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IFN-α Directly Promotes Programmed Cell Death-1 Transcription and Limits the Duration of T Cell-Mediated Immunity

Seigo Terawaki,*,1,2 Shunsuke Chikuma,*,1 Shiro Shibayama,†,1 Tamon Hayashi,†
Takao Yoshida,‡ Taku Okazaki,‡ and Tasuku Honjo*

Programmed cell death-1 (PD-1) is an inhibitory coreceptor for T lymphocytes that provides feedback inhibition of T cell activation. Although PD-1’s expression on T cells is known to be activation dependent, the factors that determine the timing, intensity, and duration of PD-1 expression in immune reactions are not fully understood. To address this question, we performed a fine mapping analysis of a conserved 5′-flanking region of the PD-1 gene and identified a putative IFN stimulation response element, which was responsible for PD-1 transcription in the 2B4.11 T cell line. Consistent with this finding, activation by IFN-α enhanced both the induction and maintenance of PD-1 expression on TCR-engaged primary mouse T cells through an association IFN-responsive factor 9 (IRF9) to the IFN stimulation response element. Furthermore, PD-1 expression on Ag-specific CD8+ T cells was augmented by IFN-α in vivo. We propose that strong innate inflammatory responses promote primary T cell activation and their differentiation into effector cells, but also cause an attenuated T cell response in sustained immune reactions, at least partially mediated by IFN-α.

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*Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Yoshida-Konoe, Sakyo-Ku, Kyoto, 606-8501 Japan; †Exploratory Research Laboratories, Tsukuba Research Institute, Ono Pharmaceutical Company, Tsukuba, Ibaraki, 300-4247 Japan; and ‡Division of Immune Regulation, Institute for Genome Research, University of Tokushima, Karamoto, Tokushima, 770-8503 Japan

E-mail address: honjo@mfour.med.kyoto-u.ac.jp

Abbreviations used in this article: IRF9, IFN-responsive factor 9; ISGF, IFN-stimulated gene factor; ISRE, IFN-stimulated regulatory element; PD-1, programmed cell death-1; PD-L1, PD-1 ligand-1; UTR, untranslated region.

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suggesting that PD-1 expression induces and maintains their anergic state by continuously blocking the activation signals.

It is therefore important to examine how PD-1 expression is regulated in activated T cells. However, only a few studies have been carried out on the mechanisms of PD-1 induction. It was reported that, in naïve T cells, TCR-mediated calcium influx initiates PD-1 transcription by activating NFATc1 (NFATs, cytoplasmic, calcineurin-dependent 1), which binds to the 5′-promoter region of the PD-1 gene (18). Others have suggested that common γ-chain–associated cytokines, such as IL-2, IL-7, IL-15, and IL-21 induce PD-1 expression (19). However, the mechanisms for maintaining such a high level of PD-1 expression on chronically activated (“exhausted”) T cells are still not clear because a comprehensive promoter analysis of PD-1 in T cells has not been performed.

Thus, it is likely that other transcriptional mechanisms, in addition to NFATc1 and γ-chain cytokines, contribute to PD-1’s up-regulation and/or maintenance in activated T cells. In this study, we therefore performed an extensive analysis of the PD-1 promoter region using a mouse T cell line. We found that, not only TCR-mediated signaling, but also an IFN-stimulated regulatory element (ISRE), which has been suggested to drive PD-1 transcription in macrophages (20), is responsible for PD-1 expression in T cells. Unlike in macrophages, PD-1 transcription was synergistically regulated by signaling cascades involving TCR activation and type I IFN in activated T cells, providing strong feedback inhibition in T cell-mediated immunity.

