Effector Th1 cells under PD-1 and CTLA-4 checkpoint blockade abrogate the upregulation of multiple inhibitory receptors and by-pass exhaustion

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Abstract
Immune checkpoint inhibitor (ICI) immunotherapy relies on the restoration of T-cell functions. The ICI receptors are not only found on exhausted T cells but also upregulated upon activation and reach high levels on effector T cells. In an ex vivo model, this study explored the consequences of PD-1 and cytotoxic T-lymphocyte antigen (CTLA-4) blockade applied during specific time frames of T-cell stimulation that coincide with distinct functional phases in type 1 helper T (Th1) cells. When applied at an early stimulation stage, the checkpoint blockade interfered with the upregulation of multiple inhibitory receptors such as PD-1, LAG3, TIM-3 and CTLA-4. Moreover, extension of the blockade period restricted the hyporesponsiveness in T cells. Alternatively, a short-term ICI treatment was advantageous when applied at late time frames of Th1 cell stimulation. Here, a transition phase from effector to exhausted state, which coincided with the late time frames of Th1 stimulation, was clearly determined together with the transcriptomics data demonstrating the initiation of significant alterations in metabolic pathways, genetic information processes, effector and exhaustion specific pathways. Applied in this transition phase, PD-1 and/or CTLA-4 blockade downregulated the inhibitory receptors which were already present on the effector Th1 cells, potentially through endocytic pathways. Therefore, the efficacy of ICI therapy was modulated by the functional status of T cells and can be improved by modifying the timing and duration of PD-1 and CTLA-4 blockade. In conclusion, the ICI therapy not only supports the reactivation of T cells but can also constrain de novo exhaustion.

KEYWORDS
activation, checkpoint blockade, differentiation, exhaustion, Th cells

INTRODUCTION
The efficacy of immunotherapy approaches has been associated with the presence of a baseline inflammatory response and the potential of invigorating pre-existing immunity [1, 2]. Even though inflamed tumours, which are characterized with high T-cell infiltration and noticeable interferon-γ (IFN-γ) signalling, tend to better respond to the immune checkpoint inhibitors (ICI), the functional status of T cells and suppressive assets of the
tumour microenvironment determine the success of the therapy [3, 4]. Type 1 helper T (Th1) cells play a critical role in anti-tumour immune responses by enhancing antigen presentation and cytotoxic responses [5–9]. Nevertheless, both cytotoxic T lymphocytes (CTL) and Th1 cells are prone to immune regulation which occurs due to continuous stimulation and chronic exposure to inflammatory factors. Under these circumstances, the T cells acquire a hyporesponsive or exhausted state [8, 10, 11]. Exhaustion in Th1 cells, which is functionally characterized by progressive loss of IFN-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-2 cytokine production and proliferation, are also identified with a unique epigenetic landscape [12, 13]. The presence of multiple inhibitory receptors such as PD-1, cytotoxic T-lymphocyte antigen (CTLA-4), TIM-3, TIGIT and LAG3 has been acknowledged as an asset of exhausted T cells [10, 14]. Nevertheless, many inhibitory receptors are expressed upon T-cell activation and become highly upregulated on effector T cells in order to establish checkpoints that limit inflammatory damage and auto-reactivity [15]. Therefore, not only the hyporesponsive T cells but also the T cells at distinct phases of functional responsiveness can be susceptible to ICI therapeutics such as anti-PD-1 and anti-CTLA-4 monoclonal antibodies [16, 17].

The T-cell exhaustion has been broadly characterized in dysfunctional CD8⁺ T cells under persistent and sub-optimal antigen exposure [18]. Insufficient CD4⁺ T cell help has also been associated with the induction of exhaustion in CD8⁺ T cells [19]. On the other hand, further research is required to define Th cell exhaustion under distinct contexts such as alterations in functional stages and subtype differentiation [20]. Upon recognition of the antigen-MHC class II complex, a plethora of co-stimulatory signals and cytokines influence the differentiation of Th1 cells [21]. As previously demonstrated by our group, continuous exposure to co-stimulatory signals drives the Th1 cells into exhaustion [22]. Intriguingly, in this context, the inhibitory signals received through PD-1 at an early time-frame of T-cell stimulation tended to hinder the acquisition of functional exhaustion induced by the co-stimulatory signals [22]. Therefore, the impact of co-inhibitory signals on the regulation of Th1 responses remains to be better elucidated [23–25].

