Zoledronate can induce colorectal cancer microenvironment expressing BTN3A1 to stimulate effector γδ T cells with antitumor activity

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gammadelta (γδ) T lymphocytes are involved in stress responses to injured, infected or transformed cells.\textsuperscript{1,2} The most representative γδ T cell subset in the blood is the Vγ9Vδ2 (3–5% of circulating γδ T lymphocytes), while the subset bearing the Vδ1 chain is <1–2% and is mainly present in the mucosal-associated lymphoid tissue.\textsuperscript{1,3} Vδ2 T lymphocytes recognize unprocessed non-peptide molecules, including phosphoantigens (PAg) derived from the mevalonate pathway in mammalian cells, and via the 1-deoxy-D-xylulose-5-phosphate pathway, in bacterial cells.\textsuperscript{1,5} γδ T cells also recognize NKG2D ligands (MICA, MICB and ULBP\textsubscript{s}), overexpressed at the cells surface by viral infections or tumor transformation.\textsuperscript{1,3} Another activation signal can be delivered via FcR-II\textsubscript{a}/CD16 that, upon interaction with the Fc of IgG, initiates the antibody-dependent cellular cytotoxicity to destroy opsonized cells or microorganisms.\textsuperscript{1,3} After activation, γδ T cells proliferate, acquire cytotoxic ability and secrete a pattern of Th1 pro-inflammatory cytokines, such as IFN\textsubscript{γ} and TNF\textsubscript{α}.\textsuperscript{1,2} For this unconventional antigen recognition and multiple activation pathways, γδ T lymphocytes are antitumor effector cells in several cancer types, including colorectal cancer (CRC), and potential suitable tools for anticancer therapy.\textsuperscript{3–8}

Different drugs can be utilized to improve the mechanisms of γδ T cell activation,\textsuperscript{2,3,8} in particular, synthetic pyrophosphate-containing compounds have been proposed for cancer immunotherapy on the basis of their ability to stimulate γδ\textsubscript{T} cells.\textsuperscript{9–12} Moreover, aminobisphosphonates (N-BPs), in addition to their effect of inhibiting osteoclastic bone resorption,\textsuperscript{13} lead to γδ T cell activation and proliferation, with a consequent increased number of γδ T cells in peripheral blood, displaying antitumor activity.\textsuperscript{14–18} Indeed, N-BPs, such as zoledronate (Zol), are chemically stable analogs of inorganic pyrophosphate (IPP) that inhibit the mevalonate pathway and upregulate IPP accumulation, promoting antitumor Vγ9Vδ2 T cells \textit{in vitro} and \textit{in vivo}.\textsuperscript{15–20} For this reason, different N-BPs have been used in anticancer clinical trials.\textsuperscript{21–24}

In the last years, the butyrophilin 3A (BTN3A, CD277) family, structurally related to B7 co-stimulatory molecules, has emerged as important structure contributing to Vγ9Vδ2 T cell stimulation.\textsuperscript{25,26} This family in humans is composed of three...
isoforms: BTN3A1, BTN3A2 and BTN3A3, characterized by two extracellular immunoglobulin domains, a transmembrane region and all, but BTN3A2, by an intracellular signaling domain, named B30.2.27,28 It has been demonstrated that only the B30.2 domain of BTN3A1, thanks to a positively-charged pocket, can bind PAg and drive the activation of Vγ9Vδ2 T cells through conformational changes of the extracellular domains.29-31 Two recent reports describe the importance of BTN3A1 binding to the cytoskeleton, and its consequent membrane stabilization, to stimulate the PAg-induced tumor cell reactivity by human Vγ9Vδ2 T cells.32 PAg accumulation would also induce BTN3A1 conformational changes responsible for its recognition by Vδ2 T cells.33

In this paper, we show that (i) colon cancer cells, exposed to Zo, stimulate the expansion of Vδ2 T cells with effector memory (EM) phenotype and cytotoxic activity; (ii) this effect is partially related to BTN3A1 expression and cellular re-distribution; (iii) BTN3A1 is detected in CRC at the tumor site, both on epithelial and on stromal cells, close to areas infiltrated by Vδ2 T lymphocytes; (iv) Zo is effective in stimulating antitumor effector Vδ2 T cells from ex vivo CRC cell suspensions; and (v) both CRC cells and tumor-associated fibroblasts (TAF) can be primed by Zo to trigger Vδ2 T cells.