Materials and Methods

Mice

All mice were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University or the Tsukuba Research Institute, Ono Pharmaceutical Company, and used under approved protocols. C57BL/6 and BALB/c mice were from Japan SLC (Hamamatsu, Japan) or Charles River Laboratories Japan (Yokohama, Japan). DO11.10 TCR transgenic mice were kindly provided by Dr. Kenneth Murphy (Washington University), maintained by Dr. Yoshio Wakatsuki (Kyoto University), and bred with Rag2−/− mice. CD8+ 2C Tg mice were from Dr. Dennis Loh (Washington University).

Cell culture

2B4.11 cells and primary mouse T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 55 μM 2-ME, 100 U/ml penicillin and 100 μg/ml streptomycin, nonessential amino acids, and 1 M HEPES (pH 7.4) at 37°C in 5% CO2. The murine colon adenocarcinoma MC38 cells were kindly provided by Dr. Jim Allison (Memorial Sloan-Kettering Cancer Center, New York, NY) and were cultured in DMEM (Invitrogen Corporation) with 10% heat-inactivated FBS and 1% anti-biotic-antimycotic (Invitrogen Corporation).

Cytokines and Abs

Recombinant mouse IL-2, IL-6, IL-7, and IFN-γ were purchased from Wako Pure Chemical Industries (Osaka, Japan), IL-15 and IL-21 were from PeproTech (Rocky Hill, NJ), TGF-β was from R&D Systems (Minneapolis, MN), and IFN-α was from Miltenyi Biotec (Bergisch Gladbach, Germany). All cytokines except for IL-2, IL-15, and IFN-α were used at 100 U/ml; IL-2 and IL-15 were used at 25 U/ml. IFN-α was used at 100 and 500 U/ml for murine and MC38 cell culture, respectively. Anti-murine PD-1 Ab (clone 4H2) is a chimeric rat Ab with a murine IgG1 C region, which was provided by Medarex/Bristol-Myers Squibb Company (Milpitas, CA). Control mouse IgG was purchased from Thermo Scientific (Rockford, IL).

Flow cytometric analysis

FITC-conjugated anti-CD25, anti-DO11.10 TCR (KJ 1-26) Abs, PE-conjugated anti-CD3 Abs and its isotype control hamster IgG, APC-conjugated anti-CD4 Abs, APC-Cy7–conjugated anti-CD8 Ab, and Pacific blue-conjugated anti-CD4 Abs were obtained from eBioscience (San Diego, CA). PE-conjugated murine IgG2a, murine H-2Kd Abs were obtained from BD Biosciences (San Jose, CA). The 1B2 anti-2C TCR clone–specific Ab, labeled with Alexa 488 or Alexa 647, was described previously. All cells were first blocked with anti-murine FcR (2.4G2) and then stained at 4°C for 30 min. Samples were analyzed by FACScalibur or FACSCanto II (BD Biosciences).

Histology

Mouse and human genomic information was obtained from the Ensembl Genome Browser (http://www.ensembl.org). The sequences were analyzed by PipMaker (http://pipmaker.bx.psu.edu/pipmaker).

Mouse PD-1 reporter constructions

The DNA fragment containing the upstream region and 5′ untranslated region (UTR) of the B6 mouse PD-1 gene was obtained by PCR amplification using the BAC clone, RP24-223E1, from BACPAC Resources (Children’s Hospital Oakland Research Institute, Oakland, CA) as a template. Fragment truncations were achieved by PCR using restriction enzyme linker-tagged sequence-specific primers. The resultant fragments were cloned into the SacI-Xhol site of the pGL4.10 vector (Promega) for promoter analyses. The virus thymidine kinase promoter in the pRL-TK vector (Promega) was replaced with the mouse cyclophilin B gene promoter, which corresponds to 800 bp of the upstream sequence and the 5′-UTR sequence of the gene. This reporter was used as an internal control for monitoring the transfection efficiency.

