Journal of Autoimmunity 103 (2019) 102285

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Analysis of the PD-1/PD-L1 axis in human autoimmune thyroid disease: Insights into pathogenesis and clues to immunotherapy associated thyroid autoimmunity

Daniel Álvarez-Sierra[a,b,d], Ana Marín-Sánchez[a,b], Paloma Ruiz-Blázquez[b,d], Carmen de Jesús Gil[b,d], Carmela Iglesias-Felip[b,e,f], Óscar González[b,g], Anna Casteras[h], Roser Ferrer Costa[i], Paolo Nuciforo[c], Roger Colobran[a,b,d], Ricardo Pujol-Borrell[a,b,d,*]

[a] Immunology Division Hospital Universitari Vall d'Hebron, 119-129 Passeig Vall d'Hebron, 08035, Barcelona, Spain
[b] Vall d'Hebron Institute of Research (VHIR), 119-129 Passeig Vall d'Hebron, 08035, Barcelona, Spain
[c] Vall d'Hebron Institute of Oncology (VHIO), 115-117 Calle Nazaret, 08035, Barcelona, Spain
[d] Department of Cell Biology, Physiology and Immunology, Autonomous University of Barcelona, 08193, Bellaterra (Barcelona), Spain
[e] Department of Pathology, Autonomous University of Barcelona, 08193, Bellaterra (Barcelona), Barcelona, Spain
[f] Histopathology Division, Hospital Universitari Vall d'Hebron, 119-129 Passeig Vall d'Hebron, 08035, Barcelona, Spain
[g] Surgery Department, Hospital Universitari Vall d'Hebron, 119-129 Passeig Vall d'Hebron, 08035, Barcelona, Spain
[h] Endocrinology Division, Hospital Universitari Vall d'Hebron, 119-129 Passeig Vall d'Hebron, 08035, Barcelona, Spain
[i] Clinical Biochemestry Division, Hospital Universitari Vall d'Hebron, 119-129 Passeig Vall d'Hebron, 08035, Barcelona, Spain

ARTICLE INFO

Keywords:
Graves' disease
T-Lymphocytes
Immune checkpoints
Autoimmunity
Tolerances
PD-1
PD-L1
PD-L2
IFNγ

ABSTRACT

Autoimmune thyroid diseases (AITDs), i.e., Graves' disease (GD) and Hashimoto thyroiditis (HT), are the most prevalent organ-specific autoimmune diseases, but their pathogenesis is still incompletely understood. The PD-1/PD-L1 pathway is an important mechanism of peripheral tolerance that has not been investigated in AITDs. Here, we report the analysis of the expression of PD-1, PD-L1 and PD-L2 in PBMCs, infiltrating thyroid lymphocytes (ITLs) and in thyroid follicular cells (TFCs) in GD, HT and multinodular goiter (MNG) patients and healthy controls PBMCs (HC). By combining flow cytometry, tissue immunofluorescence and induction experiments on primary and thyroid cell line cultures, we show that: 1) while PD-1 + T cells are moderately expanded in PBMCs from GD vs HC, approximately half of T cells in the infiltrate are PD-1 + including some PD-1^{hi}; 2) PD-L1, but not PD-L2, is expressed by 81% of GD glands and in 25% of non-autoimmune glands; 3) PD-L1, was expressed by TFCs in areas that also contain abundant PD-1 positive T cells but; 4) co-localization in TFCs indicated only partial overlap between the smaller areas of the PD-L1 + and the larger areas of HLA class II + expression; 5) IFNγ is capable of inducing PD-L1 in > 90% of TFCs in primary cultures and cell lines. Collectively these results indicate that the PD-1/PD-L1 axis is operative in AITD glands and may restrain the autoimmune response. Yet the discrepancy between easy induction *in vitro* and the limited expression *in vivo* (compared to HLA) suggests that PD-L1 expression *in vivo* is partially inhibited in GD and HT glands. In conclusions 1) the PD-1/PD-L1 pathway is activated in AITD glands but probably not to the extent to inhibit disease progression and 2) Thyroid autoimmunity arising after PD-1/PD-L1 blocking therapies in cancer patients may result from interfering PD-1/PD-L1 tolerance mechanism in thyroid with minimal (focal) thyroiditis. Finally acting on the PD-1/PD-L1 pathway could be a new approach to treat AITD and other organ-specific autoimmunity in the future.

1. Introduction

Autoimmune thyroid diseases (AITDs) are the most prevalent organ-specific autoimmune diseases, with incidences that can reach 50/100.000 per year in the female population [1,2]. The etiology of autoimmune organ-specific autoimmune diseases is attributed to a number of well-established genetic factors and to more controversial and less well-defined environmental factors, and their pathogenesis is still incompletely understood [2–4]. According to the multiple checkpoint hypothesis of autoimmunity [5,6], immunological events in the

* Corresponding author. FOCIS Centre of Excellence, Hosp Univ Vall d'Hebron, Laboratories Building, Spain.
E-mail address: rpujol@vhebron.net (R. Pujol-Borrell).

https://doi.org/10.1016/j.jaut.2019.05.013
Received 26 March 2019; Received in revised form 10 May 2019; Accepted 14 May 2019
Available online 08 June 2019

target organ can trigger and/or maintain autoimmune responses. In the case of the two most common AITDs, i.e., Hashimoto thyroiditis (HT) and Graves' disease (GD), the thyroid glands are infiltrated by lymphocytes that, in the long term, develop lymphoid follicles with functional germinal centers [7]. The thyroid follicular cells (TFCs) exhibit extensive areas of HLA class I overexpression and *de novo* expression of HLA class II [8,9], as well as the increased expression of adhesion molecules [10]. A transcriptomic analysis of Graves' disease glands revealed a marked interferon signature that was confirmed by the demonstration of IFN-gamma transcripts by qPCR [11]. Another interesting feature of this transcriptome was the increase in the expression of inhibitory molecules and of M2 macrophages markers [11]. The activation of inhibitory circuits in spite of the predominant autoimmune response could explain the very slow progression of autoimmune thyroiditis and the chronic course of Graves' disease [12].

In the last fifteen years, it has become evident that the regulation of the immune response is much more complex than was originally envisaged, i.e., the initial activation of T cells requires several signals in addition to the recognition of HLA-peptide by the TCR complex. Moreover, there are multiple systems of modulatory receptors and ligands acting at different time points of the immune response that determine its expansion or contraction [13]. Interestingly, there is good evidence, mainly from experimental models, that the PD-1/PDL-1 axis (CD279 and CD274, respectively) may be important in modulating autoimmunity [14–16], even if attention has lately been more focused in its role in the evasion of the immune response by tumor cells [17]. In animal models of autoimmunity, e.g., in type 1 diabetes in the NOD mice, the transgenic expression of PD-L1 by islet beta cells protects against the development of diabetes, and its blockade accelerates it [18,19]. PD-L1 expression has been demonstrated in the islets of diabetic patients and is postulated to be modulated by interferons [20,21]. PD-1 and PD-L1 SNPs are also associated with GD and other autoimmune diseases [22,23].

Since immunotherapy with immune-checkpoint receptors was introduced for the treatment of cancer, there have been numerous reports on how the treatment of melanoma and other tumors with PD-1/PD-L1 axis checkpoint inhibitors nivolumab, pembrolizumab (anti-PD-1) and atezolizumab (anti-PD-L1) is often followed by inflammatory and autoimmune effects, collectively designated as Immune Related Adverse Effects (IRAEs). Among them, thyroid autoimmunity is the most common after ulcerative colitis-like symptoms. Other endocrine autoimmune diseases triggered by these treatments include hypophysitis, type 1 diabetes and autoimmune Addison disease [24,25].

Despite the above side effects of PD-1 therapy and the evidence of IFN-gamma expression in AITD glands, the PD-1/PD-L1 axis has not been systematically investigated in thyroid autoimmunity. Here, we report that PD-1+ CD4 T lymphocytes are moderately increased in PBMCs but remarkably expanded among intrathyroidal lymphocytes (ITLs) of GD patients. More importantly, PD-L1, but not PD-L2, is expressed in clusters of epithelial thyroid follicular cells (TFC) in the thyroid tissue. The modulation of PD-L1 expression in TFCs by IFN-gamma is readily demonstrated. This ectopic expression of PD-L1 in thyroid parenchyma, but not in lymphoid or myeloid cells, may critically slow the progression of AITD, explain the remarkable chronic course of AITD and suggest new therapeutic approaches for this group of diseases.

2. Materials and methods

2.1. Patients and samples

Thyroid tissue samples were collected from patients undergoing partial or total thyroidectomy at Hospital Universitari Vall d'Hebron and Hospital Universitari Germans Trias i Pujol. Samples from patients with solitary thyroid nodules or multinodular goiter (MNG) were collected as non-autoimmune controls. Serum TSH, T3 and T4 thyroid hormones and antibodies to thyroid peroxidase (TPO) and to thyroglobulin (TG) were measured by ADVIA Centaur® immunoassay (Siemens Healthcare GmbH, Erlangen, Germany), and antibodies to TSH-R were measured by TRAK human KRYPTOR competitive assay (B·R·A·H·M·S GmbH, Henningsdorf, Germany).

Tissues obtained from thyroidectomies were preserved in Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich, St Louis, MO, USA) at 4 °C for a maximum of 4 h before further processing. Small tissue blocks were snap frozen in isopenthane cooled in an acetone-dry ice bath and kept at −70 °C until used. The rest of the tissue was digested for *in vitro* experiments (see 2.2). Peripheral blood and infiltrating thyroid lymphocytes (ITLs) matched samples was collected from 10 GD patients and 10 age/sex matched healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep®, Alere Technologies AS, Oslo, Norway).

For cell culture experiments, human thyroid cell lines HT93, TPC-1 and HTH-83 were also used. HT93 is a thyroid follicular cell line transformed by transfection with SV-40 large T antigen [26], while TPC-1 and HTH-83 are thyroid cancer cell lines derived from papillary and anaplastic thyroid carcinomas, respectively [27,28].

This study was approved by the local ethical committee, and all patients included gave their written consent before tissue donation (Protocol PR(AG)77/2018).

2.2. Tissue processing

Thyroid tissue samples were weighted, minced, and digested as previously described [10]. Briefly, minced tissue was washed several times in HBSS and digested at 37 °C with mechanical stirring in 20-min steps using 0.8 mg/mL collagenase type IV (Life Technologies, CA, USA) until near total digestion. Dispersed cells were washed, and samples with > 90% viability, as assessed by trypan blue exclusion staining, were used for subsequent experimental procedures. As infiltrating thyroid lymphocytes (ITLs) are shed into the washing solution during tissue mincing and washing, they were recovered by centrifugation. Lymphocytes were then isolated by density gradient centrifugation, and samples with > 90%, viability were directly used or cryopreserved for subsequent experiments. In a few experiments, Liberase® was used instead of Collagenase to assess the possible effect of the digestion enzymes or LPS contamination on the PD-L1 and PD-L2 expression levels.

2.3. Immunofluorescence staining

Thyroid frozen tissue blocks were cut into 5 μm cryosections, PD-1, PD-L1 and PD-L2 expression was investigated by indirect immunofluorescence (IFL). Demographic features of all patients along with experiments performed for each one are summarized in Supplementary Table 1. To assess the cell distribution of PD-L1 and PD-L2 staining, double and triple staining protocols were applied. To avoid cross-reaction, Ig isotype or species-specific secondary labeled antisera were used. In each protocol, controls to assess background included sections incubated with the conjugated secondary anti-sera without the corresponding primary antibody and sections in which each of the primary antibodies in the protocol was omitted in turn to detect possible cross-reactions. Antibodies used are detailed in Supplementary Table 2. For quantitative assessment, each section was examined by two independent observers. Sections were first examined under a UV photomicroscope equipped with the adequate filters, and selected slides were examined under a confocal microscope (FV1000, Olympus Corporation, Tokyo, Japan). To identify and quantify the cells that expressed PD-1, ImageJ analysis software [29] with the Cell Counter plugin was used on sections stained for PD-1 and CD4 or CD8.

2.4. Cell culture

Thyroid dispersed cell preparations were cultured in 25 cm^2 flasks

with 5 mL RPMI culture medium containing 2 mM L-glutamine (Gibco, Waltha, Massachusetts, USA), 600 U/mL penicillin, 0,5 mg/mL streptomycin, 10 mM HEPES (Lonza, Basel, Switzerland) and 10% FBS (HyClone, Chicago, Illinois, USA) in a 37 °C humidified 5% CO2 incubator. Thyrocytes were allowed to attach to the plastic for 24 h; then, the monolayers were washed to remove intrathyroidal lymphocytes and other non-adherent cells, and medium was replaced. Thyroid cell monolayers were stimulated with 0, 25, 50, or 100 UI/mL IFNγ (Roche, Basel, Switzerland) for 24 and 48 h. For flow cytometry analysis and qPCR experiments, monolayers were trypsinized and cells were used for the subsequent experiments after washing by centrifugation. HT93, HTH-83 and TPC-1 cell cultures were harvested during logarithmical growing phase. Once viability was confirmed to be > 90%, cells were seeded in 25 cm^2 flasks at a concentration of 300,000 cells/flask. When 60–70% confluence was reached, cultures were stimulated and recovered for flow cytometry and qPCR analysis as for primary thyroid cultures.