Results

**CRC exposed to Zo stimulate the expansion of Vδ2 T cells with antitumor cytotoxic activity**

Fourteen different established CRC cell lines (Colo205, Colo741, Colo320, SW620, HCT15, HCT116, DLD1, WiDr, LoVo, LS180, HT29, CaCo2, SW48 and SW480) were co-cultured with peripheral blood T cells from healthy donors, at the T:CRC ratio of 10:1, in the presence or absence of 5 μM Zo and IL2. As shown in Fig. 1, many of these CRC cell lines (LS180, LoVo, WiDr, Colo741, Colo320 and to a lesser extent SW620, HT29, DLD1 and Colo205), when exposed to Zo, were able to induce the expansion of γδ T lymphocytes, after 20 d of culture (Figs. 1A and 1C, 4 representative CRC cell lines; Fig. 1B, all the cell lines tested, mean ± SD from six experiments with six different T cell donors for each cell line). Indeed, the percentage of γδ T lymphocytes raised from less than 5% in the starting T cell populations (range 2–5%, not shown), up to 80% in the co-cultures with Zo-treated CRC (Fig. 1A lower panels vs. IL2 alone in upper panels; Fig. 1B dark gray columns), values superimposable to those obtained using monocytes exposed to Zo (Fig. 1B). No expansion of γδ T cells was detected in the co-cultures set up in the absence of Zo (Fig. 1A upper panels and Fig. 1B white columns). Zo added to purified T cells alone did not exert any stimulating effect (Fig. 1A, lower left panel, one representative experiment).

As it has been reported that several tumor cell lines require higher doses of Zo to exert their stimulating activity of γδ T cells,34 three low-stimulating (SW620, HCT15, DLD1) and one stimulating (LS180) CRC cell lines were pre-treated (4 h) with high doses (100 μM and 50 μM) of Zo, washed and co-cultured with purified T cells as above. As shown in Fig. 1C, high doses of Zo were effective on LS180 cell line, while the other cell lines did not acquire the ability to stimulate γδ T cell growth even at the highest dose of Zo.

We chose the LS180 cell line as an example of stimulating CRC cells, to analyze the phenotype of the γδ T cell populations obtained after 20 d of culture. In particular, we focused on the distinction of naive (N), bearing CD27 and CD45RA molecules, central memory (CM), showing only CD27, EM that are double negative, and terminal-differentiated memory cells (TEMRA) that are surface positive CD45RA.35,36 We found that upon Zo
treatment, LS180 cells could drive the expansion of γδT cells showing the characteristics of EM T lymphocytes (i.e., absence of CD27 and CD45RA) (Fig. 2A, two representative experiments; Fig. 2B mean of eight experiments). Of note, this γδ T cell population exerted a cytotoxic activity against the stimulating LS180 cell line (Fig. 2C, left panel white columns) and against two other cell lines (Fig. 2C white columns: HCT15, central panel; DLD1, right panel); the cytolytic effect was enhanced by exposing the CRC cell lines used as targets to Zol (Fig. 2C, dark gray columns). Similar results, were obtained with WiDr, Colo320 and LoVo stimulating cell lines in two experiments for each cell line (not shown). These data indicate that Zol added to CRC cells, besides determining their sensitization to cytotoxicity exerted by activated γδ T cells, induces the expansion of EM γδ T lymphocytes able to kill both the stimulating tumor cells and other cancer cell lines.