Luciferase assay

2B4.11 cells (3 × 10⁴) were introduced with 1 μl of the reporter plasmid and Renilla luciferase control vector by Amaxa Nucleofection (Lonza group, Basel, Switzerland). Half of the transfected cells were stimulated in the wells of a round-bottom 96-well plate, which were precoated with anti-CD3 Ab (2C11 3 μg/ml), in triplicate (5 × 10⁵/well equivalent). The remaining half was cultivated in noncoated wells. Benzyloxycarbonyl-val-val-arg-asp(Ome)-fluoromethylketone (50 μM; Peptide Institute, Minoh, Japan) was added to avoid the massive apoptosis of 2B4.11 cells. After 8 h, the luciferase activity was measured by the Dual-Glo Luciferase assay system (Promega) using an EnVision luminometer (PerkinElmer, Waltham, MA). The PD-1 promoter activity was normalized to the Renilla luciferase activity.

Transcription factor prediction

The prediction of transcription factors was performed using two online databases, TFSERCH (http://inbs.cbrj.jp/research/db/TFSERCHJ.html) and Transcription Element Searching System (http://www.cbl.iupem.edu/cgi-bin/ess/estess).

Gel shift assay

cDNA oligos (biontin-5′-CTA TTA TGA GGT TCC TTG TTT TTG TTG TTG-3′) and biotin-5′-AAA AAG GAA GAC G AA ACT GCT AAT ATT AG-3′ for the probe; 5′-CAG TTT CCT GGT TCC-3′ and 5′-GGG AAA ACG AAA CTG-3′ for the competitor) were mixed at equal concentrations, denatured at 95°C, and then annealed by gradually decreasing the temperature, to prepare the probe and its competitor. The nuclear proteins of stimulated 2B4.11 cells were extracted with the NE-PER nuclear extraction reagent (Pierce, Rockford, IL) in the presence of protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan) and phosphate inhibitor mixture (Santa Cruz Biotechnology, Santa Cruz, CA). Then 20 fmol biotinylated probe was mixed with 4 μg nuclear extracts prepared from 2B4.11 cells, and the mixture was incubated for 20 min at room temperature in the presence or absence of 4 pmol unlabeled competitor. After the probes were resolved on a 6% acrylamide gel in 0.5 × Tris-borate-EDTA buffer and then transferred to a nylon membrane, the transferred probes were cross-linked by UV irradiation at 120 mJ/cm². The probe migrations were visualized by the Chemiluminescent Nucleic Acid Detection Module (Pierce).

In vitro T cell assay

In vitro T cell assay

T cells were isolated from the splenocytes of DO11.10 TCR/Rag2−/− mice by a mouse T cell enrichment column (R&D Systems) after erythrocyte lysis at about 90% purity, and were cocultured with mitomycin C-treated BALB/c spleenocytes in the presence of OVA peptide (329–337) for 72 h. After priming, the T cells were extensively washed to interrupt Ag presentation by removing excess peptide and by disrupting APC–T cell interactions. The primed T cells were further cultured in the presence or absence of cytokines or inhibitors.
Quantitative PCR

Total RNAs from cytokine-treated mouse T cells were extracted by NucleoSpin RNA (Macherey-Nagel, Düren, Germany). One microgram of the total RNAs was then subjected to reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The PD-1 transcripts were quantified by 7900HT using the TaqMan Gene expression assay system (Applied Biosystems). PD-1 expression was normalized to the mouse cyclophilin expression.

In vivo T cell assay

CD8+ T cells from 2C TCR Tg mice were purified by AUTO MACS (Miltenyi Biotech). C57BL/6 recipients were i.p. injected with 2 × 10^5 cells and s.c. immunized with the CFA-emulsified cognate peptide (SIYRYYGL; 10 μg) together with 100 μg L-Aβ3-restricted human hepatitis B virus core Ag-derived peptide (TPPAYRPPNAPIL) on the same day. Eight days after the immunization, the mice were i.p. injected with rIFN-α (50,000 units), and they were analyzed the next day.