The expression level and assortment of inhibitory receptors on Th1 cells are considerably altered according to the activation and differentiation status [10]. This study aims to monitor the molecular and functional aspects of effector Th1 responses and to better recognize the association between Th1 cells’ functional status and responsiveness to checkpoint inhibitors. In a co-culture model enabling functional exhaustion of Th1 cells, which was previously established by our research group [22, 26], the impact of timing and duration of PD-1 and/or CTLA-4 blockade was assessed in Th1 cells at distinct phases of effector responses.

MATERIALS AND METHODS

Th cell isolation and purification

Peripheral mononuclear cells from healthy adult donors (Hacettepe University Local Ethics Committee, approval no. GO 17/932-14) were isolated by centrifugation over Ficoll–Hypaque (Sigma-Aldrich) and enriched for CD4⁺ Th cells using negative selection by magnetic-activated cell sorting (MACS) (Miltenyi Biotec) according to manufacturer’s instructions. Additional experiments were performed with CD45RA⁺ CD4⁺ T cells which were further purified by CD3-untouched fluorescence-activated cell sorting (FACS) until a purity of >98% was obtained.

Ex vivo generation of Th1 cells

The human monocytic cell line THP-1 and freshly-purified Th cells (2.5 × 10⁴ cells) were co-cultured with THP-1 cells (5 × 10⁵) in 96-well round-bottom plates in 200 μl RPMI 1640 supplemented with 25 ng/ml functional grade purified anti-CD3 (aCD3; clone, HIT3a) (BioLegend), 10% fetal bovine serum (FBS; Biological Industries), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 g/ml) at 37°C in a humidified 5% CO₂ incubator. In order to maintain continuous stimulation and to prevent possible metabolic stress, the coculture media were daily refreshed at 1:1 ratio. Briefly, 100 μl of the media was carefully pipetted out from each well and an equal volume of fresh media containing anti-CD3 mAb (25 ng/ml) was gently added [22]. As a control, the Th cells were also stimulated with anti-CD3/28 beads where required (Miltenyi Biotec).

For the studies on immune checkpoint blockade, anti-CTLA-4 mAb ipilimumab (10 μg/ml; Yervoy, Bristol-Myers Squibb) and anti-PD-1 mAb nivolumab (10 μg/ml; Opdivo, Bristol-Myers Squibb) or a control IgG1x antibody (10 μg/ml; BioLegend) were applied at 24, 72 and 96 h of the co-cultures. The blocking mAbs were also refreshed together with the co-culture media. In order to assess the impact of endocytosis on the downregulation of PD-1 and CTLA-4 receptors upon treatment with ipilimumab and nivolumab, respectively, the incubations were also performed in the presence of monensin (2 μM, BioLegend) which was added 16 h prior to the analysis.
Flow cytometry

Immunophenotyping of Th cells was performed by flow cytometry. The cells obtained at different time points of the co-cultures were resuspended in isotonic buffer (CellWash, BD Biosciences) containing 1% FBS and incubated for 20 min at 4°C with appropriate combinations of fluorochrome-labelled antibodies mouse anti-human CD3 (clone SK7; BioLegend), CD4 (clone RPA-T4; BioLegend), CD13 (clone WM53; BD Biosciences), CD45RA (clone HI100; Sony Biotechnology), CD45RO (clone UCHL1; Sony Biotechnology), CD62L (clone DREG56; BD Biosciences), CD197 (clone G043H7; BioLegend), CD154 (clone TRAP1; BD Biosciences), CD69 (clone FN50; Sony Biotechnology), CD25 (clone BC96; Sony Biotechnology), LAG3 (clone 11C3C65; BioLegend), TIM-3 (clone F38-2E2; BioLegend), CTLA-4 (clone L3D10; BioLegend) and PD-1 (clone EH12.2H7; BioLegend). Autofluorescence values or samples incubated with isotype-matched antibodies were used as technical controls. Following the removal of the unbound antibodies, the samples were run on a FACSAria II flow cytometer (BD Biosciences) and analysed using FlowJo software (version 10, TreeStar).