2.5. Flow cytometry

PD-1 expression was assessed in CD4 and CD8 T lymphocyte memory subsets in PBMCs from HC and GD patients, and paired ITL samples from the GD glands. The results are shown as mean ± SD. Clinical and laboratory data of these patients are summarized in Table 1.

Cells from primary thyrocyte, HT93, HTH-83 and TPC-1 cell line cultures were recovered after stimulation with IFNγ, washed with PBS and stained for HLA class I, HLA-DR, PD-L1 and PD-L2 at different time points. Flow cytometry analysis was carried out in a FacsCanto II flow cytometer (BD Biosciences, CA, USA), and data were analyzed with FlowJo software (FlowJo LLC, Ashland, Oregon). Antibodies used are detailed in Supplementary Table 1. T cell subsets were defined as originally proposed by Sallusto et al. [30] following the protocols recommended in Holden et al. [31]. Results are expressed as the means ± SD of percentage or MFI. Routinely, > 5×10^4 PBMCs and > 10^4 ITLs were acquired for the analysis.

2.6. Relative gene expression analysis

RNA was extracted from frozen samples of the same tissues analyzed for PD-L1 and PD-L2 expression by IFL using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. Relative gene expression for GAPDH, IFNA1, IFNA4, IFNB1, IFNG and SOCS1 was measured using TaqMan® Gene Expression Assays (Applied Biosystems – Thermo Fisher Scientific, New York, NY, USA) and TaqMan® Gene Expression Master Mix following the manufacturer's instructions. Plates with triplicate reactions were run on a LightCycler® (Roche, Basel, Switzerland), and average Ct values were used for further statistical analysis.

Cells from HT93, HTH-83, TPC-1, and primary thyrocyte cultures were collected after stimulation with IFNγ and washed twice by centrifugation in PBS. RNA was extracted as above. Relative expression levels for GAPDH, CD274, PDCD1LG2 and HLA-DRA genes were measured by qPCR as above. Plates with triplicate reactions were run on a 7900HT Sequence Detection System (Applied Biosystems), and average Ct values were used for further statistical analysis. The standard deviation was always below 10% of the mean value. The relative abundance of each molecule was calculated by the 2DCT algorithm and expressed as arbitrary units (-ΔΔCtx1000) using GAPDH expression for normalization.

2.7. Statistical analysis

Pearson's correlation coefficient, together with parametric (Student's t-test) and nonparametric (Mann-Whitney) tests, were applied to normal and non-normal distributed data, respectively, using GraphPad Prism software (GraphPad Software, La Jolla, CA). A level of significance of 5% was applied in all the statistical evaluations.

3. Results

3.1. PD1+ T lymphocytes are expanded in ITLs and PBMCs from GD patients, and is associated with thyroid infiltration by effector and memory CD4 and CD8 cells

Analyses were carried out in PBMCs from 10 HCs and 10 GD patients, and in the case of GD, were also carried out in the paired ITLs obtained at the time of surgery.

In our GD series, the proportion of CD4 naïve T cells in PBMCs showed a trend to be lower than in HCs, and conversely, the effector subpopulations were higher ($p = 0.054$ and $p = 0.112$, respectively). T lymphocytes constituted 55.0 ± 11.2% of ITLs in this series of GD thyroid glands, and they were composed of similar proportions of $CD4^+$ (48.1 ± 18.1) and $CD8^+$ (42.8 ± 14.8) cells, both predominantly effector and memory. Naïve and memory T cell subpopulations were defined by the expression of CD45RA and CCR7 [30].

The differences in the distribution of T cell subpopulations between ITLs and PBMCs in the same GD patient were obvious and significant, i.e., $CD4^+$ effectors: 49.18 ± 15.47 in ITLs vs. 29.94 ± 8.43 in PBMCs ($p < 0.001$); $CD8^+$ effectors: 53.82 ± 4.59 in ITLs vs. 30.14 ± 8.75 in PBMCs ($p < 0.0001$). Conversely, CD4 and CD8 naïve cells were significantly reduced in ITLs from GD patients compared with their

Table 1
Clinical data at the moment of diagnosis of GD patients included for PD-1 expression analysis on PBMC and ITL paired samples.

Sample	Sex	Age	Time course[1]	TSH	Free T3	Free T4	Anti-TG	Anti-TPO	Anti-TSH-R
			(months)	(mU/L)	(pg/mL)	(ng/dL)	(UI/mL)	(UI/mL)	(UI/L)
THV-33	F	25	24	NA	NA	NA	NA	NA	NA
THV-34	F	26	18	< 0,008 ↓	> 20 ↑	7.63 ↑	240 ↑	187 ↑	8.3 ↑
THV-35	F	26	48	< 0,008 ↓	7.01 ↑	2.2 ↑	NA	NA	5.2 ↑
THV-36	F	37	12	< 0,008 ↓	14.15 ↑	3.5 ↑	< 15	> 1300 ↑	11.2 ↑
THV-37	M	61	24	< 0,008 ↓	7.17 ↑	2.22 ↑	24.5	237.4 ↑	58 ↑
THV-38	M	43	60	< 0,008 ↓	NA	3.58 ↑	255 ↑	91 ↑	55.4 ↑
THV-40	M	47	24	0.012 ↓	> 20 ↑	4.74 ↑	1130 ↑	< 6500 ↑	14.5 ↑
THV-41	F	51	> 240	< 0,008 ↓	5.43 ↑	1.76 ↑	73.6 ↑	> 6500 ↑	23.5 ↑
THV-49	F	52	18	< 0,008 ↓	> 20 ↑	5.53 ↑	32	> 6500 ↑	9.3 ↑
THV-51	F	48	44	< 0,008 ↓	NA	6.92 ↑	< 20	22.2	7.2 ↑

M: male, F: female, NA: not available, ↑: above reference levels, ↓: below reference levels.
[1]Clinical course, time elapsed between clinical diagnosis and therapeutic thyroidectomy was performed.
Reference values: TSH 0.55–4.78 mU/L, free T3 2.3–4.2 pg/mL, free T4 0.8–1.76 ng/dL, anti-TPO antibodies 0-60 UI/mL, anti-TG antibodies 0-60 UI/mL, anti-TSH-R antibodies 0–1.8 UI/L.

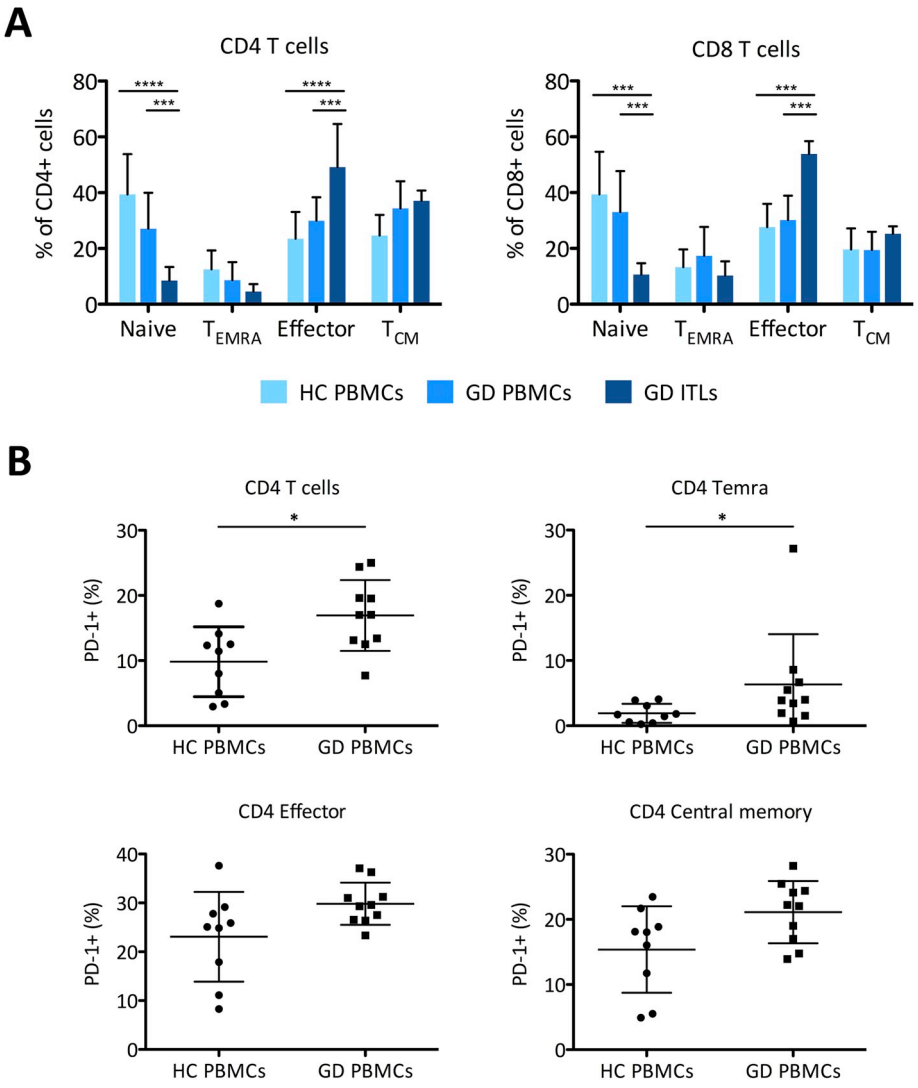

Fig. 1. PD1+ positive cell distribution in PBMCs and ITLs from GD and HC. (A) Distribution of the main T lymphocyte memory subsets for both $CD4^+$ and $CD8^+$ cells in PBMCs from HCs, and GD patients compared to intrathyroidal lymphocytes. (B) PD-1+ cells among the T lymphocyte memory subsets in PBMCs; there is a moderate increase in PD-1+ total CD4 and TEMRA T cells in GD compared to HC. *$p < 0.05$.

PBMCs; $CD4^+$ naïve: 8.49 ± 4.89 in ITLs vs. 27.10 ± 12.84 in PBMCs ($p < 0.001$); $CD8^+$ naïve: 10.66 ± 4.05 in ITLs vs. 33.02 ± 14.71 in PBMCs ($p < 0.0001$). The changes in T_{EMRA} and T_{CM} populations were minor and no significant (Fig. 1A).

The proportion of PD-1+ cells in PBMCs tended to be higher in GD than in HCs in the $CD4^+$ T cells (16.9 ± 5.4 vs. 9.8 ± 5.4, $p < 0.05$), but not in the CD8 T cells (Fig. 1B). Among $CD4^+$ T cells, the proportion of PD-1+ cells tended to be increased in the T_{EMRA} (6.3 ± 7.7 vs. 1.9 ± 1.5, $p < 0.05$), effector (29.8 ± 4.3 vs. 23.1 ± 9.2, $p = 0.054$) and central memory subsets (21.1 ± 4.8 vs. 15.4 ± 6.6, $p = 0.054$); there is therefore a tendency for PD-1 expression to be higher in the three subsets of CD4 memory cells (Fig. 1B).

Next, we evaluated in detail PD-1 expression in ITLs with respect to the paired PBMC samples. Circulating T cells, when positive for PD-1, showed a relatively low and continuous distribution of MFI, while PD-1 in ITLs showed a widespread expression intensity. Based on this expression, we defined three PD-1+ T cell categories: PD-1 negative, PD-1+ intermediate ($PD\text{-}1^{int}$), and PD-1 high ($PD\text{-}1^{hi}$) (Fig. 2A). ITLs showed a higher frequency of PD-1+ cells than PBMCs among both $CD4^+$ (48.6 ± 14.9 vs. 16.9 ± 5.4, $p < 0.001$) and CD8 T cells (44.5 ± 16 vs. 15.9 ± 10.1, $p < 0.001$) (Fig. 2B). The frequency of $PD\text{-}1^{hi}$, $PD\text{-}1^{int}$ and total PD-1+ ($PD\text{-}1^{hi}$ + $PD\text{-}1^{int}$) cells was significantly increased in the main CD4 and CD8 subsets in ITLs with respect to PBMCs (Fig. 2C–E and Table 2). Interestingly, $PD\text{-}1^{hi}$ cells were only detected in ITLs in the memory and effector subsets of both CD4 and CD8 T lymphocytes.