Antitumor efficacy studies

Nine-week-old C57BL/6 mice were treated with s.c. implants of 2 × 10^5 MC38 in the right flank on day 0. The mice then received 4 × 10^6 units IFN-α injected s.c. daily from days 1–13. Abs were given as i.p. injections of 6 or 20 mg/kg on days 0 and 3, or of 10 mg/kg three times with a 6-d interval. Tumor growth and survival were monitored during the experiments. The tumor volume was evaluated by measuring the long diameter, r(l), and short diameter, r(s), with a caliper, and calculated by the ellipsoid volume formula: v = (r(l) × r(s)^2) × 0.5. The mice were sacrificed when the tumor volume exceeded the predetermined size of 1500 mm^3 or when they developed ulcerating wounds.

Results

The 2B4.11 T cell line expresses PD-1 by TCR-dependent and -independent mechanisms

To establish a system suitable for PD-1 promoter analysis, we first examined several mouse-derived T cell lines for their PD-1 expression, including DO11.10, 3A9, WR19, and 2B4.11. Among them, we found that 2B4.11 cells, from which PD-1 cDNA was originally isolated (2), constitutively expressed PD-1 protein on their surface (Fig. 1). Although nonstimulated 2B4.11 cells possessed endogenous PD-1, TCR stimulation with an anti-CD3 Ab or PMA/ionomycin treatment further augmented the PD-1 expression (Fig. 1). No increase in PD-1 expression was observed in the other cell lines tested (data not shown). These data indicated two advantages of using these cells to clarify the expression mechanisms for PD-1: first, the 2B4.11 cell line has intact molecular machineries that can drive PD-1 expression, and second, the basal and inducible expression of PD-1 on 2B4.11 cells is useful for identifying TCR-dependent and -independent pathway(s) that regulate PD-1 expression. Thus, we used the 2B4.11 cell line for the following promoter analyses.

Establishment of a PD-1 luciferase assay using the 2B4.11 cell line

A comparison of human and mouse PD-1 genes by computational analysis revealed that, in addition to the exons, the 5’-flanking sequence up to ~3000 bp was significantly conserved between human and mouse (Fig. 2A, arrowheads). To determine the cis-regulatory elements in the 5’ region of the mouse Pdcd1 gene that are responsible for PD-1 transcription, we established a luciferase-reporter assay in the 2B4.11 T cells. Initially, we made a basic reporter construct that included the 3000-bp upstream sequence of the PD-1 gene and the 5’-UTR sequence (+1 to +63 bp), which was fused to the firefly-luciferase gene (Fig. 2B, top). Then we made a series of truncated constructs from the basal reporter (Fig. 2B). TCR engagement by a plate-coated anti-CD3 Ab caused a significant induction of luciferase activity in 2B4.11 T cells that were transfected with the basic reporter construct. A truncation of the promoter region from −1500 to −1000 bp dramatically reduced the TCR-induced luciferase activity (Fig. 2C). Interestingly, the same truncation also resulted in a small but significant loss of the luciferase activity in nonstimulated cells (Fig. 2C, dotted line). Because 2B4.11 cells can express PD-1 with or without the TCR signal, these data suggested that the DNA sequence between −1500 and −1000 bp contained cis-regulatory elements that are responsible for the PD-1 transcription in both TCR-stimulated and nonstimulated 2B4.11 T cells.

To dissect this 500-bp region, we made more reporter constructs with deletions of 100 bp each between the −1500 and −1000 bp positions. As shown in Fig. 2D, truncation of the sequence from −1200 to −1000 bp resulted in the loss of both the TCR-dependent and -independent luciferase activities. Further deletion analysis of the −1200 to −1000 bp sequence (Fig. 2E) suggested that the regions from −1120 to −1100 bp and from −1080 to −1040 bp may contain cis-regulatory elements (shown as regions 1 and 2 in Fig. 2E, respectively). Ligation of the 100-bp fragment corresponding to the −1100 to −1000 bp sequence to the luciferase gene augmented the luciferase activity, but the fragment corresponding to −1200 to −1100 bp did not (Fig. 2F), suggesting that the sequence between −1080 and −1040 bp (region 2) contained active cis elements.

