ARTICLE

Fine tuning of the DNAM-1/TIGIT/ligand axis in mucosal T cells and its dysregulation in pediatric inflammatory bowel diseases (IBD)

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De-regulated T-cell activation and functions are pivotal in the orchestration of immune-mediated tissue damage in IBD. We investigated the role of DNAM-1 (co-activating)/TIGIT (co-inhibitory)/ligand axis in the regulation of T-cell functions and its involvement in IBD pathogenesis. We show that DNAM-1 and TIGIT display a peculiar expression pattern on gut mucosa T-cell populations, in a microenvironment where their shared ligands (PVR and Nectin-2) are physiologically present. Moreover, DNAM-1 family receptor/ligand system is perturbed in IBD lesions, in a disease activity-dependent manner. The expression profile of CCR6 and CD103 mucosa addressins suggests that microenvironment-associated factors, rather than skewed recruitment of circulating T-cell populations, play a more relevant role in supporting the establishment of DNAM-1 and TIGIT expression pattern in mucosal T-cell populations, and may explain its alteration in IBD. Although both co-receptors mark functionally competent T cells, DNAM-1 and TIGIT segregate on T cells endowed with different proliferative potential. Moreover, their opposing role in regulating T-cell proliferation exquisitely depends on ligand availability. All together, our data propose a role for DNAM-1 and TIGIT in regulating mucosal T-cell activation and immune homeostasis, and highlight the involvement of an imbalance of this system in IBD.

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INTRODUCTION

T-cell effector functions are regulated by the interaction between co-stimulating and co-inhibitory receptors for cell-associated ligands, whose expression is subjected to tight regulation. This mechanism tailors T-cell responses, proliferation and survival, and allows a rapid adaptation to changing cell–microenvironment and cell–cell interactions.

DNAX accessory molecule (DNAM)-1 family members represent a regulation system for both T and natural killer (NK) cells. The family includes five identified transmembrane Ig-like glycoproteins so far: DNAM-1 (CD226), TIGIT, CD96 (TACTILE), CRTAM, and CD112R (PVR-related immunoglobulin domain containing, PVRIG).^{1–4}

DNAM-1 and TIGIT are the best-characterized members of the family and exert opposite functions upon binding to shared ligands belonging to the nectin family: PVR (CD155) and Nectin-2 (CD112).^{1–4} These are widely expressed on epithelial and endothelial cells, tumor cells, and on activated T cells and dendritic cells (DCs).^{1–6} TIGIT binds both ligands with higher affinity than DNAM-1 and also interacts with DNAM-1 in cis.^{1,4,7,8}

DNAM-1, expressed on a fraction of circulating T cells, a large fraction of NK cells and on monocytes, is physically and functionally associated to LFA-1 integrin; its ligand- or antibody-dependent engagement has been shown to upregulate T-cell activation, proliferation, and cytotoxicity.^{9–13} TIGIT is expressed on activated T cells and also on a subset of NK cells.^{1,3,14} Its presence

on lymphoid tissue-resident and on regulatory T-cell (Treg) subsets has also been reported.^{15–17} TIGIT displays two immunoregulatory domains (ITIM and ITT) in its cytoplasmic region, which support its proposed inhibitory activity, whose mechanisms are nevertheless still elusive. TIGIT may inhibit T-cell functions through several mechanisms: competition with DNAM-1 for their shared ligands, disruption of DNAM-1 homodimerization, interference with activating intracellular signaling cascades, and indirectly by either inducing Treg responses or by stimulating DC to produce interleukin (IL)-10.^{7,16–23} This system, where two different receptors with opposite functions are engaged by the same ligands, potentially expressed on the same target cell, suggests an intriguing role of DNAM-1 and TIGIT in the fine-tuning of T-cell functions in distinct microenvironments, characterized by different ligand availability.

The regulation of effector and memory T-cell presence and functional role bears particular relevance in the gut mucosa compartment, under both physiological and pathological conditions. “Conventional” T-cell subsets, such as CD4⁺ T helper and Treg, and CD8⁺ cytotoxic T cells (CTLs), as well as many different “innate-like” T-cell subsets, such as invariant Natural Killer T, mucosa-associated invariant T, and $\gamma\delta$ TCR⁺ T cells, are abundantly represented in the gut mucosa microenvironment. These specialized T-cell populations play crucial functions in the intestinal mucosa homeostasis and maintenance of epithelial barrier efficiency, other than defending the host from pathogens. The

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Fig. 1 TIGIT is more expressed than DNAM-1 on mucosal T-cell populations of healthy pediatric individuals and pediatric IBD patients. Percentage of DNAM-1⁺ (a) and TIGIT⁺ (b) cells, and co-receptor surface density (expressed as net mean fluorescence intensity, net MFI, c, d), on CD4⁺, CD8⁺, and CD4⁺ CD8⁺ (DN) T cells isolated from colon biopsies of active (A, *n* = 24 a, c; *n* = 36 b, d) and remitting (R, *n* = 14 a, c; *n* = 26 b, d) IBD patients, and age- and sex-matched non-IBD controls (C, *n* = 16 a, c; *n* = 22 b, d). Bars represent median and 10–90 percentile range. *P*-values refer to Kruskal–Wallis non-parametric test

presence of diet antigens and commensal flora molecular components makes the issue of their fine regulation even more crucial to preserve immune homeostasis in the gut mucosa microenvironment.^{21,24–28}

Inflammatory bowel diseases (IBDs) are a spectrum of chronic inflammatory diseases, sharing the localization (gut mucosa), the chronicity, and the immune-mediated damage. The two main IBD forms are Crohn's disease (CD) and ulcerative colitis (UC), which display shared characteristics as well as peculiarities.²⁹ Multiple gene polymorphisms, together with environmental factors, commensal flora dysbiosis, and deregulation of the immune system cooperate in the initiation and chronicization of IBD inflammatory condition.^{26,30–33} Pediatric IBD accounts for 10–25% of IBD cases and, although displaying characteristics distinct from the adult onset form, it may provide a privileged point of view to dissect the initial steps of IBD pathogenesis, in the relative absence of many environmental factors.^{31,34,35}

Many studies have put the focus on the role of T-cell dysregulated responses, elicited by the loss of physiological tolerance against commensal flora molecular components and by weakened regulatory mechanisms, in IBD onset and chronicization.^{25,27,36–38} Our work characterizes several aspects of the biology of the DNAM-1 family receptor/ligand axis on peripheral blood and mucosal T-cell populations, and its perturbation in inflamed gut microenvironment of pediatric IBD patients.

