Functional expression of CD137 (4-1BB) on T helper follicular cells

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Abbreviations: APC, antigen presenting cells; DC, dendritic cell; IHC, immunohistochemistry; LN, lymph node; mAb, monoclonal antibody; NK, natural killer; TFH, T helper follicular.

CD137 (4-1BB) is a surface protein initially discovered to mark activated T lymphocytes. However, its broader expression pattern also encompasses activated NK cells, B cells and myeloid cells, including mature dendritic cells. In this study, we have immunostained for CD137 on paraffin-embedded lymphoid tissues including tonsils, lymph nodes, ectopic tertiary lymphoid tissue in Hashimoto thyroiditis and cancer. Surprisingly, immunostaining mainly decorated intrafollicular lymphocytes in the tissues analyzed, with only scattered staining in interfollicular areas. Moreover, pathologic lymphoid follicles in follicular lymphoma and tertiary lymphoid tissue associated with non-small cell lung cancer showed a similar pattern of immunostaining. Multispectral fluorescence cytometry demonstrated that CD137 expression was restricted to CD4+ CXCR5+ follicular T helper lymphocytes (TFH cells) in tonsils and lymph nodes. Short-term culture of lymph node cell suspensions in the presence of either an agonistic anti-CD137 monoclonal antibody (mAb) or CD137-ligand stimulated the functional upregulation of TFH cells in 3 out of 6 cases, as indicated by CD40L surface expression and cytokine production. As a consequence, immunostimulatory monoclonal antibodies targeting CD137 (such as urelumab and PF-05082566) should be expected to primarily act on this lymphocyte subset, thus modifying ongoing humoral immune responses in patients with autoimmune disease and cancer.

Introduction

CD137 (4-1BB, Tnfrsf9) is a surface glycoprotein originally discovered as an activation marker of CD4+ and CD8+ T lymphocytes. It belongs to the tumor necrosis factor receptor (TNFR) family and, upon crosslinking of the receptor by natural or artificial ligands, is endowed with co-stimulatory properties for T-cell activation.

Tissue distribution is broader than originally described, as CD137 has also been found to decorate activated natural killer (NK) cells, B cells, dendritic cells (DCs), myeloid precursors, mastocytes, and tumor capillary endothelial cells.

There is only one described ligand, namely CD137L or 4-1BBL, which acts as an agonistic moiety that crosslinks 4-1BB giving rise to activation signals. CD137L is expressed on activated professional antigen presenting cells (APCs) such as activated B cells, macrophages and DCs. CD137 ligation with agonistic monoclonal antibody (mAb) has been exploited to amplify CD8+ T-cell immunity against experimental tumors leading to tumor eradication, findings propelling ongoing clinical trials with humanized anti-CD137 mAbs urelumab and PF-05082566 (NCT01471210 and NCT01307267, respectively).

Interestingly, the same anti-CD137 antibodies that cure transplantable mouse tumors have paradoxically been reported to decrease hyper antibody-mediated humoral responses and to ameliorate autoimmunity driven by auto-reactive CD4+ T lymphocytes in mouse models of disease.

Very little is known regarding CD137 expression in steady state conditions in human tissues. Here, we immunostained secondary lymphoid organs to ascertain which cell subsets and histological compartments express CD137 in healthy conditions or under naturally occurring inflammation. Our data indicate a clear pattern of immunostaining of lymphocytes selectively located in secondary follicles in lymph nodes and tonsils and...
identified as T helper follicular (TFH) cells.\textsuperscript{17,18} We further discovered that CD137 actively signals in this lymphocyte subset since short-term culture of such cells in the presence of anti-CD137 agonistic mAb led to functional upregulation of markers and cytokines of T and B cell cooperation.

**Results**

**CD137 detection in lymphoid follicles from human lymph nodes and tonsils**

Figure 1 shows representative immunostaining of patient lymph node (LN) tissue with anti-CD3 (Fig. 1A), anti-CD4 (Fig. 1B), anti-CD8 (Fig. 1C) and anti-CD137 (Fig. 1D) antibodies. Microphotographs from serial sections show a secondary lymphoid follicle and interfollicular areas representative of at least 5 independent cases from different donors.

In every case, the majority of immunostaining detected with the anti-CD137 polyclonal antibody was found inside the follicles with a pattern of staining compatible with CD4\textsuperscript{+} T cells as well as some activated B cells.\textsuperscript{20} Interestingly, staining in interfollicular areas was very scattered, whereas expression in the marginal zone around and inside the germinal center of the follicles was more prominent (Fig. 1D).