Identification of an ISRE as a cis element for PD-1 induction in 2B4.11 T cells

A computer analysis using online database tools (TFsearch and TESS) predicted putative acute myeloid leukemia-1, ISRE, and C/EBPα binding sites within the −1080 to −1040 bp sequence (Fig. 3A). Among them, we found the ISRE was one of the most well-conserved motifs among different species, including human, rat, mouse, gorilla, among others (data not shown). Furthermore, we found that the introduction of two base mutations (TT to GG) into the 15-bp ISRE consensus sequence in the PD-1 reporter (−1500 bp to the 5’ UTR) mostly abolished the reporter activity (Fig. 3B), demonstrating that the ISRE is the critical cis-acting element for PD-1 expression in 2B4.11 T cells. To examine whether the identified ISRE-like sequence functions in response to TCR-mediated signaling in 2B4.11 T cells, we performed an electromobility gel-shift assay using a synthesized dsDNA oligo of the ISRE (5’-CTA ATATA GCA GTT TCG TTT TCC TTT TT-3’). As shown in Fig. 3C, nuclear extracts prepared from nonstimulated 2B4.11 cells contained several binding proteins for the ISRE oligo. Notably, the nuclear extracts from 2B4.11 cells stimulated for 30 min

![FIGURE 1. Constitutive and inducible PD-1 expression on 2B4.11 T cells. 2B4.11 cells were left untreated (dashed line), or stimulated with PMA/ionomycin (upper panel) or with plate-coated 2C11 (lower panel) for 12 (thin line), 24 (thick line), or 36 h (extra thick line). PD-1 expression was determined by FACS. Shaded histograms represent nonstained controls.](http://www.jimmunol.org/DownloadedFrom/10.4049/jimmunol.2774191-fig1)
reproducibly generated an additional band with a different electric mobility (Fig. 3C, left side). This TCR-signal–dependent association between an NF and the ISRE motif was completely abolished in a competition assay with dsDNA oligonucleotides of the ISRE consensus sequence (Fig. 3A, right side). These data together suggested that the ISRE was responsible for the PD-1 expression in TCR-triggered 2B4.11 cells.

**Enhancement of PD-1 expression by IFN-α in vitro**

ISRE is well-known as a binding motif for IFN-stimulated gene factor 3 (ISGF3), a type I IFN receptor-activated trimolecular complex of the STAT1, STAT2, and cytosolic IFN-responsive factor 9 (IRF9) proteins. Given the importance of the ISRE element in the 2B4.11 T cells, which mimic quasi-activated T cells, we hypothesized that type I IFN-mediated signaling influences PD-1 expression on activated primary T cells through the activation of the ISRE element. Thus, we examined the stimulatory effect of rIFN-α on PD-1 expression in TCR-stimulated primary mouse T cells. As shown in Fig. 4A, the addition of exogenous IFN-α at the time of initial TCR stimulation significantly enhanced the TCR-driven PD-1 expression on DO11.10 TCR transgenic T cells without affecting the upregulation of CD25, another activation marker. However, the addition of IFN-α alone did not induce PD-1 expression (Fig. 4A, bottom panel).

Therefore, we next examined whether adding IFN-α after the initial TCR-mediated induction of PD-1 would affect the duration...
of its expression. T cells were initially activated through TCR and then maintained in the presence or absence of the survival factor IL-2, with or without IFN-α. The addition of exogenous IFN-α 3 d after TCR activation sustained the PD-1 on activated T cells until at least day 7, when the PD-1 expression had returned to almost the level in naive T cells (Fig. 4B). Unexpectedly, the addition of IL-2, which is reported to drive PD-1 expression on human T cells (19), did not clearly enhance the PD-1 level on mouse T cells. It is likely that γ-chain cytokines have different effects on PD-1 expression in humans versus mice. In agreement with the protein expression, IFN-α treatment on day 3 significantly increased the PD-1 transcripts on day 4 (Fig. 4C) and maintained this increased level up to day 7 (data not shown). This enhancement was not observed using the other cytokines we tested (IL-2, IL-6, IL-15, etc.) in the same system. These data strongly supported the idea that IFN-α could trigger PD-1 gene transcription in activated primary T cells.