Proliferation and cell cycle assays

For the assessment of Th cell proliferation, prior to the co-culturing, purified CD4⁺ T cells were stained with cell proliferation dye eFluor670 (5 μM; Invitrogen) according to the manufacturer’s instructions. Following 72 h of co-culturing, the percentage and MFI of the cells were measured by flow cytometry according to the dilution of proliferation dye.

Quantitation of DNA content was used for the assessment of cell cycle phases. Briefly, ice-cold 70% ethanol was added dropwise onto the purified Th cells and fixed for 30 min at 4°C. Following, the washing steps with phosphate-buffered saline (PBS, 1X), the cells were treated with of RNase I (100 μg/ml, for 30 min at 37°C; Sigma-Aldrich), washed and then incubated with propidium iodide (PI, 100 μg/ml, for 10 min at room temperature; Sigma-Aldrich). The intensity of intercalated PI was analysed by flow cytometry with a stringent gating strategy on singlet events according to FSC-A, FSC-H and FSC-W values.

Quantitation of cytokine secretion

The supernatants were collected from the co-cultures at different time points and the concentration of Th1-related cytokines IL-2, IFN-γ and TNF-α were determined by a flow cytometric bead array and data were analysed in accordance with the manufacturer’s protocol (LEGENDplex, BioLegend).

Bulk RNA sequencing and transcriptomic analysis

Total RNA was isolated from highly-purified Th cells from different time points of the co-cultures (RNaseasy, QIA-GEN). The RNA samples obtained from 10 independent experiments were pooled. Gene expression levels of over 2 × 10⁴ human RefSeq genes were measured by AmpliSeq Human Gene Expression Panel (Thermo Fisher Scientific) on an Ion Torrent next-generation sequencing platform (Ion S5, Thermo Fisher Scientific). The genes with read counts/million <0.5 were excluded by pre-processing and iDEP software was used for expression analyses. Heat maps were generated according to the average expression level for each gene. Global gene expression patterns of Th populations obtained from different time points were compared by principal component analysis. Differentially expressed genes (DEGs) were identified with the DESeq2 package using a threshold of false discovery rate <0.01 and fold-change >4 with comparisons wherein the data from unstimulated naïve Th cells (obtained from 0 h of the co-cultures) were used as normalizer. The most significant and time-characteristic genes of Th cells were highlighted on volcano plots. A search tool for the retrieval of interacting genes/proteins (STRING) was employed to construct protein–protein interaction network with a high-level of confidence. Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses were performed to determine the change in pathway expression patterns in at different time points by manual examination of total read count data.

Immunofluorescence

From the 120-h co-cultures, the Th cells were purified and mounted (Universal 320 Cytospin, Hettich). The cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 10% BSA. The slides were incubated with anti-human-PD-1 (QA19A98, 1/200; BioLegend) and anti-human-CTLA-4 (D4E9I, 1/200; Cell Signaling Technology) primary antibodies and then with Alexa488- and Alexa555-conjugated secondary antibodies (1:1000, Abcam). Following the 4,’6-diamidino-2-phenylindole (DAPI, 300 nM; Sigma-Aldrich) counterstaining, the cells were examined under an epifluorescence microscope (Olympus), and the images were processed with ImageJ software (NIH Image).
Statistical analysis

Otherwise noted, all data were presented as the mean ± standard error. The results from immunophenotyping and functional assays were obtained from at least six independent experiments repeated with Th cells from different donors whereas RNAseq data were acquired from 10 independent experiments performed with Th cells from different donors. Each experiment was performed in duplicates or triplicates per time point. Two-tailed Student’s t-test, Wilcoxon/Mann–Whitney U-tests and analysis of variance were used to assess the statistical significance. A p value of 0.05 or lesser was considered significant. Statistical analyses were performed by Graphpad v8 software (Prism).

RESULTS

Monitoring and characterization of functional transition in effector Th1 cells

We have previously demonstrated that the functional exhaustion in Th1 cells, which was preceded by a potent effector phase, is intensified by a plethora of co-stimulatory signals derived from myeloid leukaemia cells [22, 26]. In this study, we used a similar ex-vivo coculture model, where continuum of primary signals was provided by anti-CD3 stimulation whereas costimulation was derived from the monocytic cell line, THP-1. CD86 and ICOS-LG co-stimulatory molecules are constitutively expressed by THP-1 [22, 26]. The negative influence of metabolic activities and nutrient deprivation was also avoided by refreshment of the culture media containing anti-CD3 mAb (Figure 1a). The presence of THP-1 cells did not interfere with T-cell functions, expansion or viability (Figures S1 and S2).