Expression of PD-1 by infiltrating T cells was further evaluated by IFL on cryostat sections from selected glands with lymphocytic infiltration (GD n = 9, HT n = 5, and MNG n = 1). A high proportion of infiltrating CD4 and CD8 T lymphocytes expressed PD-1 (Table 3). In AITD glands, lymphocytes can be found in small and large infiltrates that are organized as lymphoid follicles and may contain functional germinal centers [32]; in them, the distribution of lymphocytes is reminiscent of secondary lymphoid organs with a predominance of $CD4^+$ T and B cells, while $CD8^+$ T cells tended to be found dispersed throughout the parenchyma and in the small and large nonorganized infiltrates (Fig. 3A–B). Overall, in GD, only a small proportion of the parenchyma was replaced by the infiltrates, while in Hashimoto thyroiditis, there is a progressive loss of the thyroid follicular epithelial architecture [32]. There were no differences on PD-1 expression between GD and HT samples regarding proportion of positive T cells or the intensity of the staining. PD-1+ staining was confined to cells with the morphology of lymphocytes that were both in dense infiltrates and in the smaller groups of cells in the interstitium among thyroid follicles.

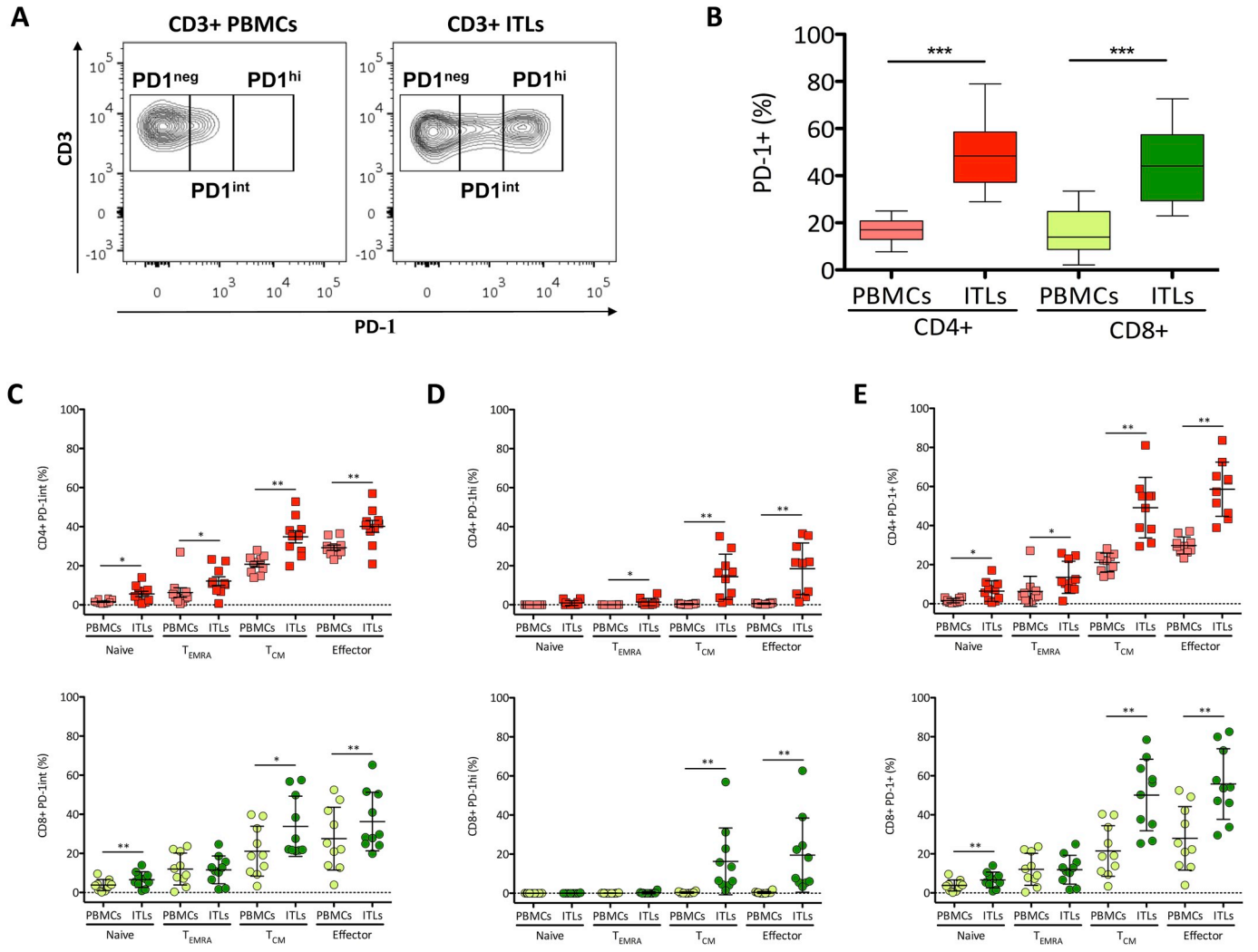

Fig. 2. PD-1 expression in PBMCs and ITL paired samples from GD patients. (A) Representative flow cytometry analysis showing the different patterns of PD-1 expression in peripheral and infiltrating T cells in GD; gates are set to separate the subpopulations defined by PD-1 MFI: negative (PD-1neg), low (PD-1int), and high expression (PD-1hi). PD-1 is expressed in peripheral T cells with low to intermediate intensity, while in ITLs there is a proportion of PD-1hi cells. (B) Comparison of the degree of PD-1 expression between PBMCs and ITLs in $CD4^+$ and $CD8^+$ T lymphocytes from GD patient paired samples. (C–E) Distribution of CD4 and CD8 memory subpopulations in PBMCs and paired ITLs samples from GD patients among PD-1 expression groups previously defined by MFI: (C) low expression (PD-1int), (D) high expression (PD-1hi), and (E) total PD-1 expression (PD-1int + PD-1hi) *p < 0.05, **p < 0.01, ***p < 0.001.

Table 2
Statistical analysis of previously defined PD-1 expression groups on CD4 and CD8 memory subpopulations between PBMC and ITL paired samples from GD patients shown in Fig. 2C.

	PD-1^{int}			PD-1^{hi}			PD-1+ (PD-1^{int} + PD-1^{hi})		
	PBMCs (Mean ± SD)	ITLs (Mean ± SD)	p-value	PBMCs (Mean ± SD)	ITLs (Mean ± SD)	p-value	PBMCs (Mean ± SD)	ITLs (Mean ± SD)	p-value
CD4	16.66 ± 5.36	33.83 ± 10.08	< 0.01	0.28 ± 0.18	14.73 ± 11.45	< 0.01	16.94 ± 5.44	48.56 ± 14.86	< 0.01
CD4 Naive	1.61 ± 1.14	5.60 ± 4.31	< 0.05	0.01 ± 0.01	0.91 ± 1.27	0.11	1.62 ± 1.14	6.50 ± 5.23	< 0.05
CD4 Temra	6.31 ± 7.67	12.18 ± 7.02	< 0.05	0.03 ± 0.05	1.35 ± 1.88	< 0.05	6.33 ± 7.71	13.53 ± 8.21	< 0.05
CD4 Effector	29.24 ± 4.26	40.15 ± 9.66	< 0.01	0.59 ± 0.36	18.46 ± 13.22	< 0.01	29.83 ± 4.32	58.61 ± 13.86	< 0.01
CD4 CM	20.78 ± 4.63	34.82 ± 9.984	< 0.01	0.33 ± 0.27	14.36 ± 11.59	< 0.01	21.11 ± 4.77	49.18 ± 15.47	< 0.01
CD8	15.76 ± 9.98	29.91 ± 12.62	< 0.01	0.18 ± 0.20	14.63 ± 15.54	< 0.01	15.94 ± 10.06	44.54 ± 16.07	< 0.01
CD8 Naive	3.79 ± 2.84	6.62 ± 4.01	< 0.01	0.00 ± 0.01	0.03 ± 0.09	1	3.79 ± 2.84	6.64 ± 4.03	< 0.01
CD8 Temra	12.01 ± 8.13	11.60 ± 7.11	0.85	0.06 ± 0.09	0.25 ± 0.54	0.13	12.06 ± 8.18	11.85 ± 7.39	0.92
CD8 Effector	27.52 ± 16.06	36.29 ± 14.98	< 0.05	0.40 ± 0.53	19.48 ± 19.02	< 0.01	27.93 ± 16.28	55.76 ± 18.11	< 0.01
CD8 CM	21.12 ± 12.73	33.85 ± 15.42	< 0.01	0.35 ± 0.40	16.27 ± 17.09	< 0.01	21.47 ± 12.97	50.12 ± 18.31	< 0.01

Wilcoxon signed-rank test for matched samples was used to compare groups.

In view of the flow cytometry data, it was not surprising to find by microscopy that in AITD glands, 61.4 ± 6.9 of $CD4^+$ T lymphocytes expressed PD-1, a high proportion of which were PD-1^{hi}. This population of cells was not detected in PBMCs from HCs or from AITD patients.

The proportion of PD-1+ cells was moderately higher in sections

Table 3
Summary of PD-1 + cells in thyroid autoimmune glands as assessed by direct microscopy examination.

	OF	[1]CD4 +	CD4 + PD-1 + (%)	OF	[1]CD8 +	CD8 + PD-1 + (%)	CD4/CD8 index
GD SAMPLES							
TB-446	6	312	55.8	6	155	69	2.0
TB-450	3	178	57.3	5	185	49.2	1.0
THV-68	6	263	57.4	7	271	66.8	1.0
TB-403	5	1721	54.7	8	683	58.3	2.5
TB-443	3	40	65	4	58	65.5	0.7
TB-442	6	73	72.6	5	86	66.3	0.8
TB-430	3	27	63	4	49	65.3	0.6
TB-421	4	559	67.6	5	556	62.1	1.0
TB-464	4	21	76.2	4	20	75	1.1
Mean	4.4	354.9	63.3	5.3	229.2	64.2	1.2
SD	1.3	541.7	7.7	1.4	236.6	7.2	0.6
HT SAMPLES							
TB-441	6	1123	60.3	8	586	56.5	1.9
TB-444	5	1368	59.6	6	682	49.3	2.0
TB-447	6	760	59.3	7	819	51.4	0.9
THV-63	5	451	58.8	5	809	74.4	0.6
TB-290	3	501	51.5	4	767	53.7	0.7
Mean	5	840.6	57.9	6	732.6	57.06	1.2
SD	1.2	397.3	3.6	1.6	98.2	10.1	0.7
GD + HT SAMPLES							
Mean	4.6	528.4	61.4	5.6	409.0	61.6	1.2
SD	1.3	536.1	6.9	1.5	316.3	8.7	0.6

OF: optical fields; GD: Graves' disease; HT: Hashimoto thyroiditis.
[1] Total number of CD4 or CD8 cells on all OF examined.

than in tissue analyzed by flow cytometry, but strong adhesion of activated T cells to TFCs during the processing and dispersion of the thyroid tissue may explain this result. The finding of remarkably high PD-1 expression in the infiltrating T cells in AITD glands prompted us to look for the expression of its ligands, PD-L1 and PD-L2, in both parenchymal and infiltrating cells, see 3.2.

3.2. PD-L1, but not PD-L2, is expressed by TFCs in GD and HT thyroid glands

Pilot flow cytometry experiments on freshly dispersed cell suspensions from two GD and two MNG glands showed PD-L1 expression in 12.7% and 2.7% of TFCs, respectively (Fig. 4A). In the same cell preparations, TFCs from GD glands showed a strong HLA class I expression, and moderate to high HLA class II expression compared to MNG (32.2 vs. 1.5%); these are well-known features of TFCs in autoimmune thyroid glands [8,33]. PD-L2 expression was not detected on TFCs from either GD or MNG samples (data not shown). The use of Liberase® instead of collagenase to digest the tissue samples did not modify the level of fluorescence (Data not shown).