RESULTS

Divergent expression pattern of DNAM-1- and TIGIT on circulating and gut mucosa-resident T-cell populations, and its alteration in IBD

The expression of DNAM-1 and TIGIT co-receptors on tissue-resident T-cell populations has been poorly explored so far. We

evaluated the expression of the two co-receptors on gut mucosa T-cell subsets, isolated from colon biopsies of either pediatric IBD patients or age- and sex-matched non-IBD controls. Notably, increased T-cell infiltrate characterized inflamed mucosa lesions of active IBD patients and non-inflamed samples of remitting patients, mostly accounted for by “classical” CD4⁺ and CD8⁺, rather than “innate-like” CD4⁺ CD8⁺ (double negative, DN) cells (Supplementary Fig. 1a).

Here we show that DNAM-1 was physiologically less expressed than TIGIT on CD4⁺, CD8⁺, and DN main mucosal T-cell subsets (Fig. 1a, b). The percentage of DNAM-1⁺ T cells was comparable in control and IBD samples, independently of disease activity; differently, the frequency of TIGIT⁺ cells was selectively and significantly reduced on CD4⁺ T cells of patients undergoing clinical remission, when compared with non-IBD controls (Fig. 1b); indeed, TIGIT⁺CD45⁺ total leukocytes were not appreciably different in controls and active or remitting IBD patients (data not shown).

Interestingly, TIGIT surface levels, evaluated as receptor mean fluorescence intensity, were lower on both CD4⁺ and CD8⁺ T lymphocytes obtained from inflamed mucosa samples of active IBD patients, as compared with non-inflamed tissue samples of remitting IBD patients or with control samples (Fig. 1d). DNAM-1 expression levels were also significantly downregulated on CD4⁺ and, less markedly, on CD8⁺ T cells of active IBD patients (Fig. 1c), whereas no alteration of either co-receptor could be appreciated on DN T cells of IBD patients (immunocytofluorimetric analysis of representative patients and control is reported in Supplementary Fig. 1b).

These data first show that TIGIT is more prevalent than DNAM-1 on mucosal T-cell populations, and that its expression is more severely perturbed in IBD condition, in a disease activity-dependent manner.

Fig. 2 Distinct expression pattern of DNAM-1 and TIGIT on circulating and mucosa-resident T-cell populations. Percentage of DNAM-1⁺ (**a**) and TIGIT⁺ (**b**) cells on mucosal and peripheral blood CD4⁺ and CD8⁺ T-cell subsets of age- and sex-matched non-IBD controls (C, thin border, *n* = 15) and IBD patients (IBD, thick border, *n* = 32 **a**, *n* = 49 **b**). Bars represent median and 10–90 percentile range. *P*-values refers to Wilcoxon's non-parametric test for paired samples. **c** DNAM-1 (left) and TIGIT (right) expression on mucosal (upper panels) and peripheral blood (lower panels) CD4⁺ and CD8⁺ T cells of a representative control individual: isotype control mAb (shaded profile), DNAM-1, or TIGIT (black line)

The expression pattern of the two co-receptors was distinctly different on peripheral blood T-cell subsets (Supplementary Fig. 2) and mostly comparable between pediatric controls and IBD patients, except for a lower frequency of DNAM-1-expressing DN T cells. The paired analysis of peripheral blood and gut mucosa-infiltrating T-cell subsets in the same individuals confirmed that the percentage of mucosal DNAM-1⁺CD4⁺ and CD8⁺ T cells was strikingly lower, with respect to their circulating counterparts (Fig. 2a). Conversely, the percentage of TIGIT⁺ cells was significantly higher on mucosal T cells, when compared with peripheral blood populations (Fig. 2b). Interestingly, the divergent expression pattern of DNAM-1 and TIGIT between blood and mucosa populations was preserved in IBD patients.

Collectively taken, these data highlight a compartmentalized expression pattern of DNAM-1 and TIGIT on circulating and gut mucosa-infiltrating T-cell populations. They also suggest that IBD-associated alteration of these receptors is tightly linked to the gut microenvironment; indeed, DNAM-1 and TIGIT perturbation on tissue-infiltrating CD4⁺ and CD8⁺ T populations is not observed in their circulating counterparts.

Evaluation of the expression of mucosa-addressing receptors on DNAM-1⁺ and TIGIT⁺ circulating T cells
The peculiar representativity of DNAM-1⁺ and TIGIT⁺ mucosal T cells may depend on selective recruitment of peripheral blood populations. To test this hypothesis, we analyzed the expression pattern of CCR6 and CD103 mucosa-addressing receptors on circulating DNAM-1⁺ and TIGIT⁺ CD4⁺ and CD8⁺ T-cell populations. Indeed, CCR6 binds mucosa-restricted CCL20 chemokine and CD103 (αEβ7) integrin–E-cadherin interaction is involved in lymphocyte recruitment and retention to the gut epithelium.^{25,39–41} The percentage of peripheral blood CD4⁺ and CD8⁺ T cells expressing these two mucosa-addressing receptors was comparable in IBD patients and non-IBD controls (Supplementary Fig. 3a, b).