To ascertain if the pattern observed in LNs was also seen in other secondary lymphoid organs, we immunostained for CD137 expression on serial sections of human tonsils derived from infant patients who had undergone tonsillectomy. As clearly seen in Figure 2, a similar distribution pattern was observed. In this case CD3, CD4 and CD8 marker profiles indicated a clear co-distribution of CD137 staining with intrafollicular CD4\textsuperscript{+} T cells. Interfollicular areas were again consistently devoid of CD137 immunostaining in the tissues of at least 5 donors (Fig. 2D).

**Lymphoid follicles in ectopic or pathologic tertiary lymphoid tissue contain CD137\textsuperscript{+} T cells**

Hashimoto thyroiditis is an autoimmune condition that is typically characterized by ectopic tertiary lymphoid tissue formation.\textsuperscript{21} We immunostained excised tissue derived from 3 cases of diagnosed Hashimoto thyroiditis (Figs. 3A, B, C and D) to study the pattern of CD137 distribution. Again, a weak but detectable immunostaining pattern was only apparent within the lymphoid follicles. This was mainly located in the geriminal center, but not in the rest of the inflammatory infiltrate (Fig. 3D). The distribution pattern in serial sections remained consistent with selective expression on follicular CD4\textsuperscript{+} T cells (Figs. 3–C).

Pathological follicles are also a feature of follicular lymphomas. In order to ascertain the follicular CD137 expression pattern in other diseased tissues, we immunostained a small series of lymph nodes resected from patients suffering from follicular lymphoma (Figs. 4A–D). A scattered and weak CD137-specific pattern of staining was observed, again suggesting expression on CD4\textsuperscript{+} T cells located inside the malignant follicle structures (Fig. 4D).

Moreover, structured lymphoid tissue is frequently formed in the tumor bed and periphery surrounding malignancies, including those associated with non-small cell lung cancer.\textsuperscript{22} Staining tissue derived from 3 cases of squamous lung cancer encompassing such structured lymphoid follicles revealed a similar staining pattern (Figs. 5A–D).

**Expression of CD137 is restricted to follicular helper T cells**

In order to identify the subset of lymphocytes expressing CD137, we used multicolor fluorescence cytometry to study the CD4\textsuperscript{+} CXCR5\textsuperscript{+} subset in human tonsils and lymph nodes. Dot plots in Figure 5 show that this was the only T cell subset homogeneously expressing surface CD137 in fresh cell suspensions derived from excised tonsils (Fig. 6A) and lymph nodes (Fig. 6B). The vast majority of TFH lymphocytes were indeed CD137\textsuperscript{+}. Data are separately presented for gated memory TFH (CD45RO-positive) and antigen naïve (inexperienced) TFH (CD45RO-negative) populations showing that both subsets expressed CD137 although those...
cells expressing the alternatively spliced CD45R0 did so more frequently and with brighter intensity. Moreover, we detect that CD137-positive T_{FH} cells co-express surface PD-1 (CD279) in over 70% of the gated events (Fig. S1). This important finding indicates that in clinical trials with a combination anti-PD-1 and anti-CD137 mAbs (such as NCT02253992) both immunostimulatory antibodies will meet their targeted molecule on this T_{FH} subset.

Of note, B cells from the same samples showed detectable levels of expression of CD137L on their plasma membrane, indicating the potential for functional molecular interactions in the immune contexture of the germinal center (Fig. S2).

In conclusion, CD137 immunostaining seems to be focally localized in intrafollicular specialized T lymphocytes residing in both physiologically normal or pathological human secondary lymphoid tissue.

**CD137 is functional on T_{FH} cells**

To assess if CD137 ligation was able to modulate T_{FH} lymphocyte functions, we established 48h-cell cultures in the presence of plate-bound agonistic anti-CD137 mAb (6B4) or control antibody (Fig. 7A). As can be seen in Figure 7B in 3 out of 6 LN samples, anti-CD137 mAb induced the expression of CD40L, CD25, CD69 on gated CXCR5^{+} CD4^{+} T lymphocytes, as well as increased Ki67 levels (Fig. 7B and C). These markers clearly indicate functional activation of such T lymphocytes. Reasons for the lack of activity in the other 3 individual samples are unclear, since all the lymphocyte suspensions were similarly viable. Human individual variability may underlie our observation of responder inconsistency. In addition, tissue culture supernatants of the LN cell suspensions showed that in such responder cases, CD137 was able to induce the secretion of IL-4, IL-10 and IL-13 (Fig. 7D). All these 3 cytokines are known to be involved in T_{FH} functions at the germinal center. Similar results were observed using an additional lymph node cell suspension

![Figure 2. Selective expression of CD137 on lymphocytes in human germinal centers from tonsils. Similar microphotographs showing IHC staining as in Figure 1A performed on 5 tonsil surgical specimens excised from children (age 7-10 years).](image1)

![Figure 3. Selective expression of CD137 on lymphocytes in tertiary lymphoid tissue of Hashimoto thyroiditis patients. IHC staining for CD3 (A), CD4 (B), CD8 (C) and CD137 (D) in a representative section of 3 cases of Hashimoto thyroiditis undergoing surgery.](image2)
that was incubated with anti-CD137 agonist antibody or with plate-bound CD137-ligand fusion protein (mCD8-CD137L). The extent of functional activation was comparable between the antibody and the recombinant CD137-ligand (Fig. S3).

