Phenotype and tissue distribution of CD28H\(^+\) immune cell subsets

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ABSTRACT
CD28H is a newly discovered co-receptor of the human B7 family. CD28H interacts with its ligand B7-H5 and regulates T cell response. Here we showed that CD28H was not expressed on granulocytes, monocytes, myeloid dendritic cells (MDCs), and B cells, but constitutively expressed with moderate levels on memory T cells and with high levels on naive T cells, innate lymphoid cells (ILCs), natural killer (NK) cells, and plasmacytoid dendritic cells (PDCs) in human peripheral blood. Similar CD28H\(^+\) cell profile existed in secondary lymphoid organs and pathological tissues including multiple types of cancers. Further analysis demonstrated that CD28H\(^+\) naive and CD28H\(^+\) memory T cells were characterized with increased naive feature and less effector functional phenotype, respectively. High levels of constitutive CD28H expression on naive T cells and innate immune cells suggest a potential role of CD28H in innate and adaptive immunity.

Introduction

The B7-CD28 co-signaling pathway is crucial in acquisition of effector functions by the immune system. Recent studies have demonstrated that inhibitory B7 family members and their receptor signaling pathways mediate dysfunctional T cell immunity in the tumor microenvironments\(^1\)\(^-\)\(^3\) and blockade of PD-L1 (B7-H1) and PD-1 pathway induces important objective clinical responses in many types of human cancer.\(^4\)\(^,\)\(^5\) It is well known that CD28 ligation in the context of TCR engagement is regarded as the most important co-signaling pathway to activate T cells and to prevent T cell anergy. In addition, CD28 may promote NK cell cytolytic activity and cytokine production.\(^6\)\(^,\)\(^7\) A recent report shows that ILCs can express CD28 at different levels in inflammatory conditions,\(^8\) however, the potential immunological role of CD28 in ILCs remains unknown.

In an effort to identify novel B7-CD28 co-signaling members and particularly explore potential new B7 target for cancer immunotherapy, CD28H (TMIGD2) is a newly discovered co-receptor of the human B7 family that interacts with its ligand B7-H5 (B7-H7, HLHA2) and regulates T cell responses.\(^9\) CD28H is constitutively expressed on naive T cells. Repetitive antigenic exposure induces a complete loss of CD28H on T cells.\(^9\) It has been reported that CD28H signaling pathways promotes T cell proliferation and effector responses.\(^9\) However, additional studies demonstrate an inhibitory role of B7-H5 signaling in T cell activation\(^10\) and show a negative association between tumor tissue B7-H5 expression and patient outcomes.\(^11\)\(^,\)\(^12\)\(^,\)\(^13\) In this study we systematically analyzed CD28H expression profile, the phenotype and the distribution of CD28H\(^+\) cells in different immune cell subsets in peripheral blood, secondary lymphoid organs, and multiple types of human pathogenic tissues including cancer tissues. Our study suggests a potential role of CD28H in innate and adaptive immunity.

Results

**CD28H expression on immune cell subsets in human peripheral blood**

We systemically examined the CD28H expression profile across immune cell subsets in healthy human peripheral blood following mononuclear cell enrichment. We found that CD28H was negligible on granulocytes, monocytes, myeloid dendritic cells (MDCs), and B cells (Fig. 1A-C). As expected, NK and T cells expressed CD28H,\(^9\) and naive T cells expressed higher levels of CD28H than memory T cells (Fig. 1C-E). Interestingly, we detected high levels of CD28H expression on plasmacytoid dendritic cells (PDCs) and innate lymphoid cells (ILCs), which had not been previously reported (Fig. 1B-D). We compared the phenotypic and potential functional characteristics of CD28H expression on different immune cell subsets in peripheral blood, secondary lymphoid organs, and pathological tissues including human cancer tissues.

**CD28H\(^+\) naive T cells possess enriched naive characteristics**

6–15% naive T cells were CD28H\(^-\) (Fig. 1D, E). We analyzed and compared the potential phenotypic differences between...
CD28§ and CD28± naïve T cells. We found that the majority of CD28§, but not CD28± naïve T cells expressed CD31 (Fig. 2A), a recent thymic emigrant marker. In line with this, CD28± naïve T cells expressed higher levels of T£1-regulating transcription factor T-bet (Fig. 2B), as well as effector cytokines IFNγ (Fig. 2C) and TNFα (Fig. 2D). Similar levels of IL-2 and CXCL8 were detected in CD4§CD28§ and CD4±CD28± naïve and memory T (Sup. Fig. 1A-D). Interestingly, CD28 surface expression was lower on CD28§ than CD28± naive cells (Fig. 2E). Furthermore, enriched naïve CD4± T cells cultured in vitro progressively lost CD28§ surface expression (Fig. 2F). Taken together, compared with CD28± naïve T cells, CD28§ naïve T cells show enriched naïve characteristics.