In the co-cultures, 72 h was determined as a peak point for effector T-cell responses, Th1 responses were maintained for 96 h; however, a significant decrease was observed in functional responses at later time points (Figure 1b–d,g). T-cell proliferation had a significant rate at 72 h (proliferation, 60.9 ± 21.8% and S-G2/M phase of cell cycle, 55.8 ± 18.4%) (Figure 1b,c). Accordingly, IL-2, TNF-α and IFN-γ secretions were maximal at 72 h, respectively (Figure 1d). At 96 h, the effector functions of Th cells such as proliferation (proliferation, 12.2 ± 7.1% and S-G2/M phase of cell cycle, 12.4 ± 2.1%) and cytokine secretion were considerably decreased. Particularly, at 120 and 144 h, Th cell expansion and cytokine production were almost completely abolished (Figure 1b–d,g).

Throughout the co-culture period, multiple inhibitory receptors were determined on the majority of Th cells. At 72 h, the percentage of Th cells bearing the inhibitory receptors PD-1, CTLA-4, LAG3 and TIM-3 was at a high level (PD-1+, 81.7 ± 11.3%; CTLA-4+, 59.8 ± 11.9%; LAG3+, 75.2 ± 5.8%; TIM-3+, 50.1 ± 3.1%) (Figures 1e,f and S3). Moreover, the expression of these inhibitory receptors was slightly upregulated on Th1 cells as the co-culture period was extended to 144 h (Figure 1e,f).

Collectively, the Th1 cells were functionally responsive during 72 h of co-culturing; whereas at 120–144 h, they acquired a hyporesponsive state, lost proliferation and cytokine secretion capacities and upregulated multiple inhibitory molecules. Moreover, 96 h of co-culturing coincided with initiation of the decline in the functional responses (Figure 1g).

Specific molecular signatures validate the functional status of Th1 cells

Next, transcriptomic changes in the Th cells purified from the co-cultures at the end of different incubation periods were studied. Th cells obtained from the co-cultures at 72–96 h and at 120–144 h were distributed in two distinct clusters (Figure 2a). The DEGs were determined with stringent filtering (adjusted p value <0.05, [fold change] >4). The expression of the genes which were previously associated with T-cell hyporesponsiveness were found to be upregulated in the Th cells collected from the co-cultures at 120 and 72 h (Figure 2b). A gradual increase was noted especially in the expression of ARHGEF1, DGKA, EOMES, GPR56, ICAM2, JAZF1, KLF2, KLF3, MAF, NFATC1, STK38, TMC6 and TOX genes as the duration of co-culture was extended (Figure 2b).

The total read counts of each KEGG gene set indicated that the pathways related to genetic information processing, cellular processes, metabolism and immunity dominated molecular signature of the effector Th cells at 72 h. At 120 and 144 h, the gene expression patterns were associated with the exhaustion and memory phenotype as well as with the attenuation in signalling, metabolism and genetic information pathways (Figures 2c and S4). To gain a better insight into dynamics of biological pathways during Th cells’ functional status, STRING analysis was employed to construct protein–protein interaction network for the upregulated DEGs. Expectedly, the number of clustered networks within the DEGs of the Th cells at 72 h were higher than those of the cells at 120 and 144 h (Figure 2d). In particular, the DEGs of Th cells purified from the co-cultures at 120th and 144th hours formed a network cluster for chromatin remodelling which indicates the formation of an epigenetic landscape. Notably, the Th cells obtained from the 96-h co-cultures carried certain gene expression signatures of T-cell
hyporesponsiveness (Figures 2b–d and S4) which was in accordance with the decline in functional responses at this time point (Figure 1b–g). Therefore, 96 h represented a late time frame of Th1 cell stimulation which may signify a transition phase from effector state to exhaustion.

Timing and duration of checkpoint blockade define the responsiveness of Th1 cells at distinct functional phases

Since the inhibitory molecules were upregulated on both responsive and hyporesponsive Th1 cells, next, we sought to investigate the impact of PD-1 and CTLA-4 checkpoint blockade applied at distinct time periods of co-culturing. Anti-PD-1 mAb, anti-CTLA-4 mAb or their combination were applied at 24, 72 or 96 h.

