo-SCT hematologic cancer patients after 7-day coculture with peptide-loaded allogeneic DCs, either lipofected with Luci siRNA (top graphs) or PD-L siRNA (top graphs) and either mock-electroporated (left graphs) or IL15/IL15Rα mRNA-electroporated (right graphs). (**A**) Gated cells were selected for viable HA-1-tetramer–positive $CD3^+CD8^+$ cells. **B,** Absolute counts were calculated by multiplying total cell count, % $CD8^+$ T cells and % MiHA-specific T cells. Absolute counts were transformed to relative counts compared with those of the corresponding mock EP No siRNA DC, which were set to 100. ns, not significant; **, $P < 0.01$, repeated-measures one-way ANOVA with Bonferroni *post hoc* test. Abbreviations: APC, allophycocyanin; EP, electroporation; Luci, Luciferase; PE, phycoerythrin; PD-L, programmed death-ligand.

(GVHD), to prevent relapse of cancer in patients. In this context, DC vaccination has gained much interest, because these cells can be loaded with specific antigens to be presented to T cells and, express costimulatory molecules that promote tumor-reactive T-cell immunity (18).

Although DC vaccination has proven to be safe and immunogenic in clinical trials, optimization is needed to improve durable clinical responses, which remain scarce (3). We adapted conventional IL4 DC with two major changes, namely electroporation of *IL15/IL15Rα* mRNA and siRNA interference with the PD1/PD-L pathway. We adopted this protocol for four reasons. First, IL15 is a promising immunotherapeutic drugs with great potential for broad usage in cancer therapy (5). Cells expressing both IL15 and IL15Rα can activate T cells more effectively than DCs expressing IL15 alone (19). Second, interference with the PD1/PD-L-pathway has already proven to be effective in reducing tumor growth and improving overall survival in multiple clinical trials (20). Third, a more targeted "*in situ*" delivery carries a lower risk of autoimmunity and immune-related adverse effects than systemic delivery of IL15/IL15Rα complexes and PD-L1–blocking antibodies (21, 22). Therefore, we opted to transfect DCs with mRNA encoding for IL15/IL15Rα and siRNAs to prevent PD-L1/2 expression. Fourth, a combination of lipofection and electroporation was chosen over a double-electroporation strategy to introduce PD-L silencing and IL15 transpresentation, because double electroporation of DCs diminishes their yield and viability after harvest, whereas combining lipofection and electroporation is clinically feasible (10).

While both IL15-transpresenting DCs (19, 23) or Langerhans type DCs, which also use the IL15 transpresentation mechanism (24, 25), and PD-L–silenced DCs (10, 11) separately have been described to have an increased capacity to activate and expand tumor-reactive T cells *in vitro*, this work additionally shows the superior effects of combining IL15 transpresentation and PD-L silencing in a single DC preparation. Next to successfully implementing both mechanisms into conventional IL4 DCs, we showed that PD-L–silenced IL15-transpresenting DCs were the best stimulators of IFNγ and TNFα production by allogeneic T cells compared with DCs where only one strategy was implemented. In a target population of allo-SCT–treated hematologic cancer patients, the combination of these two modifications in our DCs resulted in the highest *in vitro* activation and expansion of MiHA-specific $CD8^+$ T cells. This underscores the superior specific T cell–stimulatory potential of our DC protocol in which IL15 transpresentation and interference with the PD-1/PD-L-pathway are combined. Because monocytes from the corresponding stem cell donors were not available for analyses of the MiHA-reactive T cell–stimulatory potential in a host–donor setting, we performed this study with partly HLA-matched third-party donor DC. This is in concordance with our previous studies, in which we demonstrated the superior potency of allogeneic and autologous PD-L silenced DC vaccines for priming and boosting of MiHA-reactive T-cell responses, as well as for inducing highly effective and selective cytolytic T-cell activity (reflected by degranulation and killing) toward $MiHA^+$ target cells (10, 11, 14, 26). Although we did not perform cytotoxicity experiments in this study, it is unlikely that T cells expanded with IL15-transpresenting PD-L-silenced DC vaccines would perform worse than expanded with one of these manipulations. Altogether, we believe that IL15-transpresenting PD-L–silenced DCs hold great potential for boosting tumor-reactive T-cell responses in cancer patients. These findings contribute to the growing body of evidence that both immunotherapy and combination therapies are valuable key players in the search for efficient cancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.M.J. Van den Bergh, E.L.J.M. Smits, Z.N. Berneman, W. Hobo

Development of methodology: J.M.J. Van den Bergh, H. Dolstra, W. Hobo

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M.J. Van den Bergh, E.L.J.M. Smits, T.J.A. Hutten, H. De Reu, H. Dolstra

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M.J. Van den Bergh, T.J.A. Hutten, V.F.I. Van Tendeloo

Writing, review, and/or revision of the manuscript: J.M.J. Van den Bergh, E.L.J.M. Smits, Z.N. Berneman, V.F.I. Van Tendeloo, H. Dolstra, E. Lion, W. Hobo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M.J. Van den Bergh, T.J.A. Hutten

Study supervision: J.M.J. Van den Bergh, E.L.J.M. Smits, Z.N. Berneman, V.F. Van Tendeloo, E. Lion, W. Hobo

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