**CD28§ T cells exist in lymphoid organs and pathological tissues**

CD28§ is expressed in the majority of naïve T cells. Naïve T cells are largely located in secondary lymphoid organs. CD28§ ligation by B7-H5 may have a stimulatory or inhibitory effect on T cells. We analyzed CD28§ T cells in the human secondary lymphoid organs including tonsil and spleen. CD28§ expression was not different between blood, tonsil, and spleen (Fig. 4A-B). Next we examined CD28§ expression on T cells from ovarian cancer patient blood, ovarian cancer tissues, colon cancer tissues, and colon colitic tissues. We found CD28§ T cells in different pathological tissues (Fig. 4C-D). The levels of CD28§ T cells were slightly or moderately reduced in different pathological tissues as compared with healthy blood and ovarian cancer patient blood (Fig. 4C-D). We further evaluated the effector state of CD28§-expressing tissue-infiltrating T cells. We observed that the expression levels of CD57 (Fig. 4E) and IFNγ (Fig. 4F) were significantly lower in CD28§ T cells than CD28± T cells in different tissues. Thus, CD28§ T cells can be recruited into tumor and inflammatory tissue sites and tissue CD28§ T cells show less activation and differentiation.

**CD28§ ILCs and NK cells exist in lymphoid organs and pathological tissues**

Similar to peripheral blood, we analyzed CD28§ expression on NK cells in secondary lymphoid organs including tonsil and spleen and different types of human cancer tissues. We detected high levels of CD28§ expression on NK cells in tonsil and...
spleen (Fig. 5A, B) and several types of cancer tissues (Fig. 5C). It appeared that the percentage of CD28H⁺ NK cells was lower in tissues than in peripheral blood (Fig. 5C). We also analyzed CD28H expression on ILCs in secondary lymphoid organs including tonsil and spleen and different types of human cancer tissues (Fig. 5D). ILCs expressed high levels of CD28H in secondary lymphoid organs (Fig. 5E) and different types of human cancer tissues (Fig. 5F). Notably, the percentages of CD28H⁺ ILCs were comparable in different pathological tissues (Fig. 5F). Further studies should measure the functional consequences of CD28H ligation on pDCs, ILCs and NK cells.

**Discussion**

In this work we have systemically examined CD28H expression in different immune cell subsets. In line with the previous report,⁹ we have shown that CD28H is constitutively expressed on the majority of naïve T cells and a small fraction of memory T cells in human peripheral blood, secondary lymphoid organs, and different pathological tissues including breast cancer, colon cancer, lung cancer, ovarian cancer, and colitic colon tissues. Furthermore, we have not detected CD28H expression on B cells. Based on the phenotype and effector cytokine profile, we have found that CD28H⁺ memory T cells show less effector function and minimal differentiation features. It has been described that engagement of CD28H⁹ and its ligand B7-H5¹⁰ result in T cell activation and inhibition, respectively. This discrepancy is poorly understood in literature. Non-existence of murine CD28H and B7-H5 and lack of reliable, consistent, and reproducible reagents including specific neutralizing antibodies against human CD28H and B7-H5 significantly dampen our efforts toward a comprehensive understanding of this B7 family pathway. Nonetheless, CD28H expression profile and tissue distribution suggest a potential role of CD28H in adaptive T cell immunity.

In addition to T cells and B cells, we have examined CD28H expression on antigen presenting cell subsets and innate immune cell subsets. Granulocytes, monocytes, and MDCs do not express CD28H. However, 50% pDCs express CD28H. As peripheral blood¹⁸ and tumor associated¹⁹ PDCs are major type-I IFN producers, PDCs are considered innate immune cells. CD28H expression is highly expressed on NK cells and ILCs. CD28H can support NK cell function.⁶,⁷ ILCs may express different levels of CD28 but the effect of its ligation has not yet been elucidated.⁵ Given the
homology of CD28 and CD28H, CD28H may carry out a potential stimulatory and/or survival signal to ILCs and NK cells. Although high levels of CD28H expression on PDCs, ILCs, and NK cells in blood, secondary lymphoid organs, and pathological tissues suggest a potential role of CD28H in innate immunity, functional studies are in urgent need to define biologic and pathological relevance of CD28H and B7-H5 signaling pathway. Similar to the CD28 and B7 signaling pathway, we speculate that the CD28H and B7-H5 signaling pathway may add a novel layer of immune regulation in innate and/or adaptive immunity. Nonetheless, functional studies are essential to determine the basic immunological activity of this interaction and to assess whether targeting this pathway is an effective therapeutic approach for cancer immunotherapy.