Blockade of CTLA-4 and PD-1 significantly enhanced the effector responses of Th1 cells in terms of IL-2, IFN-γ and TNF-α production (Figure 3). The combination of anti-PD-1 and anti-CTLA-4 mAbs led to a rather effective restoration of the cytokine secretion. Especially, IL-2 levels were explicitly augmented (~10-fold) once the checkpoint blockade was started at 96 and lasted for 24 h (Figure 3). For TNF-α, the timing of the blockades, either initiated at early or late phases of co-culturing, resulted in a compatible upsurge which was potentially due to the extension of the incubation periods. The maximum amount of IFN-γ was detected when the anti-PD-1 and anti-CTLA-4 mAbs were added at 24 h of the co-cultures for a 96 h-long incubation (Figure 3). Compared to anti-CTLA-4 mAb, anti-PD-1 mAb alone was more successful at restoring the secretion of IL-2 (started at 96 h of co-culturing continued for 24 h), TNF-α and IFN-γ (started at 24 h of co-culturing continued for 96 h) (Figure 3).

The expression of inhibitory receptors CTLA-4, PD-1, LAG3 and TIM-3 were assessed. Intriguingly, under the CTLA-4 blockade, Th cells obtained at 120 h expressed lower percentages of PD-1, LAG3 and TIM-3 compared to the co-cultures treated with a non-specific IgG antibody (Figures 4a and S5). Moreover, a similar phenomenon was observed with the PD-1 blockade where the percentage of Th cells positive for CTLA-4, LAG3 and TIM-3...
were significantly reduced at 120 h. The combination of anti-CTLA-4 and anti-PD-1 mAbs more consistently decreased the amount of Th cells expressing multiple inhibitory receptors (Figures 4a and S5). Next, the kinetics of inhibitory receptor expression was assessed following the initiation of the blockade at 24 h. Interfering with PD-1 and/or CTLA-4 at an early time point restricted the upregulation of multiple inhibitory receptors on Th1 cells (Figure 4b). The percentage of LAG3+ and TIM-3+ cells were significantly declined after the effector phase at 72 h; however, PD-1 and CTLA-4 positivity was more drastically decreased on the effector Th1 cells at early time points (Figure 4b). These data indicate that, when applied at an early phase of effector Th1 responses, CTLA-4 and PD-1 blockade hinders the upregulation of multiple inhibitory receptors, hence interferes with the development of exhaustion. Alternatively, when applied at a late period of effector responses, the blockade can maintain the Th1 functions through downregulation of CTLA-4, PD-1, LAG3 and TIM-3.

In order to monitor the impact of receptor downregulation, endocytic trafficking was inhibited in the Th cells obtained from 96-h co-cultures and the anti-PD-1 or anti-CTLA-4 mAbs were applied. The reciprocal downregulation of PD-1 and CTLA-4 molecules upon the treatment with anti-CTLA-4 mAb and anti-PD-1 mAb, respectively, was related to an internalization process (Figure 4c). Moreover, upon anti-PD-1 checkpoint blockade, PD-1 and CTLA-4 receptors could be detected in same endocytic vesicles in the cytoplasm of Th1 cells (Figure 4d).

**DISCUSSION**

Effector capacities of exhausted T cells can be restored by the blockade of negative signals from inhibitory receptors...
such as PD-1 and CTLA-4 [18]. Even though the exhausted CD8+ T cells have been regarded as preferential targets for ICI immunotherapy, inadequate help from CD4+ T cells is another challenge to overcome the immune suppression in cancer [27]. The functional impairment in Th1 cells disrupts the orchestration of anti-viral and anti-tumour immunity at many levels such as antigen presentation, production of high-affinity antibodies and cytotoxicity [25, 28]. CD4+ T cells are also prone to exhaustion; however, there is no consensus for defining the phenotypic hyporesponsiveness in various Th subsets and the influence of Th exhaustion on immune responses in different pathologies and in ICI immunotherapy needs to be better elucidated [23–25]. This study focused on the Th1 subset which provides a major support for CD8+ T-cell-mediated responses. Here, CD4+ T cells were induced into effector Th1 subtype, and then they were driven into a hyporesponsive state, ex vivo. This setting enabled a clear monitoring of functional and transcriptomic changes in distinct time frames of CD4+ T-cell stimulation. Since the inhibitory receptors were highly upregulated on effector Th1 cells and on exhausted Th1 cells, next, the ICI was applied for different durations at the critical time frames determined. Collectively, our results validated the functional and transcriptomic changes in Th1 cells, identified an effector-to-exhaustion transition phase in Th1 cells and demonstrated modulation of ICI therapy according to the functional and transcriptomic status of T cells (Figure 5).