DNAM-1⁺ CD4⁺ and CD8⁺ T cells expressed higher levels of both homing receptors, as compared with DNAM-1[−] counterpart (Fig. 3a, b). TIGIT⁺CD4⁺ T cells expressed more CCR6 and CD103 than TIGIT[−] counterpart, whereas TIGIT⁺CD8⁺ T cells were expressing more CD103 and less CCR6 (Fig. 3c, d). The pattern of mucosa-addressing receptors on DNAM-1- and TIGIT-expressing T cells was largely preserved in IBD patients.

In sum, DNAM-1⁺ and TIGIT⁺ T cells are both equipped with homing receptors to allow their preferential recruitment to the gut mucosa compartment. This evidence implies that selective recirculation only partially contributes to the establishment of the peculiar pattern of the two co-receptors in healthy gut mucosa T cells and does not explain the alterations observed in inflamed and non-inflamed tissue samples of IBD patients.

Modulation of DNAM-1 and TIGIT expression by mucosa microenvironment-related factors

Several factors in the mucosa microenvironment may concur to modulate DNAM-1 and TIGIT co-receptor expression. Healthy gut-resident T-cell populations are continually exposed to activating stimuli, thanks to the abundant presence of commensal- and diet-derived antigens, a wide variety of growth factors and cytokines, and cell–cell interactions with both immune and stroma components.^{21,24–28}

We observed that the expression of DNAM-1 and TIGIT, evaluated as the percentage of positive cells (Supplementary Fig. 4a, d), and co-receptor surface intensity (Supplementary Fig. 4b, e), markedly augmented on peripheral blood CD4⁺ and CD8⁺ T cells upon a 3-day polyclonal stimulation with anti-CD3 plus anti-CD28 monoclonal antibody (mAb), thus confirming and expanding previous evidence of DNAM-1 and TIGIT upregulation during T-cell activation.²⁰

Among other cytokines, IL-15 plays a critical role in gut immune homeostasis and in chronic intestinal inflammation.⁴² Here we

Fig. 3 Distribution of CCR6 and CD103 mucosa-addressing receptors on DNAM-1- and TIGIT-expressing T cells. Percentage of CCR6⁺ (a, c) and CD103⁺ (b, d) cells on DNAM-1-positive or DNAM-1-negative (a, b), and on TIGIT-positive or TIGIT-negative (c, d) fractions of peripheral blood CD4⁺ and CD8⁺ T lymphocytes in non-IBD controls (C, thin border, $n = 7$ a, $n = 13$ b, $n = 7$ c, $n = 15$ d) and IBD patients (IBD, thick border, $n = 36$ a, $n = 38$ b, $n = 35$ c, $n = 53$ d). Bars represent median and 10–90 percentile range. *P*-values refer to Wilcoxon's non-parametric test for paired samples

show that a 24 h treatment with IL-15 induced the upregulation of TIGIT, but not of DNAM-1, in terms of either percentage of positive cells (Fig. 4a, d), and surface receptor density (Fig. 4b, e), on circulating CD4⁺ and CD8⁺ T cells; IL-2 and IL-21, members of the same cytokine family, showed a similar effect (data not shown).

Prolonged contact with cell-associated PVR and Nectin-2 shared ligands may also affect DNAM-1 and TIGIT expression. Our analysis shows that PVR was physiologically expressed on the vast majority of EpCAM⁺ colon epithelial cells (Fig. 5a). Differently, Nectin-2 was basally present on a low percentage of epithelial cells and tendentially augmented on inflamed mucosa samples of active IBD patients (Fig. 5c). Notably, PVR and Nectin-2 were basally expressed on a limited fraction of T cells (Fig. 5b, d). Interestingly, Nectin-2, but not PVR, was significantly upregulated on T cells of active IBD patients.

Interestingly, we found that in vitro treatment with IL-10 or transforming growth factor (TGF)- β negatively regulated Nectin-2 expression on HT-29 gut epithelial cell line (Fig. 5e), without affecting PVR levels (data not shown). Indeed, IL-10 and TGF- β anti-inflammatory factors are abundantly present in healthy gut mucosa and their diminution is a feature of IBD condition.³⁷

Altogether, these results report the in vitro upregulation of TIGIT by IL-15, characteristic constituent of gut mucosa tissue. They also first describe the asset of PVR and Nectin-2 ligands on both epithelial and hemopoietic components in the healthy gut mucosa environment. Further, our data suggest a role for anti-inflammatory cytokines in negatively controlling Nectin-2 expression and indicate that Nectin-2 upregulation on epithelial and T cells is a feature of inflamed gut microenvironment. Our data support a role for microenvironment-associated signals in the establishment of the physiological pattern of co-receptor-expressing mucosal T cells and indicate that upregulation of ligand expression correlates with the diminution of DNAM-1 and TIGIT in IBD inflamed tissue.

DNAM-1 and TIGIT interplay in the regulation of CD4⁺ and CD8⁺ T-cell proliferation and functional capability

It has been suggested that DNAM-1 and TIGIT regulate T-cell proliferation in opposite ways.^{11,20} In this scenario, DNAM-1/TIGIT/

ligand axis may play a role in the homeostasis of the mucosal T-cell pool and its disturbance may be involved in IBD. To gain more information on the dynamics of the two co-receptors during T-cell proliferation, we analyzed the expression of DNAM-1 and TIGIT co-receptors on proliferating and non-proliferating peripheral blood T cells, upon a 3-day stimulation of healthy donor peripheral blood mononuclear cell (PBMC) with anti-CD3/CD28 mAbs. Interestingly, DNAM-1 was enriched in the proliferating fraction, whereas TIGIT⁺ cells were more abundant in the non-proliferating fraction, in both CD4⁺ and CD8⁺ T-cell subsets (Fig. 6a, b). This suggests that the two co-receptors mark cell populations endowed with different proliferative capability, under our experimental conditions.