**IFN-α stimulates direct ISGF binding to the ISRE in the PD-1 promoter**

Among the three components of type I IFN-induced ISGF3 (STAT1, STAT2, and cytosolic IRF9), IRF9 contains the DNA-binding motif for ISRE (21). To demonstrate that IFN-α signaling induces activation of the ISRE in the PD-1 promoter, we performed a chromatin-immunoprecipitation assay using TCR-stimulated primary mouse T cells. Purified T cells were activated with plate-bound anti-CD3 and anti-CD28 Abs with or without recombinant IFN-α for 20 h. The chromatin precipitated by anti–IRF-9 was enriched in the PD-1 promoter of IFN-α-treated but not nontreated, T cells, suggesting that the ISGF3 complex bound directly to the PD-1 promoter in activated primary T cells (Fig. 4D).

Interestingly, treatment of ex vivo naive T cells with IFN-α alone did not cause IRF9 to bind the PD-1 promoter (data not shown), suggesting that TCR and IFN-α are both required to activate the ISRE in the PD-1 promoter. This is consistent with the absence of PD-1 induction with IFN-α treatment alone. In a preliminary study, we noted that the initial PD-1 induction on naive T cells was inhibited by the addition of FK506, a calcineurin inhibitor, supporting a previous finding (18) that the TCR-mediated calcium-calcineurin-NFAT cascade is required for PD-1 induction (data not shown). It is possible that the activation of naive T cells via TCR signaling is necessary for PD-1 induction, and that IFN-α modifies its duration secondarily. In any case, taken together, our data suggest that the ISRE contributes to the augmentation of PD-1 expression on both the 2B4.11 T cell line and primary T cells.

**IFN-α enhances the PD-1 expression on Ag-stimulated T cells in vivo**

Physiologically, IFN-α is produced by plasmacytoid dendritic cells and provides early protection against infections (22). IFN-α also directly contributes to the differentiation of CD8+ T cells into CTLs (23) or memory T cells (24). Given the contribution of IFN-α signaling to PD-1 transcription in vitro, we next examined whether it promoted the PD-1 expression on Ag-specific T cells in vivo. We adoptively transferred CD8+ T cells from MHC class I-restricted 2C TCR Tg mice (2C T cells) into C57BL/6 recipients and challenged the mice with a cognate peptide, SIYRYYGL, and assayed the expansion of IFN-α-stimulated T cells by IFN-α treatment compared with the level in T cells.
shown as a fold increase (mean 

tative PCR and normalized to the cyclophilin B transcript. The data are 

munoprecipitation (IP) was performed as indicated. The data are repre-

indicated for the following 4 d. The PD-1 expression on KJ26-1+ cells was 

examined by FACS.

A

B

C

D

FIGURE 4. IFN-α stimulates and prolongs the PD-1 expression on mouse primary T cells. A. IFN-α enhances the PD-1 expression on activated mouse T cells. CD4⁺ T cells isolated from DO11.10/Rag2 knockout mice were primed with different concentrations of OVA peptide with BALB/c splenocytes for 72 h in the presence (bold line) or absence (dashed line) of rIFN-α. PD-1 and CD25 were examined by FACS. B. T cells from DO11.10/Rag2 knockout mice were preactivated with OVA-pulsed splenocytes for 3 d and then cultured as indicated for the following 4 d. The PD-1 expression on KJ26-1⁺ cells was examined. C. The mRNA expression from B was determined by quantitative PCR and normalized to the cyclophilin B transcript. The data are shown as a fold increase (mean ± SD) over the control sample, which was treated only with IL-2. D. Purified primary mouse T cells were stimulated in the presence or absence of exogenous IFN-α for 20 h. Chromatin immunoprecipitation (IP) was performed as indicated. The data are repre-

representative of more than two independent experiments.