In a previous study, we demonstrated the impact of continuous and potent co-stimulatory signals on the induction of Th1 exhaustion [22]. Classically, IL-12 has been regarded as the essential mediator for Th1 differentiation. On the other hand, IL-12-independent generation of Th1 subtype can be achieved through strong co-stimulatory signals or exposure to high levels of IL-2 [26, 29, 30]. Excessive co-stimulation leads to progressive loss of effector functions, elevated expression of multiple (PD-1, CTLA-4, TIM-3 and LAG3) inhibitory receptors and reduced production of IFN-γ, TNF-α and IL-2 cytokines in Th1 cells [31]. The effector Th1 cells generated ex vivo, which were identified through transcriptomics and functional assets upon 72 h of stimulation, displayed significant activity in the pathways related to plethora of cellular processes, genetic information processing and metabolism. On the late time-frames of Th1 stimulation (at 120 and 144 h) the transcriptional program on genetic information processing and metabolism was negatively altered; however, the pathways related to environmental information processing, cellular processes and immunity were in a transcriptionally active state. According to this transcriptomic signature, the hyporesponsive Th1 cells obtained from the late time-frames are compatible with the precursor exhausted T cells that retain the capacity to respond to PD-1 and/or CTLA-4 inhibition [27, 28].

Intriguingly, Th1 cells in a specific frame of time (96 h in our setting) were identified to carry the biological assets of both exhausted and effector Th1 cells. Even though a reduction in proliferation and cytokine secretion was observed, these cells were significantly more responsive than the Th1 cells obtained after 120 h of stimulation. The transcriptomics data from 96-h Th1 cells, which were mixed of both responsive and hyporesponsive signatures, indicated a transition phase from effector to exhausted state. Albeit being open to extracellular signals through the pathways of environmental information processing, the Th1 cells in this transition phase displayed a drastic decrease in genetic information

**Figure 3** The amount of IFN-γ, TNF-α and IL-2 secreted by Th1 cells upon blockade of CTLA-4 and PD-1 inhibitor receptors. Anti-PD-1 and/or anti-CTLA-4 antibodies were refreshed daily together with anti-CD3 mAb and culture media. The amount of cytokines was assayed by flow cytometric bead array from the supernatants collected at the 120 h of co-culturing. The co-cultures established with isotype IgG antibody served as controls (n = 6–10 biologically independent samples for each condition). Data are presented as mean ± SEM, colour-coded lines and asterisks indicate significance in two-tailed Student t-test (*p < 0.05; **p < 0.01).
FIGURE 4 Impact of PD-1 and CTLA-4 checkpoint blockade on inhibitory receptor expression in Th1 cells accumulated at distinct functional phases. (a) Percentage of Th1 cells bearing PD-1, CTLA-4, TIM-3 and LAG3 inhibitory receptors after periodic blockades ending at the 120th hour of co-culturing \( (n = 6 \) biologically independent samples for each condition). (b) The decreasing kinetics of inhibitory receptor expression on Th1 cells upon incubation with anti-PD-1 and/or anti-CTLA-4 antibodies \( (n = 6 \) biologically independent samples for each condition). The arrow indicates the time point \( (24 \) h) that the blockade was initiated. Anti-PD-1 and/or anti-CTLA-4 antibodies were refreshed daily together with anti-CD3 mAb and culture media. The percentage of T cells expressing inhibitory receptors from the control co-cultures without additional antibodies (grey line) and the co-cultures established with isotype control antibody (IgG, brown), CTLA-4 blockade (blue), PD-1 blockade (magenta), CTLA-4 and PD-1 co-blockade (black) are shown. Data are presented as mean \( \pm \) SEM, colour-coded lines and asterisks indicate significance in two-tailed Student \( t \)-test \( (* p < 0.05; * * p < 0.01) \). (c) Prior to the incubation with anti-PD-1 mAb or anti-CTLA-4 mAb, or isotype-matched control mAb at 96 h of co-culturing, the cells were treated with monensin endocytosis inhibitor. Representative flow cytometry histograms for PD-1 and CTLA-4 expression on Th cells at 120 h are demonstrated. (d) Visualization of PD-1 and CTLA-4 inhibitory receptors distribution in Th1 cells exposed to anti-PD-1 mAb at 96 h of co-culturing for 24 h. Representative immunofluorescence images are shown. White arrows indicate the co-localization of CTLA-4 and PD-1 receptors in the cytoplasmic compartment upon PD-1 checkpoint blockade. Histograms (c) and images (d) are representative of two independent experiments with similar results.
processing pathways. Moreover, the genes associated with chromatin remodelling, which potentially establish an epigenetic landscape for exhausted T cells [32, 33], were not upregulated. Therefore, this specific stage of T cell responses may create a therapeutic window for invigorating immune reactions preceding the exhaustion. Either the long-term blockade starting from an early effector phase or the short-term blockade starting from a late effector stage (the transition phase) was successful at restoring the secretion of TNF-α and IFN-γ. Nevertheless, IL-2 secretion was especially augmented when the blocking antibodies were applied at the transition stage. IL-2 expression is highly susceptible to the inhibitory signals mediated by PD-1 and CTLA-4 [34]. Moreover, IL-2 secretion is an early and transient event that peaks subsequent to Th cell activation and diminishes in the effector phases [22]. Therefore, in our study, the restoration of IL-2 secretion was regarded as the most informative feature indicating the functional restoration of Th1 responses.