All in all, CD137 seems to be functionally expressed on T<sub>FH</sub> cells modulating critical activities, albeit some of the patient samples rendered negative results. Importantly, both CD137 and CD137L are expressed by interacting lymphocytes residing in the same tissue compartment.

**Discussion**

CD137 is a major target for immunotherapeutic antibodies seeking to augment antitumor immunity in the clinic. Hence, information on the distribution of the target in healthy and pathological tissues is crucial to understand both potential therapeutic and adverse side effects. CD137 has been reported to be expressed on tumor-infiltrating lymphocytes in both mice and humans. However, few data exist on expression elsewhere in human lymphoid tissues.

We found that CD137 seems to be highly restricted to follicular structures in tonsils and lymph nodes. The pattern of immunostaining is highly consistent with selective expression on T<sub>FH</sub> cells, as confirmed by flow cytometry. Therefore, when exogenous therapeutic monoclonal antibodies are administered to patients and reach these structures, the expectation is a functional impact on the effector functions of T<sub>FH</sub> lymphocytes, particularly impacting B cell cooperation and humoral immunity. Indeed, mouse data clearly indicate that agonistic anti-CD137 mAb treatment decreases antibody responses to immunizing antigens given simultaneously with the anti-CD137 mAb. The postulated mechanism of action for the inhibition of the antibody response was T cell-dependent and involved disruption of the T-helper network with follicular DCs.

Our observations on CD137 T<sub>FH</sub> expression have been made in...
relatively few cases per condition, and because of this, we cannot rule out individual variations both in terms intensity of expression or functionality requiring further research to clarify. However, our results provide rationale for studies on the role of the CD137 pathway in the pathogenesis of various diseases involving, or potentially involving, T_{FH} lymphocytes.

The presence of CD137^{+} T cells in the pathological follicles of follicular lymphoma had been previously reported and found to be positively correlated with good prognosis.\textsuperscript{28} However, T_{FH} lymphocytes may also be cooperating in the well-being of malignant clonal B cells. Clinical trials with agonistic anti-CD137 mAb are ongoing in follicular lymphoma patients (NCT01307267 and NCT01775631) and the clinical outcome of these trials will be of great interest.

It is tempting to speculate that, as observed in mice, humans receiving treatment with CD137 mAb will downregulate ongoing humoral immune responses. This hypothesis is addressable in clinical trials, providing common vaccines are given to patients concurrently with anti-CD137 mAb treatment.

The fact that tertiary lymphoid tissue commonly associated with cancers contains CD137^{+} T_{FH} lymphocytes provides another site of action for agonistic antibodies which may act on the antitumor humoral response.

Multicolor fluorescence cytometry conclusively demonstrated that T_{FH} cells are the major subset showing detectable CD137 surface expression. Both memory and naïve lymphocytes in this location express CD137 and the nature of antigenic stimuli inducing or sustaining CD137 expression remains to be determined. Since previous experiments in mouse models strongly suggest that CD137 ligation on this T_{FH} subset by agonistic mAb impacts inhibition of T and B cell cooperation facilitating antibody responses,\textsuperscript{14} a similar behavior is predicted in humans.

However in short-term cultures, CD137 agonist ligation induced proliferation and functional activation of T_{FH} lymphocytes.
Figure 7. CD137 is functional on T<sub>FH</sub> lymphocytes of a fraction of individuals. (A) Experimental design of the short-term (48 h) cultures in the presence of plate-bound monoclonal antibody (mAbs). (B) Immunostaining represented as mean fluorescence intensity (MFI), expressed as arbitrary units, in 6 cell suspensions from mesenteric and axillary non-malignant lymph nodes (LNs) surgically excised from patients. Results are from gated CD4<sup>+</sup> CXCR5<sup>+</sup> T cells. Individual samples are color-coded according to the graph legend. (C) Representative histograms from one of the cases offering positive results. Phycoerythrin (PE)-labeled mAb specific for each antibody (blue line) and isotype-matched labeled controls (red line) were used to characterize cell surface phenotypes by flow cytometry. (D) Concentrations of the indicated cytokines measured by ELISA in the LN cell culture supernatants from the cells derived in B.
lymphocytes. Since CD40L is induced, this would predict cooperation for Ig class switching and affinity maturation of the B-cell compartment. Further research will address in detail the role of CD137 in the physiology of Tfh lymphocytes, including the apparent individual variability.