PBS-treated mice (Fig. 5A, 5B). These data confirmed that IFN-α increased the PD-1 expression on Ag-stimulated T cells in vivo.

IFN-α induces the expression of PD-1 on tumor-infiltrating T cells and PD-L1 on tumors

IFN-α has been considered for use in cancer immunotherapy for a long time because of its potential as a strong immunostimulant (25). In fact, IFN-α is widely used to treat metastatic renal cell carcinoma, but it has limited efficacy (26, 27). Based on our current findings, we propose that the use of IFN-α in cancer therapy might result in the attenuation of T cell-mediated attack, by inducing inhibitory PD-1 on T cells that could subsequently bind to PD-L1 expressed on tumor cells. To test this hypothesis, we first examined the effect of IFN-α on the tumor cells. IFN-α treatment enhanced the expression of MHC class I molecules on the cultured MC38, a murine colon carcinoma cell line, suggesting that the T cell-mediated recognition and killing of tumor cells can be facilitated (Fig. 6A, left and center). However, IFN-α–treated MC38 also showed a marked induction of PD-L1 expression, probably via similar pathways as IFN-γ, a known strong inducer of PD-L1 (4) (Fig. 6A, right). The data suggest that IFN-α treatment on MC38 cells can facilitate both tumor killing by and their evasion from the tumor-reactive T cells, by enhancing recognition of tumor Ags and via PD-L1–mediated ligation of PD-1, re-

spectively.

We s.c. transplanted MC38 cells into the flank of C57BL/6 mice and then treated the recipients with a daily injection of IFN-α. Importantly, this treatment induced strong PD-1 expression in the tumor-infiltrating T cells. As shown in Fig. 6B, the frequency of PD-1⁺ cells among the tumor-infiltrating CD8 T cells (Fig. 6B, left panel) and the strength of PD-1 expression (Fig. 6B, right panel) were enhanced in IFN-α–treated mice compared with control mice, whereas the number of CD8 T cells per tumor weight was decreased (Fig. 6C). A previous report suggested that the PD-1– PD-L1 interaction attenuates tumor-specific cytotoxic T cells during the effector phase (28). Our data strongly support the idea that IFN-α attenuates the T cell response against tumors by in-

creasing the opportunity for interactions between the PD-1 on tumor-infiltrating T cells and PD-L1 on tumor cells.

PD-1 blockade boosts the IFN-α–mediated antitumor effects

If the earlier hypothesis is correct, inhibiting the interaction be-

 tween PD-1 and PD-L1 during IFN-α therapy should overcome the potential detrimental effects and provide an effective cancer immu

notherapy. The injection of IFN-α alone until day 13 apparently attenuated tumor growth compared with untreated control mice (Fig. 7A). However, in accordance with the augmented PD-1 and PD-L1 induction, no mouse receiving IFN-α alone showed tumor rejection. In contrast, PD-1 blockade by the administration of a PD-1 blocking mAb, 4H2 alone, also resulted in an efficient suppression of tumor growth, with some mice completely rejec-

FIGURE 5. IFN-α stimulates PD-1 expression on Ag-specific T cells in vivo. A. CD8⁺ T cells from 2C TCR Tg mice were adoptively transferred into C57BL/6 mice and challenged with CFA-conjugated SIY peptide and helper peptide. Eight days later, the mice were injected with rIFN-α. The PD-1 expression on clonotype-positive cells was determined by FACS on day 9. B. A representative mean fluorescence intensity (MFI) value from three independent experiments is shown.
FIGURE 6. IFN-α treatment augments the expression of PD-1 and PD-L1 in antitumor immunity. A, MC38 cells were cultured with 500 U/ml IFN-α for 24 h; then PD-L1, H2-Dk, or H2-Kk was examined by FACS analysis. B and C, C57BL/6 mice received s.c. implants of murine colon adenocarcinoma MC38 on day 0. The mice then received a daily s.c. injection of PBS or IFN-α from days 1–11. The tumor-infiltrating lymphocytes were harvested on day 11 and stained with anti-CD8 and anti–PD-1 Abs. One representative result from two independent experiments is shown.