To further characterize PD-L1 and PD-L2 expression, cryostat sections were stained for PD-L1 and PD-L2 by IFL. PD-L2 was negative in all samples studied, independent of the diagnosis, confirming the flow cytometry data. PD-L1 expression was observed in numerous thyroid follicles in most tissues from AITD patients (19/22), but only in a few cells in a low proportion of MNG samples (4/16). PD-L1 + cells were

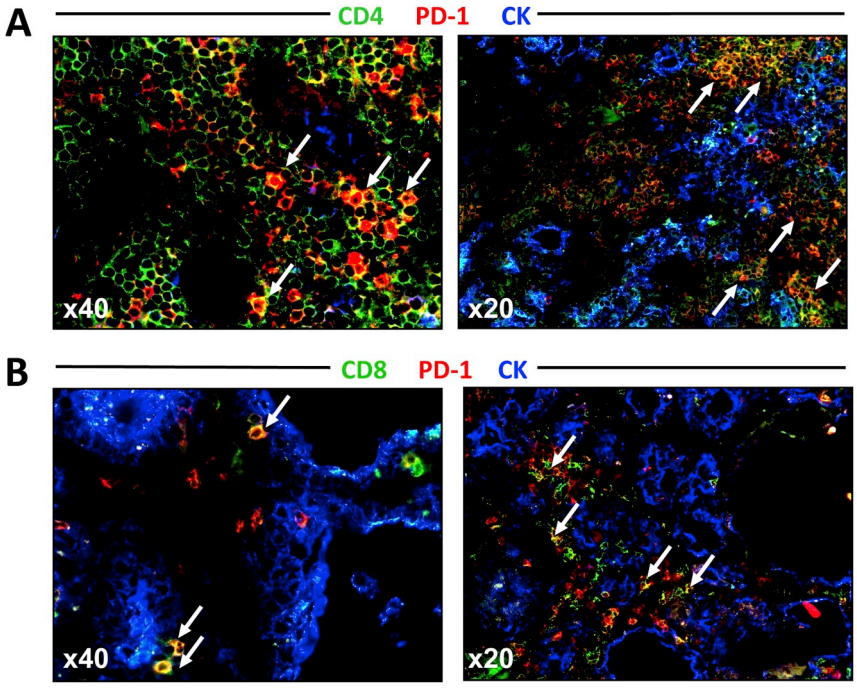

Fig. 3. PD-1 expression in infiltrating T lymphocytes in a GD thyroid gland. Representative IFL images of PD-1 expression in (A) CD4 and (B) CD8 lymphocytes on frozen sections from GD samples. White arrows mark CD4 or CD8 infiltrating lymphocytes expressing PD-1. Note the different distributions of CD4 and CD8 cells in the tissue and the high level of PD-1 expression in a high proportion of T cells. All scale bars correspond to 50 μm. CK: cytokeratin.

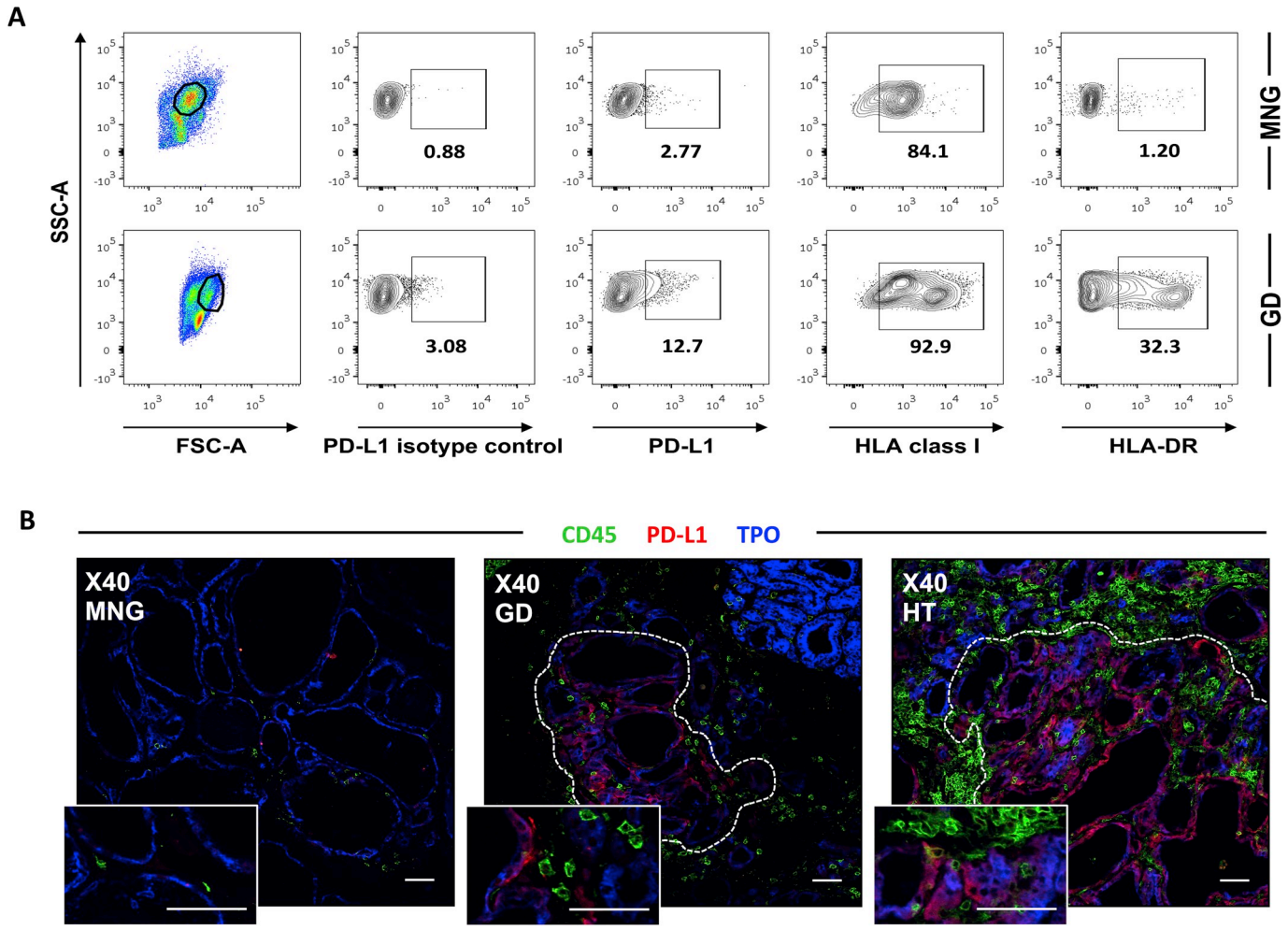

Fig. 4. PD-L1 expression in TFCs from GD and HT. (A) PD-L1 expression demonstrated by flow cytometry on freshly isolated TFCs from a GD gland compared to TFCs from an MNG gland. HLA class I overexpression and HLA-DR *de novo* expression in TFCs is also shown for comparison. (B) Expression of PD-L1 in cryostat thyroid sections stained by IFL. PD-L1 + TFCs double stained for TPO and PD-L1 (violet) are only seen in GD and HT. Dashed white lines mark the main areas of TPO + PD-L1 + TFCs. All scale bars correspond to 50 μm.

mainly TFCs and only rarely infiltrating cells or cells lining the basal pole of the thyroid follicles, presumably endothelial cells [34]; three-color IFL staining confirmed these results. The distribution of PD-L1 + TFC was patchy, and there were small areas where most TFCs were positive for PD-L1, surrounded by wide areas in which the thyroid follicles were completely negative (Fig. 4B). To analyze the relationship between PD-L1 and HLA class II expression in TFCs, and its possible association with the presence of PD-1 + lymphocytes in the surrounding infiltrates, thyroid sections were stained by three-color IFL for PD-L1, HLA-DR and TPO or PD-1, PD-L1 and TPO. The areas in which TFCs expressed HLA-DR were on the order of three-to five-fold larger than those in which they expressed PD-L1, though a systematic morphometric analysis was not performed. Interestingly, the distribution of high HLA-DR expression only partially overlapped with that of PD-L1, even if both PD-L1 and HLA class II positive TFCs were found in the same sectors of the section (Fig. 5A). PD-1 + T lymphocytes were conspicuously present in the infiltrates, but their presence did not appear to determine the expression of PDL-1 by the TFCs of the follicles in the vicinity. However, examples of PD-1 + T cells in apparent direct contact with PD-L1 + TFCs were documented (Fig. 5B).

To better assess PD-L1 expression in these glands, tissue samples from 16 GD, 5 HT and 16 MNG thyroid glands were scored blindly for PD-L1 expression by two independent observers. Based on the number of positive follicles and the intensity of PD-L1 expression, a semi-quantitative score was applied. Criteria were as follows: score 0 (negative), absence of PD-L1 + TFC in any thyroid follicle in the entire section (average 3 × 4 mm); score 1, at least one thyroid follicle in which at least 25% of the TFCs were PD-L1 +; score 2: more than one thyroid follicle, but fewer than 50% of the follicles contained at least 25% PDL-1 + TFCs; and score 3: more than 50% of the thyroid follicles in the section contained at least 25% of TFCs positive for PDL-1. Pictures in Fig. 4B correspond to glands with a PD-L1 score of 0 (MNG), 1.5 (GD), and 3 (HT), respectively. PD-L1 was detected on TFCs of 13/16 (81%) samples from GD patients and all 5 HT samples. By contrast, 12/16 non-autoimmune thyroids (75%) were negative for PD-L1, and none of them had a PD-L score > 1 (Fig. 5C).

Infiltration by PD-1 + T lymphocytes and expression of PD-L1 by TFCs occurred in the same gland but there was not a direct or inverse correlation among these two parameters (Supplementary Figs. 1A–B).

3.3. PD-1 expression in PBMCs and thyroid autoantibody levels

As PD-1 + T cells contain the Tfh subset that is involved in antibody production, we investigated the possible association of the proportion of PD-1 T cells in PBCMs and ITLs with the levels of thyroid antibodies. Anti-TG correlated positively with PD-1 + CD8 cells in PBMCs ($r = 0.81$, $p < 0.05$), and with PD-1^{int} $CD8^{+}$ cells in ITLs ($r = 0.74$, $p < 0.05$). Anti-TPO levels correlated positively with the percentage of PD-1^{int} CD4 T cells in ITLs ($r = 0\text{-}73$, $p < 0.05$).

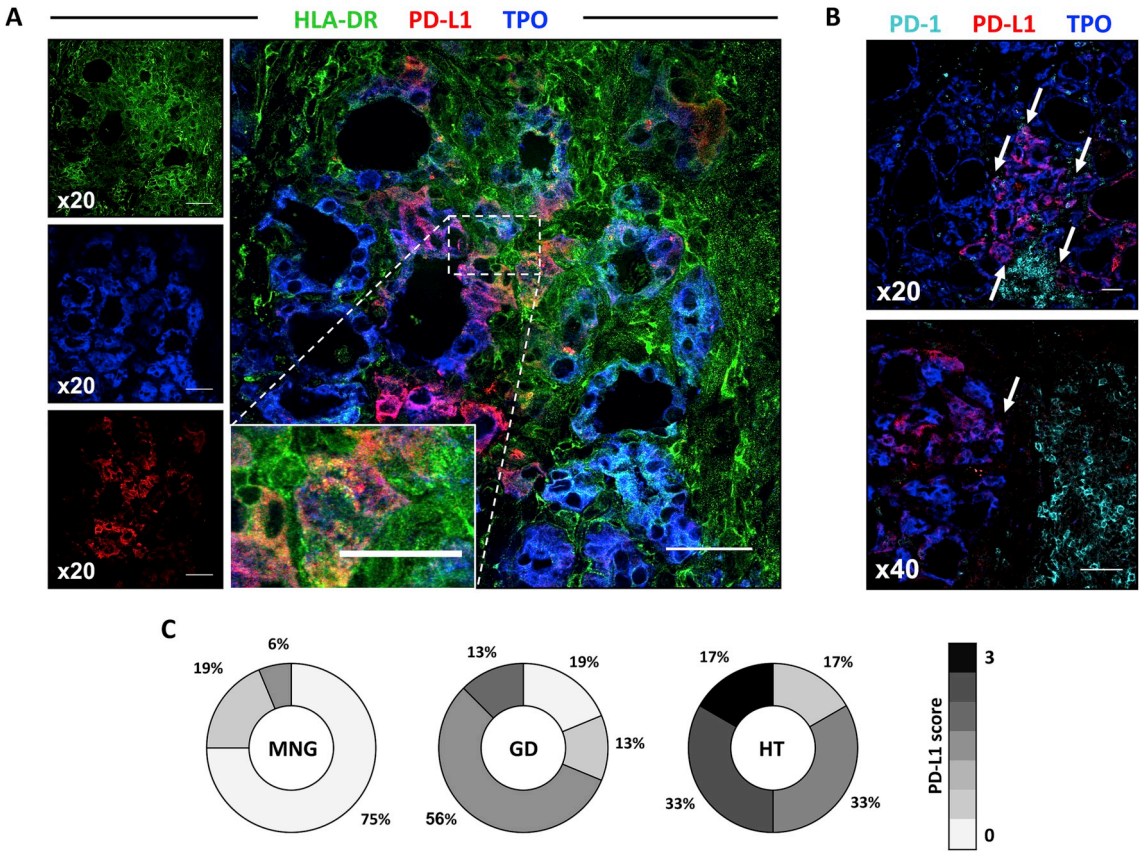

Fig. 5. PD-L1 and HLA-DR expression in TFCs from GD and HT glands. (A) Different expression patterns of *de novo* HLA-DR and PD-L1 in AITD tissue; cropped area shows TPO+ TFCs co-expressing PD-L1 and HLA-DR. (B) White arrows point to TPO+PD-L1+ TFCs in close proximity to CD45+PD-1+ infiltrating leukocytes. All scale bars correspond to 50 μm. (C) Distribution of the PD-L1 staining intensity scores in the glands by diagnosis, with 0 indicating negative PD-L1 expression on TFCs, and 3 indicating expression of PD-L1 on more than 50% of thyroid follicles.