To evaluate a direct role for DNAM-1 and TIGIT in regulating T-cell proliferation, healthy donor PBMCs were stimulated with anti-CD3/CD28 mAbs, in the presence of either plastic-bound PVR-Ig fusion protein or PVR-expressing Ba/F3 transfectant (PVR-Ba/F3). Stimulation with anti-CD3/CD28 mAbs induced the prompt upregulation of PVR, but not Nectin-2, on several BMC populations, already after 24 h of stimulation (Battella S. and Palmieri G., data not shown), as previously reported.²⁰ The addition of PVR-Ig or PVR-expressing Ba/F3 transfectant provides a significant excess of the shared ligand for DNAM-1 and TIGIT, as demonstrated by the relative diminution of the surface levels of both co-receptors on CD4⁺ and CD8⁺ T-cell populations (data not shown). The addition of PVR-Ig or PVR transfectants significantly increased the proliferative capability of CD4⁺ and CD8⁺ T cells, measured as the percentage of proliferating cells, as well as the number of cell divisions performed (Fig. 7a–d, respectively). Even in the presence of excess of PVR ligand, DNAM-1⁺ T cells were more represented in the proliferating fraction (Fig. 8a), whereas TIGIT⁺ cells were enriched among non-proliferating (Fig. 8b) CD4⁺ and CD8⁺ T cells, suggesting that DNAM-1/PVR interaction amplifies CD3/CD28-dependent T-cell proliferation. In accordance with this hypothesis, the presence of anti-DNAM-1 monoclonal antibody Fab₂ fragment abrogated the enhancement of T-cell proliferation provided by PVR-Ig fusion protein or by PVR transfectants (Fig. 8c, d, respectively). Interestingly, T cells that co-express DNAM-1 and TIGIT displayed a lower proliferation rate,

Fig. 4 IL-15 treatment upregulates TIGIT, but not DNAM-1, on T cells. Percentage of DNAM-1⁺ (a) and TIGIT⁺ (d) cells, and their surface density, expressed as mean fluorescence intensity (MFI, b, e), on CD4⁺ and CD8⁺ peripheral blood T cells after 24 h culture in the presence (gray symbols) or absence (white symbols) of IL-15. *P*-values refer to Wilcoxon's non-parametric test for paired samples (*n* = 10 independent healthy donors). Histograms of DNAM-1 (c) and TIGIT (f) expression in CD4⁺ and CD8⁺ T cells of a representative donor: isotype control mAb (clg, shaded profile), unstimulated (ns, gray line), and IL-15-stimulated (IL-15, black line) cells

when stimulated in the presence of wt-Ba/F3 cell line (Fig. 9a), thus behaving like DNAM-1⁺TIGIT⁺ cells (Fig. 9b) and not like DNAM-1⁺TIGIT⁻ cells (Fig. 9c); however, this pattern was reversed when the stimulation was carried out in the presence of PVR-expressing transfectant, for both CD4⁺ and CD8⁺ T cells.

These data suggest that DNAM-1 and TIGIT contribute, in opposite ways, to the regulation of TCR-dependent T-cell proliferative response, and that large ligand availability allows DNAM-1 to overcome the proliferative defect of DNAM⁺TIGIT⁺ co-expressing cells.

Finally, we assessed the capability to produce interferon- γ (IFN γ) of DNAM-1- and TIGIT-expressing CD4⁺ and CD8⁺ T-cell populations. Freshly isolated PBMCs, obtained from pediatric IBD patients or from non-IBD control pediatric subjects, were short-term (4.5 h) stimulated with Phorbol 12-myristate 13-acetate (PMA)/Ionomycin; in these conditions, functional response is mainly restricted to "antigen-experienced" (effector/memory) T-cell populations. DNAM-1⁺ and TIGIT⁺ CD4⁺ and CD8⁺ T cells were markedly enriched among IFN γ -competent cells, when compared with DNAM-1⁻ or TIGIT⁻ counterparts, and this pattern was not altered in IBD patients (Fig. 10a, b).

Altogether, these data clearly show that DNAM-1 and TIGIT co-receptors mark "primed" T cells, capable of providing prompt effector response, and confirm the dependence of DNAM-1 and TIGIT expression on previous activation. Nevertheless, DNAM-1 confers an advantage for, while TIGIT associates with an impairment of, the proliferative capability of peripheral blood T cells. Moreover, the net outcome of the opposing effects of the two co-receptors seems to depend on the availability of PVR shared ligand.

DISCUSSION

The regulation of T-cell activation and effector functions is a crucial issue in the gut mucosa microenvironment, where several effector/memory populations live in close proximity with a vast array of diet- and commensal flora-derived luminal antigens.^{25–28} The equilibrium between the capability to deploy a prompt reaction to pathogenic insults and the maintenance of homeostasis is physiologically guaranteed by multiple tolerance mechanisms. The dysregulation of gut mucosal T-cell responses indeed plays a crucial role in the immune-mediated tissue damage at the basis of IBD chronic inflammatory condition.^{26,30–33}

Our work focused on DNAM-1 and TIGIT T-cell co-receptors, whose respective activating and inhibitory roles in the regulation of mucosal T-cell immunity are almost completely unknown. We found that the two co-receptors display a peculiar compartmentalized expression pattern on mucosal T cells where, differently from blood counterparts, TIGIT was much more expressed than DNAM-1. Interestingly, a marked downregulation of TIGIT and, more limitedly, of DNAM-1, characterized mucosal T-cell populations of pediatric IBD patients in a disease activity-dependent manner. In particular, TIGIT intensity was significantly reduced on both CD4⁺ and CD8⁺ T-cell populations that infiltrate inflamed lesions of active IBD patients, thus representing a specific feature of the active form of the disease; in addition, a lower percentage of TIGIT-expressing CD4⁺ T cells characterized the non-inflamed tissue samples of remitting IBD patients.