ICI therapies mainly affect the hyporesponsive but reprogrammable T cells and aim to prevent functional dysregulation in newly activated T cells [11]. The CTLA-4 and/or PD-1 checkpoint blockade was also effective at downregulating LAG3 and TIM-3 inhibitory receptors. Either applied for a short period starting at the transition phase that corresponds to the initiation of hyporesponsive phase or applied at an early activation stage for a long duration, the PD-1 and CTLA-4 blockade efficiently reduced the expression of multiple inhibitory receptors. Therefore, CTLA-4 and PD-1 blockade can hinder the upregulation of multiple inhibitory receptors, hence limiting the negative signals interfering with the effector Th1 responses. Alternatively, CTLA-4 and/or PD-1 blockade can also directly lead to the downregulation of inhibitory receptors potentially through endocytic vesicle trafficking. The downregulation of TIM-3 and LAG3 together with PD-1 and CTLA-4 can be due to three major reasons: (i) the blockade might alter the T-cell activation program and reduce the expression of other inhibitory receptors; (ii) the alterations in T-cell effector responses may increase the degradation of inhibitory receptor proteins; and (iii) LAG3 and TIM-3 might be juxtaposed with PD-1 or CTLA-4 molecules and become prone to the endocytosis which might be directly induced by antibody binding [35, 36]. Nevertheless, further studies are required to better characterize the mechanisms underlying the reduction of multiple inhibitory receptors upon treatment with checkpoint blockade therapeutics.

Collectively, the prolonged targeting of PD-1 and CTLA-4 receptors on the effector Th cells can diminish the acquisition of a hyporesponsive state while a short-term blockade was found to be efficient for functional recovery of the hyporesponsive Th1 cells. Moreover, in our co-culture setting, the blockade of PD-1 or CTLA-4 alone resulted in simultaneous downregulation of multiple inhibitory receptors. In conclusion, the functional dynamics of Th1 cells must be considered as a pivotal parameter for the efficacy of checkpoint blockade immunotherapy.

AUTHOR CONTRIBUTIONS
Gunes Esendagli and Utku Horzum conceptualized the project and designed the experiments; interpreted data;
and wrote the manuscript. Utku Horzum and Hamdullah Yanik performed experiments. Utku Horzum performed bioinformatics analysis of RNA-seq data and contributed to the preparation of the figures. Ekim Z. Taskiran performed assays related to transcriptomics. All authors reviewed and approved the manuscript.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT
This study was approved by Hacettepe University and University of Health Sciences local ethics committees and conducted in agreement with the guiding principles of the Declaration of Helsinki and the good clinical practice. Informed consent was obtained from the participants and nurses who contributed to the study for providing blood samples.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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