Materials and methods

Human subjects and human samples

Peripheral blood was obtained from healthy volunteers and patients with ovarian cancer. Mononuclear cells were enriched by Ficoll-Hypaque (GE Healthcare and Life Sciences) or Lymphoprep (STEMCELL Technologies) density gradient centrifugation. Naïve CD4+ T cells were enriched from healthy donors using CD4+ RosetteSep enrichment cocktail (STEMCELL...
Technologies) followed by anti-CD45RA microbeads (Miltenyi Biotec) for in vitro T cell cultures. Pathological tissues from breast cancer, colon carcinoma, lung cancer, ovarian cancer, and ulcerative colitis were used. Human tissues were from patients presenting for diagnostic biopsy, prophylactic colectomy, or tumor debulking. Mononuclear cells from healthy or patient donors were used fresh unless described otherwise. All donors provided written, informed consent. In addition to these pathological tissues, human spleen and tonsil tissues were obtained from the tissue procurement core at the University of Michigan Hospital. Human pathological tissue cells were obtained as described.1,13,14 The Institutional Review Boards of the University of Michigan School of Medicine approved the study.

Reagents

Antibodies for flow cytometry analysis were CD3 (cat. 557943, 562280, 340662, clone UCHT1), CD4 (cat. 470049, clone RPA-T4), CD8 (cat. 557760, clone RPA-T8), CD11 c (cat. 559877, clone B-Ly6), CD14 (cat. 555397, clone M5E2), CD15 (cat. 642917, clone MMA), CD28 (cat. 557760, clone RPA-T8), CD11 c (cat. 559877, clone B-Ly6), CD14 (cat. 555397, clone M5E2), CD15 (cat. 642917, clone MMA), CD28 (cat. 557760, clone RPA-T8), CD11 c (cat. 559877, clone B-Ly6), CD14 (cat. 555397, clone M5E2), CD15 (cat. 642917, clone MMA), CD28H (Amplimmune), CD7 (cat. 470007, clone 124-1D1), CD16 (cat. 470007, clone 124-1D1), CD19 (cat. 25-0199-42, clone HIB19), CD45 (cat. MHCD4530, clone H130), CD57 (cat. 48-0577, clone NK-1), Foxp3 (cat. 48-4776, clone PCH101), HLA-DR (cat. 25-0199-42, clone LN3), IL-2 (cat. 46-7029, clone MQ1-17H12) (ThermoFischer), Armenian Hamster isotype control (cat. 400916, clone HTK888), and anti-Armenian Hamster (cat. 405503, clone Poly4055) (Biolegend). Antibodies for T cell activation were anti-CD3 (cat. 555329/14-0038-82, clone UCHT1) and anti-CD28 (cat. 555728/16-0289-81, clone CD28.2) (BD Biosciences and ThermoFischer).

Flow cytometry

Single-cell suspensions from tissue were stained for extracellular surface antigens with specific antibodies, followed by fixation and permeabilization using Perm/Fix solution (ThermoFischer), then stained against intracellular antigens as described.14,15 Viable single cells were analyzed.

T cell culture

Naïve CD4+ T cell subsets were stimulated with 2.5 μg/mL anti-CD3 and 1.25 μg/mL anti-CD28 mAb and 3 ng/mL rhIL-2 for up to 20 d. Medium was refreshed every 3 d with 2.5 ug/mL anti-CD3, 1.25 ug/mL anti-CD28 and rhIL-2 3 ng/mL. T cells were then measured against CD28H at indicated time-points.
**T cell stimulation**

Freshly isolated mononuclear cells were stimulated in vitro with PMA/Ionomycin, GolgiStop and GolgiPlug (BD Biosciences) for 4 hrs then stained against extracellular and intracellular antigens as described above.

**Disclosures**

The authors have no financial conflicts of interest.

**Conflict-of-interest disclosure**

The authors declare no competing financial interests.

**Acknowledgment**

This work was supported in part by research grants from the NIH/NCI R01 grants (W.Z) (CA217510, CA123088, CA099985, CA193136 and CA152470) and the NIH through the University of Michigan Cancer Center Support Grant (CA46592). We thank Linda Liu, D. Postiff, M. Vinco, R. Craig, and J. Barikdar for their assistance.

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