FIGURE 7. IFN-α and PD-1 blockade have a synergic antitumor effect. Mice received transplants of MC38 cells as in Fig. 6 and were given daily IFN-α from days 1–13. Anti–PD-1 Abs were i.p. injected on days 0, 6, 12, and 18. The mice were sacrificed when the tumor volume exceeded the predetermined size of 1500 mm³ or when they developed ulcerating wounds. This experiment was performed with 15 mice per group, and similar results were obtained in 6 independent experiments. A. Shown is the median per cohort for a representative experiment. B, The individual tumor volumes on day 28 are shown. Significant difference (∗p < 0.05, **p < 0.01) by Steel’s test. Number of individuals who had tumor volume lower than 100 mm³ (under dashed line) were IFN-α alone, 0; 4H2 3 mg/kg, 6; 4H2 10 mg/kg, 6; IFN-α+4H2 3 mg/kg, 5; and IFN-α+4H2 10 mg/kg, 7.

Discussion

In this study, we identified an ISRE within the Pdcd1 promoter and demonstrated that this motif was critical for PD-1 transcription in the 2B4.11 mouse T cell line. The same element was previously reported to be responsible for PD-1 transcription in IFN-α–stimulated mouse macrophages, but not in T cell lines (20). We unexpectedly found that TCR-dependent and -independent PD-1 transcription in 2B4.11 T cells required the ISRE. Thus, we examined the effects of type I IFN on PD-1 expression. Exogenous IFN-α caused IRF9 binding to the PD-1 promoter in T cells and enhanced the PD-1 expression on DO11.10 TCR Tg cells on their activation with peptide-loaded APCs. Once the T cells were activated, the late addition of IFN-α prolonged PD-1 transcription, even after the removal of TCR-mediated signals. Furthermore, the administration of IFN-α during the Ag-specific CD8+ T cell response augmented the PD-1 expression during Ag challenge. These results suggested that the ISRE functions cooperatively with TCR-mediated signaling to increase the intensity and duration of PD-1 expression.

Type I IFNs provide an early defense against infections. IFN-α has a direct effect on infected cells to attenuate viral replication and enhances class I molecules to guide CTL-mediated killing (25). IFN-α is also reported to activate CD8 T cells directly, providing a “third signal” that promotes their differentiation into full effector CTLs (23). Hence the idea of using IFN-α for cancer treatment is reasonable, both to elicit direct cancer cell attenuation...
and as an adjuvant for CTL induction. However, the current use of IFN-α therapy is restricted to only certain types of cancer, for example, metastatic renal cell carcinoma, and its overall effectiveness is limited (26, 27). This is, in part, because IFN-α suppresses lymphocyte proliferation, probably by inducing a set of genes that inhibit cellular activities (29, 30).

Our current data suggest that the attenuation of cancer-specific T cells during IFN-α treatment can be mediated by augmentation of the PD-1 and PD-L1 pathway. The PD-1 blockade, together with IFN-α, clearly improved the antitumor effect. Combined cancer immunotherapies using IFN-α with other chemotherapeutic agents are in clinical trials. However, most chemotherapeutic agents are immune-suppressive, which will probably interfere with the IFN-α–mediated antitumor activity. Our previous findings suggested that the PD-1/PD-L1 blockade shows antitumor activity in several mouse models (14, 15), and the efficacy of a fully human anti–PD-1 Ab in a clinical trial has been reported (31).