3.4. Expression of type I and type II IFNs and SOCS1 expression in autoimmune and non-autoimmune thyroid tissue

IFN-γ can induce PD-L1 expression in different cell types [35], and previous work by us and others showed a clear interferon signature in AITD [11,36]. HLA overexpression by TFC has been attributed to IFNs, and we inferred that IFNs might also be the mediators inducing PD-L1 in TFCs. Relative gene expression of IFN genes *IFNA1, IFNA4, IFNB1,* and *IFNG* and of the downstream IFN-inducible gene *SOCS1* was measured by qPCR in RNA extracted from the same glands (but not samples) previously analyzed for PD-L1 and PD-L2 expression by IFL.

A relative increase in *IFNG* expression was detected on HT thyroid tissue when compared to GD (5.6 ± 3.6 vs. 1.2 ± 2.1, $p < 0.05$) or to MNG (5.6 ± 3.6 vs. 0.8 ± 1.7, $p < 0.05$), possibly due to higher infiltration and active inflammation present in HT tissue. Although expression of *IFNG* was higher in GD tissues compared to MNG, it was not significant. No differences in the relative expression of type I IFNs were detected between GD, HT and MNG thyroid glands (Fig. 6A). There was a positive correlation between the relative expression of *IFNG* and PD-L1 expression IFL score ($r = 0.44$, $p < 0.01$), which was independent of patient diagnosis, but no relationship was found between this score and the expression of type I IFNs (Fig. 6B). This led to the conclusion that IFNγ is probably the cytokine driving PD-L1 expression in TFCs, as it is postulated to do for HLA expression.

3.5. IFNγ induces PD-L1 and PD-L2 expression in thyroid cell lines and TFCs from autoimmune and non-autoimmune thyroid glands

Three thyroid-derived cell lines (HT93, HTH-83 and TPC-1) and thyrocytes from MNG (n = 3) and GD (n = 4) were used as substrates. The three thyroid-derived cell lines analyzed expressed basal constitutive levels of PD-L1 and, to a lesser extent, of PD-L2. Treatment with IFNγ for 24 h induced PD-L1 expression in the three lines in a dose-dependent manner. PD-L2 was also upregulated, especially in TPC-1 and HT93 cell lines. The kinetics and the maximum expression levels were different for each cell line, while HT93 cells showed maximal PD-L1 expression at 48 h, HTH-83 and TPC-1 reached the maximal MFI at 24 h, with a small decline at 48 h (Fig. 7A–B).

In dispersed cell preparations from freshly digested GD and MNG glands, TFCs were positive for PD-L1 in 14.0% and 3.1% of the cells, respectively, while PD-L2 was negative. Interestingly, after overnight culture, more than half of TFCs from all types of glands exhibited spontaneously increased PD-L1 expression (Fig. 8A). When stimulated with IFNγ, PD-L1 expression was further induced. At the lowest concentration used (25 U/mL), approximately 80–90% of TFCs became PD-L1 positive after 24 h. PD-L1 expression remained stable, and only a slight increase was observed at 48 h. Regarding PD-L2, 15–25% of TFCs became positive at 48 h after stimulation, but with much lower MFIs than PD-L1. As expected, IFNγ induced HLA-DR expression at 48 h of TFC primary culture, with greater induction in GD than in MNG cultures, as described for HLA [37] (Fig. 8B).

To confirm the flow cytometry results, mRNA from the same samples was extracted, and qPCR was used to measure *CD274* (PD-L1), *PDCD1LG2* (PD-L2) and *HLA-DRA* gene expression relative to *GAPDH*. For thyroid-derived cell lines, the kinetics observed for mRNA after IFNγ stimulation reflected those observed by flow cytometry; only HT93 showed a discrepancy for PD-L2, for which mRNA levels increased in a dose-dependent manner up to 48 h, while surface protein declined after 24 h (Fig. 9A–B). TFCs showed a dose-dependent increase of PD-L1 mRNA transcription, peaking after 24 h of stimulation with

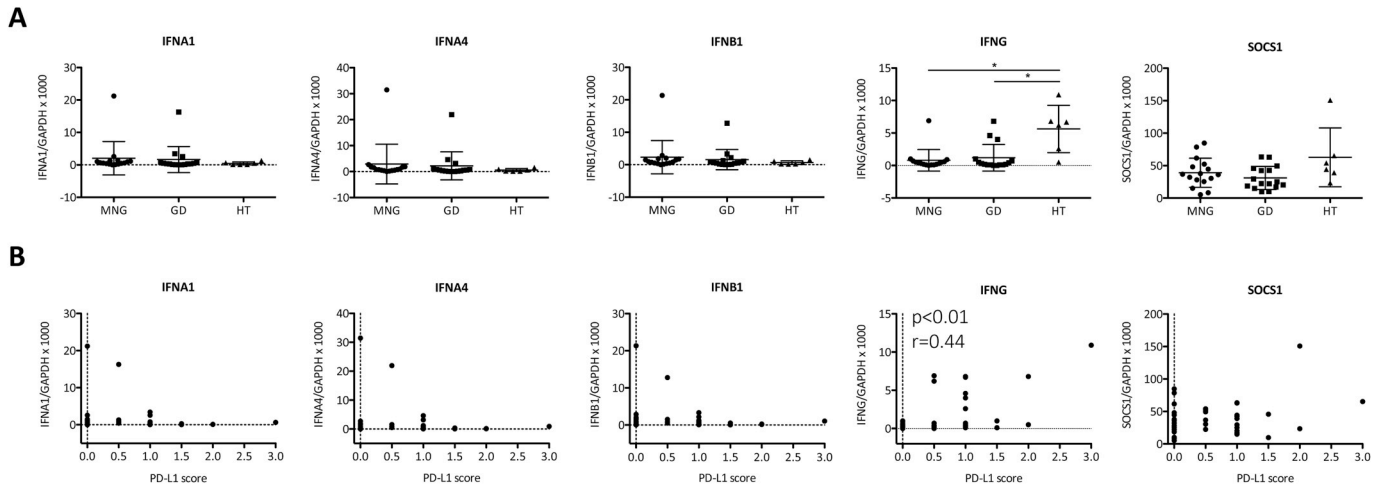

Fig. 6. Analysis of IFN expression in thyroid tissue from different diagnostic and PD-L1 score category groups. (A) Relative expression of IFNA1, IFNA4, IFNB, IFNG and SOCS1 genes in thyroid tissue from MNG, GD and HT patients. (B) Correlation between IFN relative gene expression and PD-L1 score for each tissue independent of initial diagnosis.

IFNγ, and remaining stable or decreasing after 48 h (Fig. 10A). In primary TFC cultures, there was more variability of mRNA induction among glands than for surface fluorescence, and this appeared unrelated to diagnosis (Supplementary Fig. 2). *PDCD1LG2* response to IFNγ showed similar kinetics, but relative gene expression levels were very low compared to *CD274*, but detectable (Fig. 10B).

3.6. CMTM4/6

CMTM4 and CMTM6 are membrane proteins of the chemokine-like superfamily that have been recently described as required for stable PD-L1 membrane expression [38]. To investigate whether these molecules were involved in PD-L1 expression in TFCs in AITD, we analyzed CMTM4 and CMTM6 expression by IFL in MNG and GD samples. CMTM4 and CMTM6 were highly expressed in TFCs from all samples regardless of the diagnosis (data not shown). This level of expression was in agreement with data in the GTEx transcriptomic database (https://m.gtexportal.org), in which the levels of CMTM4 and CMTM6 in thyroid tissue, as detected by RNAseq, are among the highest of all tested tissues. It is therefore unlikely that these molecules play a limiting role in the induction of PD-L1 in the TFCs of AITD glands, as shown in some melanoma cells and other malignant cell lines [39].

4. Discussion

The role of the PD-1/PD-L1 pathway in immunological tolerance and in autoimmunity has been investigated for over 15 years [40], yet its complexity has precluded reaching definitive conclusions, especially in human autoimmune diseases. It is clear that PD-1 is expressed not only by primed and “exhausted” T lymphocytes but also by Tregs, Tfh

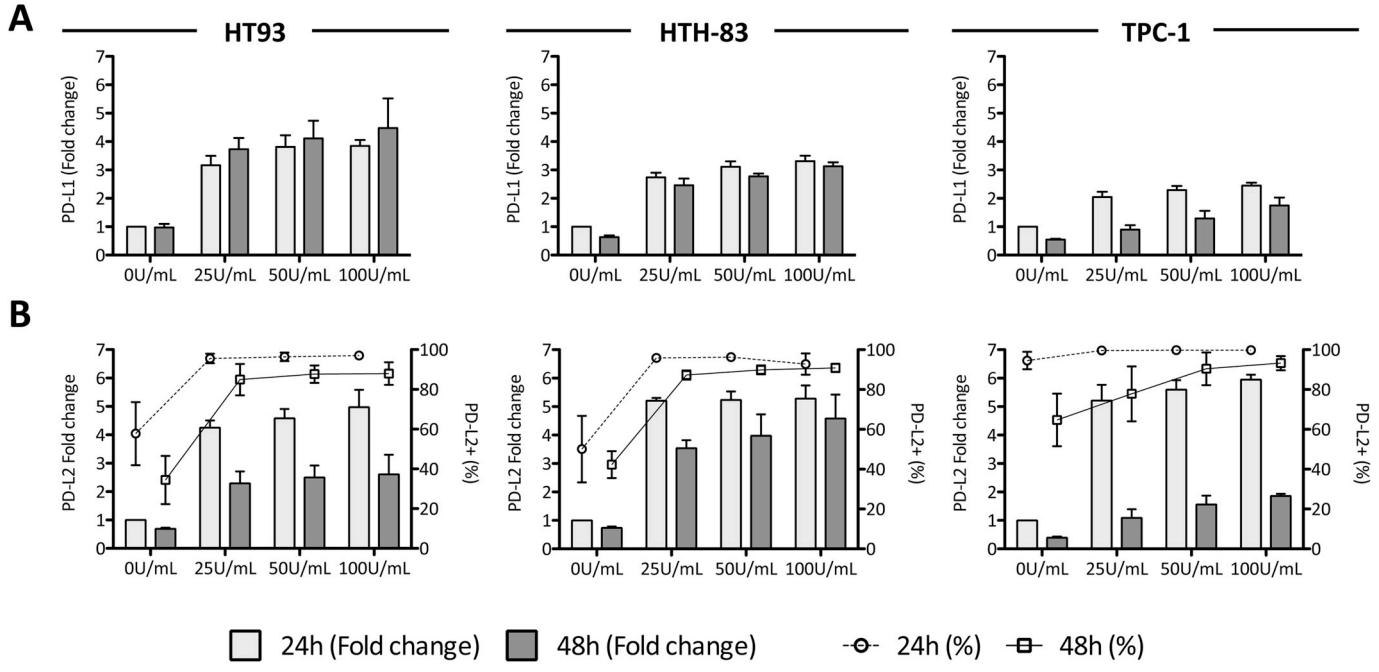

Fig. 7. Dose response and time course induction of PD-L1 and PD-L2 by IFNγ assessed by flow cytometry in thyroid cell lines. (A) PD-L1 induction is shown on top and (B) PD-L2 on lower plots for each cell line. As practically 100% of the cells were positive for PD-L1, percentages are not depicted for this molecule. PD-L2 increases over basal levels as fold changes are represented as bars with the scale of values in the left Y axis, the percentage of PD-L2 positive cells is represented by symbols and lines with the scale on the right Y axis.