Reduction of DNAM-1 and TIGIT levels on T cells in inflamed lesions of active IBD patients may be due to receptor downmodulation, fostered by larger availability of ligands, as it strictly correlates with the upregulation of Nectin-2 ligand on both

Fig. 5 Expression of DNAM-1 and TIGIT shared ligands in the gut mucosa microenvironment and modulation by anti-inflammatory cytokines. Percentage of PVR⁺ (**a**, **b**) and Nectin-2⁺ (**c**, **d**) cells on colon epithelial cell (EpCAM⁺CD45⁺, **a**, **c**), and T-cell (CD3⁺CD45⁺, **b**, **d**) fractions, isolated from mucosa samples of active (A, *n* = 10) and remission (R, *n* = 7) IBD patients and age- and sex-matched non-IBD controls (C, *n* = 6). **e** Nectin-2 expression on HT-29 colon epithelial cell line, upon treatment with either IL-10 or TGF-β (*n* = 6 independent experiments). Bars represent median and 10–90 percentile range. *P*-values refer to Kruskal–Wallis (**a–d**) and Wilcoxon's (**e**) non-parametric tests. **f** Histograms of Nectin-2 expression on HT-29 colon epithelial cell line: unstimulated (shaded profile), IL-10 (black line), and TGF-β (gray line) cells. Marker identifies Nectin-2⁺ cells, based on isotype control Ig staining. Numbers in insets are the percentage of Nectin-2⁺ cells

epithelial and hemopoietic components. In this regard, we previously reported that ligands for NKG2D T-cell activating co-receptor are also hyperexpressed on infiltrating leukocytes in active IBD tissue lesions.⁴³ On the other hand, the presence of a lower percentage of TIGIT⁺ T cells in non-inflamed tissue samples of remitting IBD patients may indicate long-lasting changes in the representativity of selected T-cell subsets and thus represent another evidence of the enduring dysregulation of the mucosal CD4⁺ T-cell compartment in pediatric IBD patients, in accordance with our previous report.⁴³ Indeed, TIGIT has been previously shown to identify a distinct, more functional, population of Treg and it has been reported to promote the stability and function of various subsets of Treg cells. Moreover, an inverted DNAM-1/TIGIT expression ratio was reported on Treg and on germinal center-associated tonsillar CD4⁺ Tfh. Further, TIGIT identified exhausted/hypofunctional CD8⁺ T cells in different pathological settings.^{1,3,4,16–18,21–23,44}

We have explored the possible mechanisms at the basis of the distinct expression profile of DNAM-1 and TIGIT co-receptors on mucosal T cells, and the factors that may contribute to the perturbation of this pattern in IBD. The analysis of mucosa-specific homing receptors on DNAM-1⁺ and TIGIT⁺ T cells only partially supports a role for differential recruitment to explain the selective enrichment of TIGIT⁺ T cells in healthy gut mucosa. The reduced presence of TIGIT⁺CD4⁺ T cells, in spite of the augmented CD4⁺ T-cell infiltrate, lends support to the hypothesis that recruitment of circulating populations does not significantly contribute to explain the loss of TIGIT⁺CD4⁺ cells in IBD patients; nevertheless, the disruption of the CCL20-CCR6 axis has been previously

implicated in IBD.⁴⁰ In addition, the percentage of DNAM-1⁺ or TIGIT⁺ T cells was not altered in the peripheral blood of IBD patients, hinting to a compartmentalized disruption of these co-receptors; interestingly, recent work reported that the frequency of circulating CD38⁺TIGIT⁺CD4⁺ correlated with IBD course, instead.⁴⁵

Altogether, our results favor the hypothesis that microenvironment-associated factors are responsible for the physiological high expression of TIGIT and low expression of DNAM-1 on mucosal T-cell populations, and that inflammation-related modifications may explain the alteration of DNAM-1/TIGIT/ligand axis in IBD. In this regard, mucosal microenvironment is characterized by the constitutive presence of a vast array of cytokines that contribute to immune homeostasis and preparedness to response. Our data show that IL-15, a typical constituent of gut mucosa microenvironment,⁴² promotes TIGIT, but not DNAM-1, upregulation on CD4⁺ and CD8⁺ T cells; on the other hand, it has been previously reported that TGF-β, a crucial mediator of intestinal homeostasis, can induce DNAM-1 downregulation on NK cells.⁴⁶

In vitro exposure to ligand-expressing Ba/F3 transfectants led to down-modulation of both DNAM-1 and TIGIT receptors from CD4⁺ and CD8⁺ T-cell surface (data not shown). Here we first show that PVR and Nectin-2, shared ligands for DNAM-1 and TIGIT, were expressed in the intestinal mucosa microenvironment, by both epithelial cells and hematopoietic components. Interestingly, a significant increase of Nectin-2-expressing T cells and a more limited upregulation of Nectin-2 on epithelial cells were observed in the inflamed mucosa of active IBD patients. The larger availability of ligands may, in turn, promote downregulation of