As a whole, we have found that unexpectedly, most of the immunohistochemistry reactivity of anti-CD137 in humans is confined to the mantle and germinal center of lymphoid follicles, both in physiological and pathological scenarios.

Materials and Methods

Tissue specimens

Paraffin embedded tissue was obtained from our clinically annotated tissue bank at the University Clinic of Navarra and processed for immunohistochemistry (IHC). Fresh surgical tissue was obtained with informed consent from axillary lymph nodes from mastectomies as well as from the tonsils of infants undergoing tonsillectomy due to hyperplasia. Sample collection was approved by our institutional ethics board. Cases refer to individual patients.

Immunohistochemistry

IHC was performed using formaldehyde-fixed and paraffin-embedded (FFPE) tissue sections 3 to 4 μm thick, a polyclonal antibody against CD137 (Thermo Fisher Scientific, #MS-621-P) and monoclonal antibodies against CD3 (DAKO, #M725401), CD4 (DAKO, #M731001) and CD8 (DAKO, #M710301). After deparaffinization and rehydration, the sections were washed in 550 mM Tris-buffered saline (TBS). Antigen retrieval for CD137 was performed in citrate buffer (pH 7) for 12 min at 98°C (PTLink Dako, #AS48030); antigen retrieval for CD3, CD4 and CD8 was performed using Tris/HCl (pH 9) for 5 min at 98°C. After blocking, the sections were incubated with the primary antibody as needed, including anti-CD137 overnight at room temperature and/or anti-CD3, anti-CD4 and anti-CD8 for 20 min at room temperature. After washing with 0.55M TBS, the sections were incubated with EnVisionFLEX/HRP (Dako, #K800021) for 20 min at room temperature. The sections were stained using the Liquid DAB + Substrate Chromogen System kit (Dako, #K500711) and were contrasted with Harris Hematoxylin. The immunoreactivity of the tumor cells was assessed qualitatively with negative declared in the absence of immunostaining and positive denoted when membrane staining was observed.

Multispectral fluorescence cytometry

The tonsils and lymph nodes were processed manually until a homogeneous cell suspension was obtained. Prior to immunostaining, cells were incubated with 50 μg/mL human IgG (Berglobina P; Behring, #654773) in phosphate buffered saline (PBS) for 10 min on ice to block Fc receptors. Subsequently, 10^5 cells were washed in cold PBS and incubated 15 min at 4°C as indicated with the following fluorescently labeled mAbs: CD4-FITC (BD Pharmingen, #555346), CD45RO-PE (eBioscience, #12-0457-42), biotinylated CD137 (Biolegend, #309806) + streptavidin-PECy5 (BD Biosciences, #554062), CXCR5-APC (BD Biosciences, #145506), CD19-FITC (Biolegend, #302206), CD20-FITC (Biolegend, #302304), PD-1-Brilliant Violet 421 (Biolegend, #329919) and CD137L-PE (Biolegend, #311504). Staining for each of the antibodies was compared with their respective isotype-matched negative control. Samples were analyzed using a FACS Calibur flow cytometer (BD Biosciences, #342975) and data analyzed via FlowJo (FlowJo, TreeStar inc.).

Short-term sterile cultures of lymph node-derived cell suspensions

Non-malignant patient lymph nodes derived from surgical resection (with informed consent) were dissociated to render single cell suspensions. Resulting cell suspensions were cultured on 6-well plates precoated with CD3/CD28 (1 μg/ml) and 10 μg/ml of the anti-CD137 agonistic mAb 6B4C6, CD137L-muCD8 (Ancell, #503-020) or control mouse IgG Ab overnight at room temperature. Follicular cells cultured 48h with the antibody-coated plates were retrieved and analyzed by multispectral fluorescence cytometry for surface expression of lymphocyte markers using the following fluorophore-conjugated antibodies: anti-CD40L-PE (BD Biosciences, #340477), anti-CD25-PE (Biolegend, #302606) and anti-CD69-PE (Biolegend, #310906) Intra-cellular staining for the proliferation marker Ki67 was performed using anti-Ki67-PE (Biolegend, #350504). Collected supernatants were assayed for the concentration of IL-4, IL-10 and IL-13 by sandwich ELISAs (BD Biosciences, #555194 and #555157; Peprotech, #900-K23) that were commercially purchased.

Disclosure of Potential Conflicts of Interest

I Melero is a consultant for Bristol-Myers Squibb, Merck Serono, Roche-Genentech, Takeda and Miltenyi Biotec and Boehringer-Ingelheim.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.
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