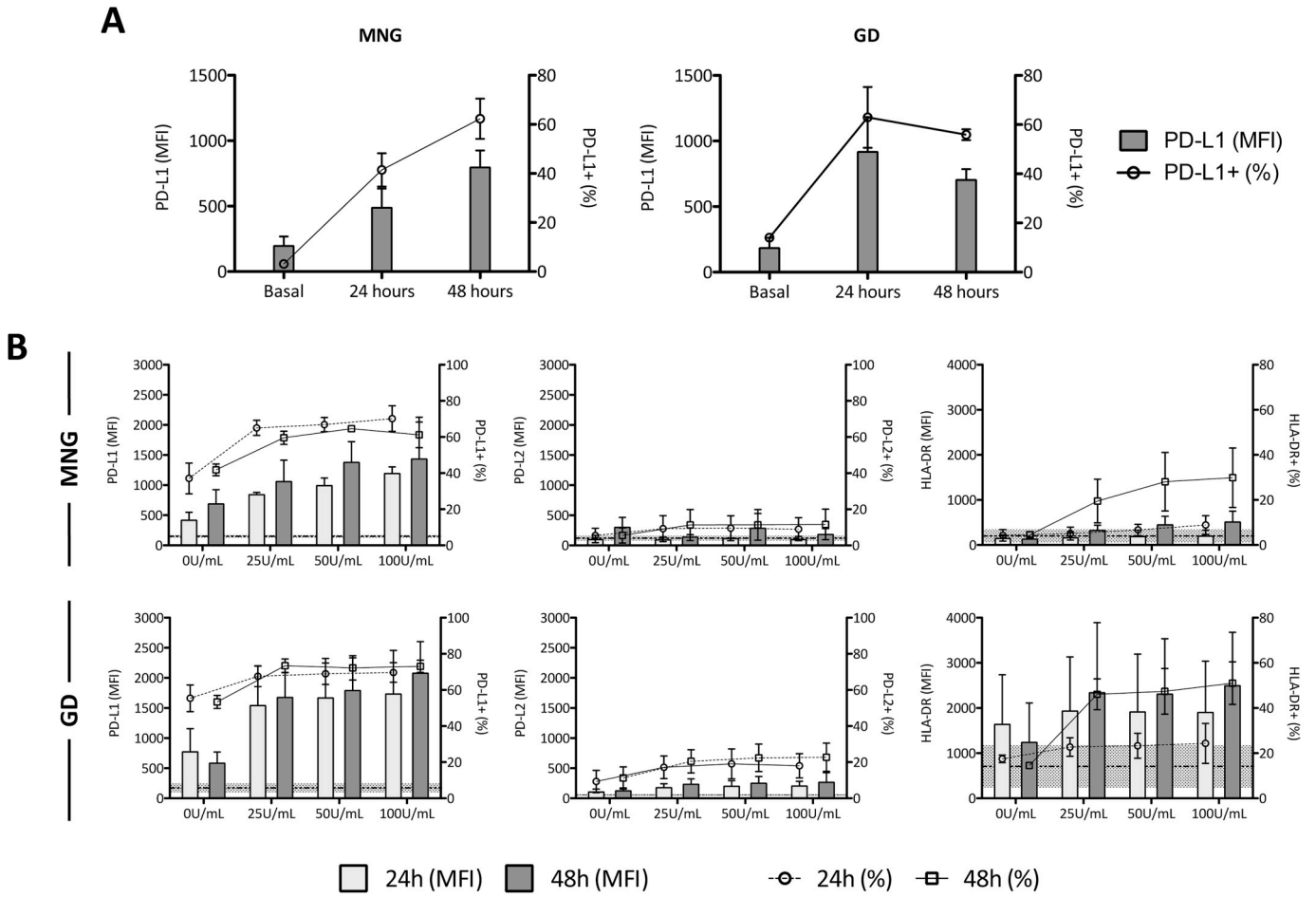

Fig. 8. Dose response and time course induction of PD-L1 and PD-L2 by IFNγ assessed by flow cytometry in primary TFC cultures. (A) Spontaneous induction of PD-L1 on GD and MNG TFCs after 24 or 48 h of culture in the absence of stimulus compared to the basal situation after the enzymatic dispersion of the thyroid. (B) Induction of PD-L1 and PD-L2 by different doses of IFNγ at 24 h and 48 h on TFC cultures from MNG and GD patients. MFI for PD-L1, PD-L2 or HLA-DR expression is represented by bars on the left Y axis; symbols connected with lines represent the percentage of positive TFCs for each protein on the right Y axis. Horizontal dashed lines and the shaded areas represent means ± SD of MFI for each marker on freshly isolated TFCs prior to culture.

and other cell types, i.e., B lymphocytes, NK cells, NKT cells, macrophages and some dendritic cells, during immune acute and chronic activation, multiplying the numbers and signs of possible interactions [41,42]. The PD-1 ligand, PD-L1, can be expressed by most myeloid and non-myeloid cells under different stimuli. PD-L2 is expressed mainly by dendritic cells and macrophages and by some nonhematopoietic cells in the lung [42]. PD-L1 interaction with PD-1 + cells is not only inhibitory, it can also qualitatively modify the immune response, e.g., the type of memory cell generated depends on the lineage of the PD-L1 + cell interacting with the T cells [43]. To better understand the role of the PD-1/PD-L1 pathway in human organ specific autoimmune disease, one initial approach is to examine the levels of expression of PD-1 and its ligands not only in PBMCs, but also in the tissue target of the disease and analyze the possible interactions. For this reason, we have undertaken the analysis of the expression of PD-1, PD-L1 and PD-L2 in PBMCs and in both the infiltrating cells and the parenchymal TFCs of the two paradigmatic diseases, GD and HT, using MNG as a non-autoimmune control tissue. By combining flow cytometry of freshly dispersed cells, tissue section IFLs and induction experiments on freshly isolated TFC and thyroid cell line cultures, we showed that the PD-1/PD-L1 pathway is probably very relevant in these diseases. The consistency between the data obtained from different substrates, techniques and blindly scoring sections support the validity of the results and conclusions. We have also ruled out that CMTM4/6 may be limiting PD-L1 expression as in some tumors [39].

The work reported here constitutes the first report of the PD-1/PD-L1 pathway in AITD and should be seen in the context of two recent reports on PD-L1 expression by islet beta cells in human type 1 diabetes, also a paradigmatic organ specific autoimmune disease [44,45]. In these two articles, the authors report results similar to ours regarding PD-L1 expression by the target cells of the immune response, but they did not analyze the phenotype of the lymphocytic infiltrate.

We have first demonstrated that PD-1 + T cells are moderately but significantly expanded in PBLs from GD patients. When comparing PD-1 expression in peripheral and intrathyroidal T lymphocytes (ITLs), we report two interesting findings: the clear expansion of PD-1 + cells in ITLs, to the point that they account for almost half of the total T cells, and the presence of a distinct population of PD-1 bright T cells. These results are reminiscent of those on the infiltrating cells in rheumatoid arthritis (RA) [46] but have not been previously reported in human autoimmune organ specific autoimmune tissue. The question that arises is that of the cell lineage or sublineages to which these PD-1 + CD4 and CD8 T cells belong. Even if a proportion could be Tregs and Tfh, both populations together would not be expected to account for nearly 50% of the infiltrating T cells. Therefore, is likely that most are chronically activated T cells and that the PD-1^{hi} population that constitutes approximately 15% of both CD4 and CD8 T cells corresponds to "exhausted" T cells, similar to those described in chronic viral infections [47]. Another PD + 1^{hi} T cell sublineage is that of peripheral helper (Tph), also described in RA and breast cancer infiltrates [48]. Further work with higher resolution tools is required to answer these questions.

As mentioned, the results presented here resemble those reported

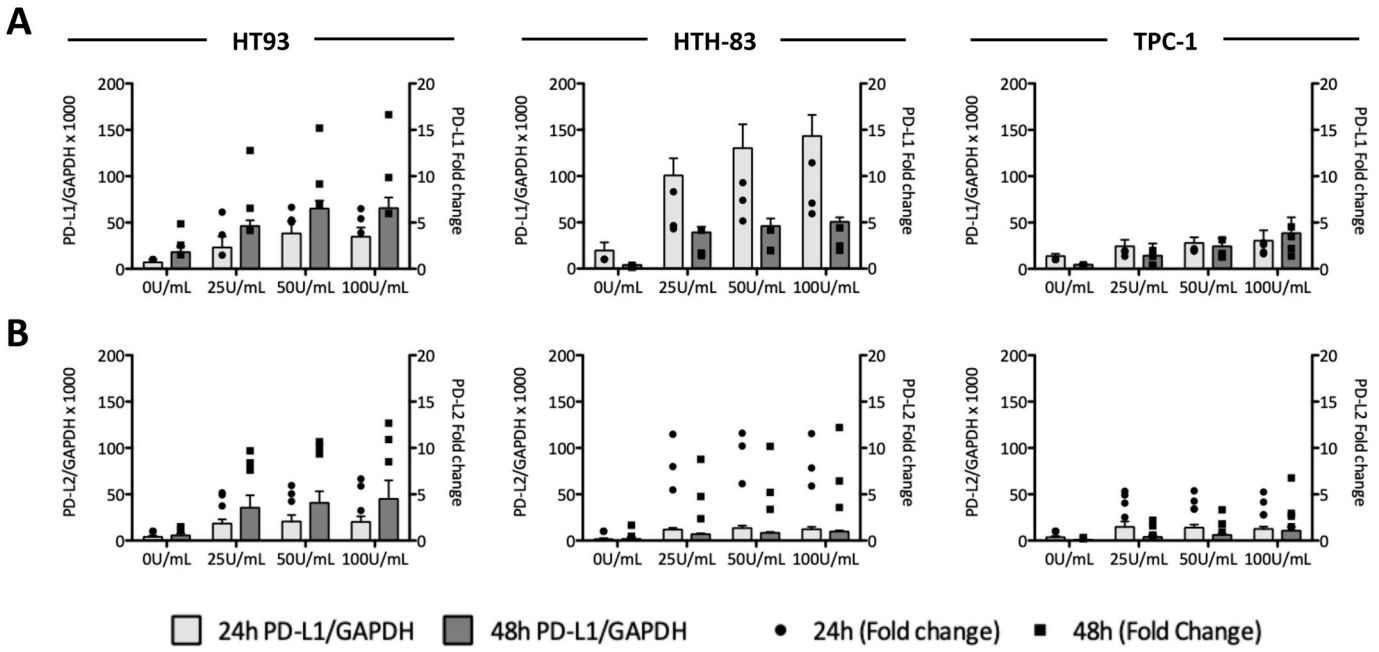

Fig. 9. Dose response and time course induction of PD-L1 and PD-L2 transcription by IFNγ assessed by qPCR in thyroid cell lines. (A) PD-L1 and (B) PD-L2 relative gene expression was normalized with GAPDH, represented by bars on the left Y axis, and as fold-change over basal level, represented by dots on right Y axis. Notice that for PD-L2 gene expression on primary cell cultures, the left Y axis is broken to improve the display of the low values.

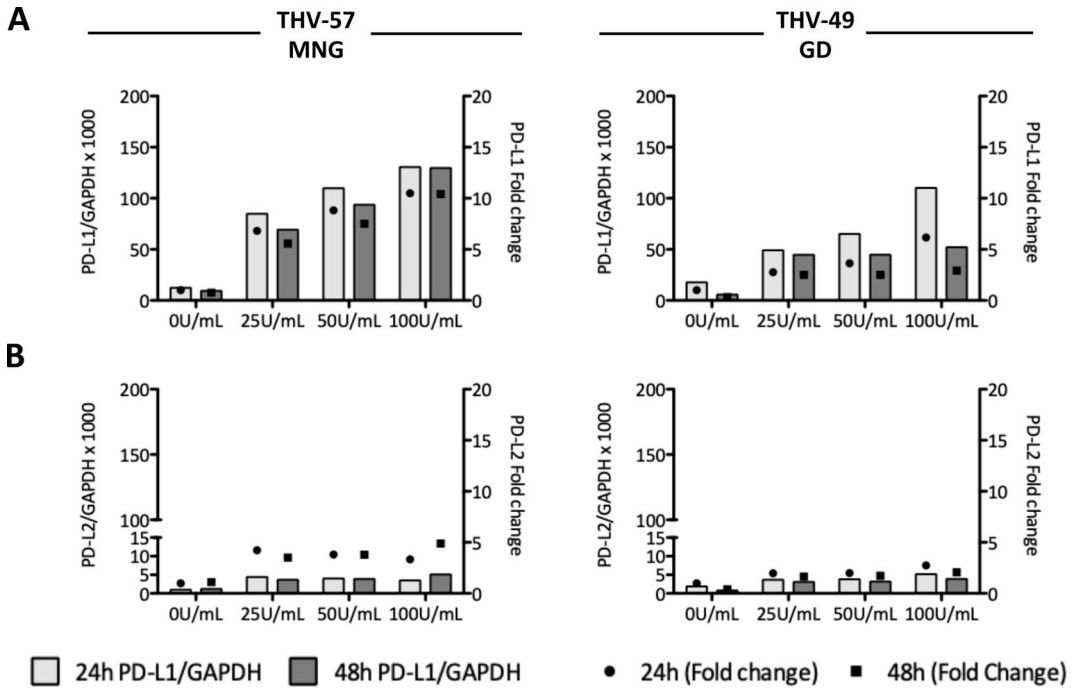

Fig. 10. Dose response and time course induction of PD-L1 and PD-L2 transcription by IFNγ assessed by qPCR in primary thyroid cells cultures. Two representative primary TFC cultures from MNG and GD glands. (A) PD-L1 or (B) PD-L2 relative gene expression was normalized with GAPDH, represented by bars on the left Y axis, and as fold-change over basal level, represented by dots on the right Y axis. Note that for PD-L2 gene expression on primary cell cultures, the left Y axis is broken to improve the display of the low values.

for Type 1 diabetes; in that, 1) there is clear expression of PD-L1 expression in the parenchymatous endocrine cells and 2) induction experiments indicate that PD-L1 expression may be attributed to IFN.