Fig. 6 DNAM-1⁺ and TIGIT⁺ T cells display distinct proliferative capability, upon CD3/CD28 stimulation. Percentage of DNAM-1⁺ (a) and TIGIT⁺ (b) cells on non-proliferating (CFSE^{high}, white symbols, NP) and proliferating (CFSE^{low}, gray symbols, P) CD4⁺ and CD8⁺ T cells, upon a 3-day stimulation with CD3/CD28 mAbs. *P*-values refer to Wilcoxon's non-parametric test for paired samples (*n* = 11 independent healthy donors). Histograms of DNAM-1 (c) and TIGIT (d) expression in CD4⁺ and CD8⁺ T cells of a representative donor: isotype control mAb (shaded profile), non-proliferating (gray line), and proliferating (black line) cells

both co-receptors, thus counteracting IL-15 capability to upregulate TIGIT, as well as the expected proliferation-dependent augmentation of DNAM-1 expression on IBD hyperactivated T cells. In this scenario, the deeper down-modulation of TIGIT than of DNAM-1 in active IBD patients may depend on the higher affinity of TIGIT for both PVR and Nectin-2 shared ligands.⁸ A complex crosstalk regulates DNAM-1 and TIGIT interaction with Nectin-2: beyond a direct competition, TIGIT may disrupt Nectin-2 homodimerization,⁸ which seems to be necessary for DNAM-1 recognition;⁴⁷ moreover, it has been suggested that TIGIT may bind Nectin-2 in cis,⁸ but the possible impact of DNAM-1 and TIGIT interaction with Nectin-2 expressed on T cells, either on receptor expression levels and on functional outcome, is unknown.

The mechanisms regulating Nectin-2 basal expression and the factors leading to its upregulation in inflamed mucosa are unknown. Our results show that IL-10 and TGF- β could specifically downmodulate Nectin-2, but not PVR, on intestinal epithelial cells, thus suggesting that the abundant presence of these immunoregulatory cytokines in the healthy mucosa microenvironment may contribute to the low Nectin-2 expression on enterocytes and, conversely, that defective presence of these factors in the inflamed mucosa of active IBD may result in ligand upregulation.

Comprehensively taken, our data describe the physiological asset of the DNAM-1/TIGIT/ligands system in the T-cell and stromal components of the intestinal microenvironment. We have also detailed the perturbation of this receptor/ligand axis in IBD, which could contribute to dysregulated T-cell activation.

Our data support a direct role of DNAM-1/TIGIT/ligand axis in the regulation of T-cell proliferation and provide evidence that the outcome of the opposing signals initiated by the two co-receptors is regulated by ligand availability. We found that DNAM-1 and

TIGIT mark T cells endowed with different proliferative capability, with TIGIT more expressed on non-proliferating T cells, whereas DNAM-1 was more represented on the proliferating fraction; interestingly, T cells that co-express both co-receptors (DNAM-1⁺TIGIT⁺) were more abundant among non-proliferating cells, hinting to a dominant-negative effect of TIGIT on T-cell proliferation capability. The presence of an excess of ligand, provided either by the co-culture with PVR transfectants, or by PVR fusion protein, markedly increased T-cell proliferation. The addition of anti-DNAM-1-blocking Fab₂ fragment abrogated this augmentation, confirming that DNAM-1 is responsible for PVR-dependent amplification of T-cell proliferation. Strikingly, the presence of high levels of PVR selectively reverted the proliferative defect of T cells that co-express both co-receptors (DNAM-1⁺TIGIT⁺). Collectively taken, these results indicate that, in the presence of ligand excess, DNAM-1 off-balances the proliferative impairment of TIGIT co-expressing cells.

The mechanisms that explain TIGIT inhibitory activity are still unclear: the interference with DNAM-1 dimerization, the higher binding affinity of TIGIT for shared ligands, and the interference with intracellular signaling pathways^{1,3,4,7,8,12,15,19,20} may all contribute to affect the proliferation capability of T cells expressing both receptors, under conditions of limiting ligand availability.

Although the two co-receptors play opposing roles on T-cell proliferation, our data clearly show that they both identified T cells capable of promptly producing IFN γ , in accordance with previous works reporting DNAM-1 and TIGIT to be preferentially expressed on "primed" (effector or memory type) and not "naïve" T cells, and in line with the presence of DNAM-1 on effector Th1 cells and CTL.^{9,11,13}

Fig. 7 The presence of PVR ligand upregulates T-cell proliferative capability. Percentage of proliferating (CFSE^{low}) CD4⁺ and CD8⁺ T cells upon a 3-day stimulation with CD3/CD28 mAbs. Stimulation was performed in the presence of either control Ig (clg, white diamonds) or PVR-Ig (gray diamonds) (**a**, $n = 13$ healthy donors), or with wt-Ba/F3 (wt, white circles) or PVR-Ba/F3 (PVR, gray circles) (**b**, $n = 11$ healthy donors). Percentage of CD4⁺ (**c**) and CD8⁺ T cells (**d**) that completed a different number of cell cycles upon stimulation with CD3/CD28 mAbs, in the presence of either wt-Ba/F3 (white symbols) or PVR-Ba/F3 (gray symbols) ($n = 11$ independent healthy donors). P -values refer to Wilcoxon's non-parametric test for paired samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$. **e** CFSE staining of a representative donor. FSC = forward scatter, gate numbers represents number of cell cycles, np: non-proliferating cells

The abundant presence of TIGIT may contribute to the fine-tuning of T-cell expansion in the ligand-rich healthy gut mucosa microenvironment and to restrain improper antigen-specific T-cell proliferation; the perturbation of TIGIT/ligand axis, together with the altered expression of other T-cell receptor/ligand systems, may contribute to T-cell dysregulation in IBD pathogenesis.^{26,30–33,43} In this regard, a pathogenic role of DNAM-1 in the immune-mediated damage in refractory celiac disease has been reported.⁴⁸ In this scenario, the role of CD96 (that binds PVR) and CD112R (PVRIG, Nectin-2 receptor), belonging to the same molecular family as DNAM-1 and TIGIT,^{1–4} in the regulation of mucosal T-cell functions is unknown and deserves additional analysis.⁴⁹

In conclusion, the peculiar characteristics of the DNAM-1 family/ligands system (i.e., the presence of activating and inhibitory receptors endowed with overlapping binding specificity and different binding affinity; the tight regulation of expression levels of both receptors and ligands by environmental cues and cell activation status) render it particularly apt to finely adjust T-cell functional responsiveness to a changing tissue microenvironment, in a timely manner, under physiological and pathological conditions.

METHODS

IBD patients and controls

All pediatric subjects were referred to the Pediatric Gastroenterology and Hepatology Unit (directed by Professor Salvatore Cucchiara, Department of Mother and Child and Urology, Sapienza University) for ileocolonoscopy. Definite diagnosis of UC or CD was based on widely agreed clinical, radiological, endoscopic, and histological criteria, as well as on the exclusion of infectious and systemic disease, food allergies, and malabsorption syndromes, as previously described.⁴³ The pediatric control population was represented by individuals investigated for unspecific colitis symptoms, in which disorders such as food allergy, malabsorption, and idiopathic inflammation had been excluded (Supplementary Table 1). Colonic biopsies and a blood sample were collected from patients and control individuals, and immediately processed. Whole peripheral blood samples of anonymized healthy donors of Transfusion Center of Sapienza University of Rome were also employed. This study was approved by our Institutional Review Board and was conducted in accordance with the regulations of health information protection policies and the declaration of Helsinki. All patients and controls gave their written informed consent.

Fig. 8 PVR-promotes upregulation of T-cell proliferation in a DNAM-1-dependent manner. Percentage of DNAM-1⁺ or TIGIT⁺ (a, b, respectively) cells on non-proliferating (CFSE^{high}, NP) and proliferating (CFSE^{low}, P) CD4⁺ and CD8⁺ T cells, upon a 3-day stimulation with CD3/CD28 mAbs, in the presence of either wt-Ba/F3 (thin border) or PVR-Ba/F3 (thick border). Bars represent median and 10–90 percentile range. *P*-values refer to Wilcoxon's non-parametric test for paired samples (*n* = 12 healthy donors). c, d Percentage of proliferating (CFSE^{low}) CD4⁺ and CD8⁺ T cells upon stimulation with CD3/CD28 mAbs, in the presence of either PVR-Ig (c, diamonds, *n* = 4 healthy donors) or PVR-Ba/F3 (d, circles, *n* = 4 healthy donors), and in the presence (gray symbols) or absence (white symbols) of anti-DNAM-1 Fab₂ fragment. *P*-values refer to *t*-test for paired samples. e CFSE histograms of representative donors, stimulated with CD3/CD28 mAbs, in the presence of PVR-Ig (upper panels) or PVR-Ba/F3 (lower panels), and in the presence (black line) or absence (gray line), of anti-DNAM-1 Fab₂ fragment; markers identify proliferating and non-proliferating cells. Numbers in insets are the percentage of proliferating (CFSE^{low}) cells in the presence (black) or in the absence (gray) of anti-DNAM-1 Fab₂ fragment

Fig. 9 The presence of excess ligand reverts the proliferative defect of DNAM-1 and TIGIT co-expressing T cells. Percentage of DNAM-1⁺TIGIT⁺ (a), DNAM-1⁻TIGIT⁺ (b), and DNAM-1⁺TIGIT⁻ (c) cells on non-proliferating (CFSE^{high}, triangles, NP) and proliferating (CFSE^{low}, circles, P) CD4⁺ and CD8⁺ T cells, upon a 3-day stimulation with CD3/CD28 mAbs, in the presence of either wt-Ba/F3 (white symbols) or PVR-Ba/F3 (gray symbols). *P*-values refer to *t*-test for paired samples (*n* = 11 independent healthy donors)

Peripheral blood mononuclear cell isolation

PBMCs from heparinized blood samples were isolated by lymphoprep (Ficoll-Hypaque, Cedarlane) density gradient centrifugation. After washing in phosphate-buffered saline (PBS), cell samples were used for in vitro stimulation, immunostaining, and cytofluorimetric assays.

Mucosal mononuclear cell isolation

Mucosal mononuclear cell suspensions were obtained by mechanical dissociation of colon biptic fragments on a 40 µm cell strainer (BD Biosciences). Undisrupted fragments were subsequently incubated for enzymatic digestion with type I collagenase (GIBCO-Invitrogen USA, 3 mg/ml in RPMI medium with 5 mM

Fig. 10 DNAM-1- or TIGIT-expressing T cells display a higher competence to promptly produce IFN γ . Percentage of IFN γ -producing cells on DNAM-1-positive or DNAM-1-negative (**a**) and on TIGIT-positive or TIGIT-negative (**b**) fractions of peripheral blood CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes in non-IBD controls (C, thin border, $n = 11$ **a**, $n = 8$ **b**) and IBD patients (IBD, thick border, $n = 18$ **a**, $n = 12$ **b**), upon short-term stimulation with PMA/ionomycin. Bars represent median and 10–90 percentile range. P -values refer to Wilcoxon's non-parametric test for paired samples. **c** Histograms of IFN γ -producing cells among DNAM-1 $^{+/-}$ (left panels) and TIGIT $^{+/-}$ (right panels) CD4 $^{+}$ and CD8 $^{+}$ peripheral blood T cells of a representative control individual: co-receptor-positive (black line), co-receptor-negative (gray line). Numbers in insets are the percentage of IFN γ -producing cells in co-receptor-positive (black) and co-receptor-negative (gray) cells

CaCl $_2$) for 30 min at 37 °C. Mechanical and enzymatic dissociation cycle was repeated twice, as previously described.^{43,50}

IL-15, IL-21, and IL-2 stimulation

Freshly isolated PBMCs were stimulated with either IL-15, IL-21 (both at 50 ng/ml, Peprotech, UK), or IL-2 (1000 U/ml, Peprotech) for 24 h at 37 °C in a 24-well plate at 1.5×10^6 cells/ml concentration, in RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum (FCS) and 1% glutamine (Euroclone, Milan, Italy) (complete medium), at 37 °C in 5% CO $_2$. After collecting, cells were washed with PBS and stained for surface antigens for 30 min at 4 °C.