We have also demonstrated that both PD-1 + T cells and PD-L1 + TFCs are present in AITD glands, implying that the PD-1/PD-L1 pathway is probably operational. Whether PD-L1 + TFCs may limit Tfh and redirect it to Tfr differentiation, as demonstrated for PD-L1 dendritic cells, is an interesting question posed by our findings [15].

PD-L1 expression regulation is only partially understood; in neoplasic cells it is linked to the activation of MAPK and PI3Kinase pathways [49], and in untransformed cells it is induced by cytokines such as type I and II IFNs, TNF-alpha, IL-10, IL-27 and some γ-chain cytokines [41,42,50]. In inflammatory situations, IFNs and the STAT-1 IRF1 pathway appear to be the dominant mechanism of induction. Our prior transcriptomic profiling of GD glands [11] and the analysis of IFNs by qPCR in the present study pointed to IFNγ, so we tested the induction of

PD-L1 by IFNγ in HT93, HTH-83 and TPC-1 thyroid cell lines and TFC primary cultures from GD and MNG thyroid glands. The five type of cultures responded with a strong induction at 24 h, with only minor differences regarding the timing of maximal induction. This was parallel to the induction of HLA class II expression, although the latter reached a higher level both in percentage of cells and intensity (MFI) in the case of GD TFCs. It is therefore probable that *in vivo*, IFNγ plays an important role in inducing both PD-L1 and HLA-DR in autoimmune thyroid glands. Very interesting was the observation by flow cytometry of the spontaneous induction of PD-L1 in cultures from MNG glands that showed a practically nil basal expression, suggesting that there is either a tonic inhibition of PD-L1 expression *in vivo* or that the release from the close contact with other TFCs in the gland triggers PD-L1 expression. In fact, TFCs in culture without TSH supplementation become undifferentiated in a couple of weeks in an epithelial to mesenchymal like-transition known to induce PD-L1 expression [51,52]. This induction is reminiscent of the transient spontaneous induction of ABH antigens by TFC once in monolayer culture [51]. A similar process may also explain the inducibility of PD-L2 by IFN-gamma *in vitro*.

From the above mentioned points, it can be deduced that PD-L1 may be a mechanism of peripheral tolerance maintenance that is induced to avoid recognition by autoreactive T cells activated in the context of inflammatory processes, as already proposed [53]. This role of PD-L1 in maintaining tolerance to thyroid cells could explain clinical thyroid autoimmunity triggered by anti-PD-1 and anti-PD-L1 MoAbs used in cancer immunotherapy which is more common in patients positive for anti-TPO or anti-TG autoantibodies [54,55].

Overall, the results reported here contribute to explaining the slow progression of thyroid autoimmune disease, which is probably restrained by the PD-1/PD-L1 pathway, in which the expression of PD-L1 by TFCs seems to play a central role. These results, which need to be further expanded, point to new mechanisms to control thyroid autoimmunity; to that end, it will be very important to establish whether PD-L1 expression by the target cells is the critical event in controlling an incipient autoimmune response triggered by environmental or microbiota-linked factors. It should be considered that the target cells, in the case of AITD, express elevated levels of HLA class I and express *de novo* HLA class II and can therefore interact with both $CD4^+$ and $CD8^+$ T cells. These findings also lead to reinterpret the ectopic HLA Class II expression hypothesis of autoimmunity postulated back in 1983 [8,56]. The concurrent expression of HLA Class I and II, PD-L1 and adhesion molecules in TFCs would in fact deliver a mix of signals to infiltrating T cells that in GD and HT would not arrest autoimmunity but restrain it; in focal thyroiditis, they would effectively arrest the process unless the interaction is disturbed, as when anti-PD-1or anti- PD-L1 immunotherapy is applied.

It can be envisioned that by increasing inhibitory checkpoint receptor expression by the target cells, they can be protected from autoimmunity in a similar manner in which viruses such as HCV evade the immune response. In this scenario, exhausting the autoreactive T cells would be desirable, as originally proposed by McKinney et al. [57]. Therefore, these results, similar to those on PD-L1 expression by human islet beta cells [44,45], open new therapeutic avenues for the treatment of autoimmunity.

Funding

This work was supported by grants PI14/00848 and PI17/00324 from the Instituto de Salud Carlos III and was co-financed by the European Regional Development Fund (ERDF).

Declarations of interest

None.

Acknowledgments

We would like to thank Dr. Jose María Balibrea, Dr. Fort, Dr. Enric Caubet, Dr, Vilallonga, and Dr. Irene Bello from the Vall d'Hebron's Surgery Dept. for the procurement of thyroid samples; Dr. Teresa Pont and Dr. Aroa Gómez, from Vall d'Hebron's Transplant Coordinator Group for organizing the circuits for organ donor tissue collection; Dr. Dolores Jaraquema who carefully reviewed the manuscript; Reyhaneh Ramezani, UAB master student for her help; Dr. Manuel Hernández, Head of Immunology Division for his support; and Dra. Mireia Jordà for her technical advice. D. A-S is supported by a doctoral scholarship from Vall d'Hebron Institute of Research (VHIR).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.05.013.

References

[1] D.S.A. McLeod, D.S. Cooper, The incidence and prevalence of thyroid autoimmunity, Endocrine 42 (2012) 252–265, https://doi.org/10.1007/s12020-012-9703-2.
[2] K. Hemminki, X. Li, J. Sundquist, K. Sundquist, The epidemiology of Graves' disease: evidence of a genetic and an environmental contribution, J. Autoimmun. 34 (2010), https://doi.org/10.1016/j.jaut.2009.11.019 J307–J313.
[3] Y. Tomer, A. Huber, The etiology of autoimmune thyroid disease: a story of genes and environment, J. Autoimmun. 32 (2009) 231–239, https://doi.org/10.1016/j.jaut.2009.02.007.
[4] R. Pujol-Borrell, D. Álvarez-Sierra, D. Jaraquemada, A. Marín-Sánchez, R. Colobran, Central tolerance mechanisms to TSHR in graves' disease: contributions to understand the genetic association, Horm. Metab. Res. 50 (2018) 863–870, https://doi.org/10.1055/a-0755-7927.
[5] I. André, A. Gonzalez, B. Wang, J. Katz, C. Benoist, D. Mathis, Checkpoints in the progression of autoimmune disease: lessons from diabetes models, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 2260–2263, https://doi.org/10.1073/pnas.93.6.2260.
[6] C.C. Goodnow, Multistep pathogenesis of autoimmune disease, Cell 130 (2007) 25–35, https://doi.org/10.1016/j.cell.2007.06.033.
[7] M.P. Armengol, M. Juan, A. Lucas-Martín, M.T. Fernández-Figueras, D. Jaraquemada, T. Gallart, R. Pujol-Borrell, Thyroid autoimmune disease:demonstration of thyroid antigen-specific B cells and recombination-activating gene expression in chemokine- containing active intrathyroidal germinal centers, Am. J. Pathol. 159 (2001) 861–873, https://doi.org/10.1016/S0002-9440(10)61762-2.
[8] T. Hanafusa, L. Chiovato, D. Doniach, R. Pujol-Borrell, R.C.G. Russell, G.F. Bottazzo, Aberrant expression of HLA-DR antigen on thyrocytes in graves' disease: relevance for autoimmunity, Lancet 322 (1983) 1111–1115, https://doi.org/10.1016/S0140-6736(83)90628-1.
[9] M. Sospedra, E. Tolosa, P. Armengol, Y. Ashhab, S. Urlinger, A. Lucas-Martin, M. Foz-Sala, D. Jaraquemada, R. Pujol-Borrell, Hyperexpression of transporter in antigen processing-1 (TAP-1) in thyroid glands affected by autoimmunity: a contributory factor to the breach of tolerance to thyroid antigens? Clin. Exp. Immunol. (1997), http://www.scopus.com/inward/record.url?eid=2-s2.0-0030871424&partnerID=MN8TOARS.
[10] E. Tolosa, C. Roura, M. Martí, A. Belfiore, R. Pujol-Borrell, Induction of intercellular adhesion molecule-1 but not of lymphocyte function-associated antigen-3 in thyroid follicular cells, J. Autoimmun. 5 (1992) 119–135, https://doi.org/10.1016/S0896-8411(05)80056-3.
[11] M. Ruiz-Riol, M. del P. Armengol Barnils, R. Colobran Oriol, A. Sánchez Pla, F.E. Borràs Serres, A. Lucas-Martin, E.M. Martínez Cáceres, R. Pujol-Borrell, Analysis of the cumulative changes in Graves' disease thyroid glands points to IFN signature, plasmacytoid DCs and alternatively activated macrophages as chronicity determining factors, J. Autoimmun. 36 (2011) 189–200, https://doi.org/10.1016/j.jaut.2011.01.002.
[12] T.J. Smith, L. Hegedüs, Graves' disease, N. Engl. J. Med. 375 (2016) 1552–1565, https://doi.org/10.1056/NEJMra1510030.
[13] L. Chen, D.B. Flies, Molecular mechanisms of T cell co-stimulation and co-inhibition, Nat. Rev. Immunol. 13 (2013) 227–242, https://doi.org/10.1038/nri3405.
[14] M.E. Keir, S.C. Liang, I. Guleria, Y.E. Latchman, A. Qipo, L.A. Albacker, M. Koulmanda, G.J. Freeman, M.H. Sayegh, A.H. Sharpe, Tissue expression of PD-L1 mediates peripheral T cell tolerance, J. Exp. Med. (2006), https://doi.org/10.1084/jem.20051776.
[15] P.T. Sage, F.A. Schildberg, R.A. Sobel, V.K. Kuchroo, G.J. Freeman, A.H. Sharpe, Dendritic cell PD-L1 limits autoimmunity and follicular T cell differentiation and function, J. Immunol. 200 (2018) 2592–2602, https://doi.org/10.4049/jimmunol.1701231.
[16] C.S. Curran, S. Gupta, I. Sanz, E. Sharon, PD-1 immunobiology in systemic lupus erythematosus, J. Autoimmun. (2018), https://doi.org/10.1016/j.jaut.2018.10.025 0–1.
[17] S.H. Baumeister, G.J. Freeman, G. Dranoff, A.H. Sharpe, Coinhibitory pathways in