PMA/ionomycin stimulation

Freshly isolated PBMCs were stimulated with PMA (50 ng/ml, Sigma-Aldrich, USA) and ionomycin (0.5 μ g/ml, Sigma-Aldrich) for 4.5 h at 37 °C, in complete medium and in the presence of Brefeldin A (10 μ g/ml, Sigma-Aldrich, USA) and Monensin (50 μ M, Sigma-Aldrich), to allow for intracellular accumulation of neo-synthesized proteins. Cells were then washed with PBS, stained for surface antigens, fixed with 2% paraformaldehyde (PFA, Sigma-Aldrich) for 20 min at room temperature, permeabilized with 0.5% saponin/1% FCS for 30 min at room temperature, and then stained for intracellular IFN γ quantification, as previously described.⁵¹

Generation of Ba/F3 transfectants

PVR-Ba/F3 (stably expressing PVR transfectants) were generated by electroporation of Ba/F3 mouse pro-B-cell line subclone⁵² with pEF6 vector carrying the cDNA for PVR (kindly provided by M. Colonna, Washington University School of Medicine, St. Louis) and stable transfectants were selected using 10 μ g/ml Blasticidin (Sigma-Aldrich). Cells were periodically tested for mycoplasma contamination by EZ-PCR Mycoplasma Test Kit (Biological Industries). Supplementary Fig. 5 reports a representative staining of parental (wt-Ba/F3) and transfectant (PVR-Ba/F3) cell lines.

Proliferation assay

PBMCs from healthy adult donors were stained with CFSE fluorescent dye (5 μ M, Sigma-Aldrich) and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (anti-CD3 OKT3 mAb clone was obtained from ATCC (Rockville, MD) and purified in our laboratory; anti-CD28 mAb was from BD Biosciences) for 3 days in complete medium, at 37 °C in 5% CO $_2$. Where indicated, PBMCs were stimulated in the presence of PFA-fixed wild-type Ba/F3 of PVR-transfected Ba/F3 cell lines, at a 2:1 ratio, or with plastic-bound PVR-Ig fusion protein (2.5 μ g/well, ThermoFisher). In some experiments, stimulation was carried out in the presence of anti-DNAM-1 Fab $_2$ fragment (5 μ g/ml). Fab $_2$ fragment was generated from 11a8 clone anti-DNAM-1 mAb, by using the Pierce™ Mouse IgG1 Fab and F(ab') $_2$ Preparation Kit (ThermoFisher), following the manufacturer's indications. Proliferating cells were identified by 5 (6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) dilution assay, as described by Quah and Parish.⁵³

Epithelial cell line treatment

EpCAM $^{+}$ HT-29 colon epithelial cell line was stimulated with IL-10 or TGF- β (50 and 25 ng/ml, respectively, Peprotech) for 24 h in a 24-well plate at 0.5×10^6 cells/ml concentration, in complete medium, at 37 °C in 5% CO $_2$. After collecting, cells were washed with PBS and stained for surface antigens for 30 min at 4 °C.

Antibodies, gating strategy, and cytofluorimetric analysis

For identification of T-cell populations in PBMCs and mucosal cells, the following antibodies: CD3 PE-Cy7, CD4 APC-H7, CD56 FITC, CD8 PerCP, CD45 FITC, CD103 FITC, and CCR6 APC, were used in various combinations; epithelial cells were identified with anti-EpCAM PE-Cy5.5; anti-IFN γ and APC was used to identify cytokine-producing cells; DNAM PE, TIGIT APC, TIGIT PE, PVR PE, and Nectin-2 APC were used to identify co-receptor or ligand positivity. All antibodies were from BD Biosciences, USA, with the exception of TIGIT PE and TIGIT APC (E-Bioscience, USA), PVR PE (BioLegend,

USA), and Nectin-2 APC (R&D Systems, USA). All antibodies were used at optimal dilutions. Sample staining was performed for 30 min at 4 °C.

The lymphocyte region was identified with physical parameters (FSC and SSC) in PBMCs and by a combination of SSC physical parameter and CD45 positivity in mucosal cell suspensions; T-cell subsets were identified by fluorochrome-conjugated mAbs positivity (Supplementary Fig. 6).

All samples were analyzed with FACSCANTO II (BD Biosciences, USA) using FlowJo (ThreeStar, Inc., USA) software.

Statistical analysis

Differences between groups were analyzed with parametric (paired *t*-test) or non-parametric (Mann–Whitney, Kruskal–Wallis, or Wilcoxon) tests, as appropriate, by using SPSS v24 (IBM, USA) and Prism v6.0 (GraphPad, USA) software.

Supplementary Material is linked to the online version of the paper at <http://www.nature.com/mi>.

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AUTHOR CONTRIBUTIONS

S.B., R.L.S., L.F., C.C., and C.P. performed the experiments. S.O. and S.I. provided clinical samples and patients' classification. A. Soriani and S.M. provided important reagents and expertise. A. Santoni provided critical input. S.B. and G.P. designed the study, performed data analysis and interpretation, and wrote the paper. All the authors revised and approved the manuscript.

ADDITIONAL INFORMATION

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