immunotherapy for cancer, Annu. Rev. Immunol. 34 (2016) 539–573, https://doi.org/10.1146/annurev-immunol-032414-112049.
[18] J. Wang, T. Yoshida, F. Nakaki, H. Hiai, T. Okazaki, T. Honjo, Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes, Proc. Natl. Acad. Sci. 102 (2005) 11823–11828, https://doi.org/10.1073/pnas.0505497102.
[19] C.J. Wang, F.C. Chou, C.H. Chu, J.C. Wu, S.H. Lin, D.M. Chang, H.K. Sytwu, Protective role of programmed death 1 ligand 1 (PD-L1) in nonobese diabetic mice: the paradox in transgenic models, Diabetes (2008), https://doi.org/10.2337/db07-1260.
[20] K.C. Osum, A.L. Burrack, T. Martinov, N.L. Sahli, J.S. Mitchell, C.G. Tucker, K.E. Pauken, K. Papas, B. Appakalai, J.A. Spanier, B.T. Fife, Interferon-gamma drives programmed death-ligand 1 expression on islet β cells to limit T cell function during autoimmune diabetes, Sci. Rep. 8 (2018) 8295, https://doi.org/10.1038/s41598-018-26471-9.
[21] M.L. Colli, J.L.E. Hill, L. Marroquí, J. Chaffey, R.S. Dos Santos, P. Leete, A. Coomans de Brachène, F.M.M. Paula, A. Op de Beeck, A. Castela, L. Marselli, L. Krogvold, K. Dahl-Jorgensen, P. Marchetti, N.G. Morgan, S.J. Richardson, D.L. Eizirik, PDL1 is expressed in the islets of people with type 1 diabetes and is up-regulated by interferons-α and-γ via IRF1 induction, EBioMedicine 36 (2018) 367–375, https://doi.org/10.1016/j.ebiom.2018.09.040.
[22] M.J. Simmonds, S.C.L. Gough, E.L. Roberts-Davies, O.J. Brand, J.M. Heward, J.A. Franklyn, P.R. Newby, Tag SNP screening of the PDCD1 gene for association with Graves? disease, Clin. Endocrinol. 67 (2007) 125–128, https://doi.org/10.1111/j.1365-2265.2007.02848.x.
[23] A.L. Mitchell, H.J. Cordell, R. Soemedi, K. Owen, B. Skinningsrud, A.B. Wolff, M. Ericksen, D. Undlien, E. Husebye, S.H.S. Pearce, Programmed death ligand 1 (PD-L1) gene variants contribute to autoimmune Addison's disease and graves' disease susceptibility, J. Clin. Endocrinol. Metab. 94 (2009) 5139–5145, https://doi.org/10.1210/jc.2009-1404.
[24] D.J. Byun, J.D. Wolchok, L.M. Rosenberg, M. Girotra, Cancer immunotherapy-immune checkpoint blockade and associated endocrinopathies, Nat. Rev. Endocrinol. 13 (2017) 195–207, https://doi.org/10.1038/nrendo.2016.205.
[25] L.-S. Chang, R. Barroso-Sousa, S.M. Tolaney, F.S. Hodi, U.B. Kaiser, L. Min, Endocrine toxicity of cancer immunotherapy targeting immune checkpoints, Endocr. Rev. 40 (2019) 17–65, https://doi.org/10.1210/er.2018-00006.
[26] A. Belfiore, T. Mauerhoff, R. Pujol-Borrell, K. Badenhoop, M. Buscema, R. Mirakian, G.F. Bottazzo, De novo HLA Class II and enhanced HLA Class I molecule expression in SV40 transfected human thyroid epithelial cells, J. Autoimmun. 4 (1991) 397–414, https://doi.org/10.1016/0896-8411(91)90155-6.
[27] M. Saiselet, S. Floor, M. Tarabichi, G. Dom, A. Hébrant, W.C.G. van Staveren, C. Maenhaut, Thyroid cancer cell lines: an overview, Front. Endocrinol. 3 (2012) 1–9, https://doi.org/10.3389/fendo.2012.00133.
[28] M.J. Jeon, W.G. Kim, S. Lim, H.J. Choi, S. Sim, T.Y. Kim, Y.K. Shong, W.B. Kim, Alpha lipoic acid inhibits proliferation and epithelial mesenchymal transition of thyroid cancer cells, Mol. Cell. Endocrinol. 419 (2016) 113–123, https://doi.org/10.1016/j.mce.2015.10.005.
[29] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (2012) 676–682, https://doi.org/10.1038/nmeth.2019.
[30] F. Sallusto, J. Geginat, A. Lanzavecchia, Central memory and effector memory T cell subsets: function, Generation, and Maintenance, Annu. Rev. Immunol. 22 (2004) 745–763, https://doi.org/10.1146/annurev.immunol.22.012703.104702.
[31] H.T. Maecker, J.P. McCoy, R. Nussenblatt, Standardizing immunophenotyping for the human Immunology project, Nat. Rev. Immunol. 12 (2012) 191–200, https://doi.org/10.1038/nri3158.
[32] M.-P. Armengol, C.B. Cardoso-Schmidt, M. Fernández, X. Ferrer, R. Pujol-Borrell, M. Juan, M. Fernandez, X. Ferrer, R. Pujol-Borrell, M. Juan, Chemokines determine local lymphoneogenesis and a reduction of circulating CXCR4+ T and CCR7 B and T lymphocytes in thyroid autoimmune diseases, J. Immunol. 170 (2003) 6320–6328, https://doi.org/10.4049/jimmunol.170.12.6320.
[33] A. Lucas-Martin, M. Foz-Sala, I. Todd, G.F. Bottazzo, R. Pujol-Borrell, Occurrence of thyrocyte HLA class II expression in a wide variety of thyroid diseases: relationship with lymphocytic infiltration and thyroid autoantibodies, J. Clin. Endocrinol. Metab. 66 (1988) 367–375, https://doi.org/10.1210/jcem-66-2-367.
[34] A.J. LaGier, J.S. Pober, Immune accessory functions of human endothelial cells are modulated by overexpression of B7-H1 (PDL1), Hum. Immunol. 67 (2006) 568–578, https://doi.org/10.1016/j.humimm.2006.04.013.
[35] A. Garcia-Diaz, D.S. Shin, B.H. Moreno, J. Saco, H. Escuin-Ordinas, G.A. Rodriguez, J.M. Zaretsky, L. Sun, W. Hugo, X. Wang, G. Parisi, C.P. Saus, D.Y. Torrejon, T.G. Graeber, B. Comin-Anduix, S. Hu-Lieskovan, R. Damoiseaux, R.S. Lo, A. Ribas, Interferon receptor signaling pathways regulating PD-L1 and PD-L2 expression, Cell Rep. 19 (2017) 1189–1201, https://doi.org/10.1016/j.celrep.2017.04.031.
[36] X. Yin, R. Sachidanandam, S. Morshed, R. Latif, R. Shi, T.F. Davies, MRNA-Seq reveals novel molecular mechanisms and a robust fingerprint in Graves' disease, J. Clin. Endocrinol. Metab. 99 (2014), https://doi.org/10.1210/jc.2014-1735 E2076–E2083.
[37] M. Sospedra, G. Obiols, L.F. Babi, E. Tolosa, F. Vargas, C. Roura-Mir, a Lucas-Martin, G. Ercilla, R. Pujol-Borrell, Hyperinducibility of HLA class II expression of thyroid follicular cells from Graves' disease. A primary defect? J. Immunol. 154 (1995) 4213–4222.
[38] D. Imamovic, S. Vranic, Novel regulators of PD-L1 expression in cancer: CMTM6 and CMTM4-a new avenue to enhance the therapeutic benefits of immune checkpoint inhibitors, Ann. Transl. Med. 5 (2017) 467, https://doi.org/10.21037/atm.2017.09.32.
[39] R. Mezzadra, C. Sun, L.T. Jae, R. Gomez-Eerland, E. de Vries, W. Wu, M.E.W. Logtenberg, M. Slagter, E.A. Rozeman, I. Hofland, A. Broeks, H.M. Horlings, L.F.A. Wessels, C.U. Blank, Y. Xiao, A.J.R. Heck, J. Borst, T.R. Brummelkamp, T.N.M. Schumacher, Identification of CMTM6 and CMTM4 as PD-L1 protein regulators, Nature 549 (2017) 106–110, https://doi.org/10.1038/nature23669.
[40] G.J. Freeman, M.F. Tomczak, B.H. Horwitz, A.H. Sharpe, Y.E. Latchman, J.E. Buhlmann, S.C. Liang, Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses, Eur. J. Immunol. 33 (2003) 2706–2716, https://doi.org/10.1002/eji.200324228.
[41] F.A. Schildberg, S.R. Klein, G.J. Freeman, A.H. Sharpe, Coinhibitory pathways in the B7-CD28 ligand-receptor family, Immunity 44 (2016) 955–972, https://doi.org/10.1016/j.immuni.2016.05.002.
[42] A.H. Sharpe, K.E. Pauken, The diverse functions of the PD1 inhibitory pathway, Nat. Rev. Immunol. 18 (2017) 153–167, https://doi.org/10.1038/nri.2017.108.
[43] S.N. Mueller, V.K. Vanguri, S.J. Ha, E.E. West, M.E. Keir, J.N. Glickman, A.H. Sharpe, R. Ahmed, PD-L1 has distinct functions in hematopoietic and non-hematopoietic cells in regulating T cell responses during chronic infection in mice, J. Clin. Investig. 120 (2010) 2508–2515, https://doi.org/10.1172/JCI40040.
[44] K.C. Osum, A.L. Burrack, T. Martinov, N.L. Sahli, J.S. Mitchell, C.G. Tucker, K.E. Pauken, K. Papas, B. Appakalai, J.A. Spanier, B.T. Fife, Interferon-gamma drives programmed death-ligand 1 expression on islet β cells to limit T cell function during autoimmune diabetes, Sci. Rep. 8 (2018) 8295, https://doi.org/10.1038/s41598-018-26471-9.
[45] M.L. Colli, J.L.E.E. Hill, L. Marroquí, J. Chaffey, R.S. Dos Santos, P. Leete, A. Coomans de Brachène, F.M.M.M. Paula, A. Op de Beeck, A. Castela, L. Marselli, L. Krogvold, K. Dahl-Jorgensen, P. Marchetti, N.G. Morgan, S.J. Richardson, D.L. Eizirik, PDL1 is expressed in the islets of people with type 1 diabetes and is up-regulated by interferons-α and-γ via IRF1 induction, EBioMedicine 36 (2018) 367–375, https://doi.org/10.1016/j.ebiom.2018.09.040.
[46] Z. Yuan, S. Li, S. Shan, S. Zhang, Y. Song, M. Chen, H. Song, W. Liao, Expression of programmed death-1 (PD-1) on CD4+ and CD8+ T cells in rheumatoid arthritis, Inflammation 37 (2013) 116–121, https://doi.org/10.1007/s10753-013-9718-8.
[47] D.L. Barber, B. Zhu, E.J. Wherry, J.P. Allison, G.J. Freeman, R. Ahmed, D. Masopust, A.H. Sharpe, Restoring function in exhausted CD8 T cells during chronic viral infection, Nature 439 (2005) 682–687, https://doi.org/10.1038/nature04444.
[48] C. Gu-Trantien, K. Willard-Gallo, Forum T FH cells play defense in cancer and offense in arthritis, Trends Immunol. 38 (2017) 875–878, https://doi.org/10.1016/j.it.2017.10.003.
[49] J. Chen, C.C. Jiang, L. Jin, X.D. Zhang, Regulation of PD-L1: a novel role of pro-survival signalling in cancer, Ann. Oncol. 27 (2016) 409–416, https://doi.org/10.1093/annonc/mdv615.
[50] G. Sciumè, A.H. Sharpe, G. Vahedi, C.A. Hunter, K. Hirahara, A. Laurence, H. Takahashi, Y. Kanno, Q. Chen, A.O. Hall, L.M. Francisco, J.J. O'Shea, K. Ghoreschi, X.-P. Yang, C.D. Dupont, H.-W. Sun, M. Tanaka, Interleukin-27 priming of T cells controls IL-17 production in trans via induction of the ligand PD-L1, Immunity 36 (2012) 1017–1030, https://doi.org/10.1016/j.immuni.2012.03.024.
[51] E.L. Khoury, Reexpression of blood group abh antigens on the surface of human thyroid cells in culture, J. Cell Biol. 94 (1982) 193–200, https://doi.org/10.1083/jcb.94.1.193.
[52] C.-Y. Ock, S. Kim, B. Keam, M. Kim, T.M. Kim, J.-H. Kim, Y.K. Jeon, J.-S. Lee, S.K. Kwon, J. Hun Hah, T.-K. Kwon, D.-W. Kim, H.-G. Wu, M.-W. Sung, D.S. Heo, PD-L1 expression is associated with epithelial-mesenchymal transition in head and neck squamous cell carcinoma, Oncotarget 7 (2016), https://doi.org/10.18632/oncotarget.7431.
[53] Q. Zhang, D.A.A. Vignali, Co-stimulatory and Co-inhibitory pathways in autoimmunity, Immunity (2016), https://doi.org/10.1016/j.immuni.2016.04.017.
[54] S. Kimbara, Y. Fujiwara, S. Iwama, K. Ohashi, A. Kuchiba, H. Arima, N. Yamazaki, S. Kitano, N. Yamamoto, Y. Ohe, Association of antithyroglobulin antibodies with the development of thyroid dysfunction induced by nivolumab, Cancer Sci. 109 (2018) 3583–3590, https://doi.org/10.1111/cas.13800.
[55] T. Kobayashi, S. Iwama, Y. Yasuda, N. Okada, T. Tsunekawa, T. Onoue, H. Takagi, D. Hagiwara, Y. Ito, Y. Morishita, M. Goto, H. Suga, R. Banno, K. Yokota, T. Hase, M. Morise, N. Hashimoto, M. Ando, H. Kiyoi, M. Gotoh, Y. Ando, M. Akiyama, Y. Hasegawa, H. Arima, Patients with antithyroid antibodies are prone to develop destructive thyroiditis by nivolumab: a prospective study, J. Endocr. Soc. 2 (2018) 241–251, https://doi.org/10.1210/js.2017-00432.
[56] R. Pujol-Borrell, T. Hanafusa, L. Chiovato, G.F. Bottazzo, Lectin-induced expression of DR antigen on human cultured follicular thyroid cells, Nature 304 (1983) 71–73, https://doi.org/10.1038/304071a0.
[57] E.F. McKinney, K.G. Smith, T cell exhaustion and immune-mediated disease — the potential for therapeutic exhaustion, Curr. Opin. Immunol. 43 (2016) 74–80, https://doi.org/10.1016/j.coi.2016.09.005.