**BTN3A1 contributes to zoledronate effect in CRC cell lines**

We then investigated the heterogeneity of the stimulating ability displayed by the various CRC cell lines. As BTN3A1 is a molecule essential for the recognition of phosphate antigens by γδ T lymphocytes,25-31 we first analyzed BTN3A1 protein by immunofluorescence and by western blot with the anti-CD277 mAb recognizing the immunoglobulin-like extracellular domain of BTN3A1, or with a rabbit polyclonal anti-BTN3A1 antiserum recognizing the C-terminal domain of the molecule. We found no significant difference in the surface expression of CD277, evaluated by cytometric analysis; however, the reactivity of this antibody in immunofluorescence is very low, also on healthy monocytes (not shown). In western blot, the same mAb could recognize the major reported bands for BTN3A1 (Fig. 3A, 57 kDa and 37 kDa) in most CRC cell lines, with the exception of CaCo2 cells; the 57 kDa band was identified also by the anti-BTN3A1 antiserum in the majority of cell lines and the 37 kDa band was evident in CaCo2 and HT29 cells (Fig 3A). These data would indicate that the efficiency in Zol stimulating effect is not directly related to the amount of BTN3A1 expressed; indeed, some stimulating cells showed low levels of BTN3A1 in immuno blotting (LoVo), whereas in some low stimulators a strong reactivity was detected (HCT15, HCT116) (Fig. 3). Thus, we asked whether the different distribution of BTN3A1, in particular its membrane localization or cytoskeletal association, was responsible for the activity of the molecule, as recently reported.32,33 We found that in DLD1 (a low-stimulating CRC cell line) a considerable amount of protein is detected in the cytosolic fraction (Fig. 3B, left blot, Cyt), probably containing also small vesicles not pelleted at low speed after nuclei isolation, while in the stimulating LS180 cell line BTN3A1 is mainly present in the membrane-enriched fraction (Fig. 3B, right blot, M). Upon Zol treatment, BTN3A1 is also detectable in the cytoskeleton-enriched fraction (that contains detergent-resistant cell-membrane fragments tightly linked to the

**Figure 2.** Expansion of effector memory antitumor Vδ2 T cell lymphocytes upon co-culture with Zol-treated LS180 CRC cell line. Panels A and B: Peripheral blood T lymphocytes were co-cultured for 20 d with LS180 CRC cell line, in the absence or presence of Zol (5 μM) and IL2. (A) Representative phenotype of Vδ2 T cells from two donors (donor 1, upper plots, donor 2, lower plots), co-cultured with LS180 and IL2 alone or with Zol-treated LS180, stained with APC-anti Vδ2, PE-anti-CD27 and PE-Cy7-anti-CD45RA. (B) Results expressed as percentage of effector memory (EM, CD45RA−CD27−) T cells, terminal-differentiated effector memory (TEMRA,CD45RA−CD27−) T cells, naive (N, CD45RA−CD27−) T cells or central memory (CM, CD45RA−CD27+) among Vδ2 T lymphocytes immediately after separation (white bars) or on day 20 of co-culture (black bars). Mean ± SD from eight experiments. ***p < 0.001 versus T lymphocytes after separation (white bars). (C): Vδ2 T cells derived from co-cultures with Zol-treated LS180 CRC cells were tested in a 4 h 51Cr release assay against untreated (white bars) or Zol-treated (5 μM for 24 h, gray bars) LS180 (left histogram) HCT15 (central histogram) or DLD1 (right histogram) cell lines at the E:T ratio of 20:1, 10:1 and 5:1. Results are expressed as percentage specific lysis, calculated as described in Materials and Methods, the mean ± SD from three experiments is shown. *p < 0.05 vs. Nil.
Zoledronate is effective in stimulating V\(\alpha\)2 effector T cells from ex-vivo CRC cell suspensions

To verify the possible effect of Zol in the tumor microenvironment, we isolated cell suspensions from different CRC specimens and performed cell cultures in the presence or absence of Zol (5 \(\mu\)M on day 0, IL2 added on day 1). As shown by immunofluorescence analysis (Fig. 6A), the starting cell population (FACS analysis in open gate on viable cells on the basis of forward and side scatter, FSC and SSC) was composed of T lymphocytes (CD3\(^+\)), epithelial cells (EPCAM\(^+\)) and monocytes (CD14\(^+\)) with potential antitumor activity. Effective in inducing the expansion of in vivo infiltrating T cells, Zoledronate significantly enhanced the proportion of CD8\(^+\) T cells (CD8\(^+\)/CD4\(^+\)) and T helper cells (CD4\(^+\)/CD8\(^-\)) in ex-vivo CRC cultures. As shown in Fig. 6D, left and right panels, TAF (collected from CRC cell cultures) and SW620 (collected from CRC cell cultures) were able to activate Zoledronate-sensitive V\(\alpha\)2 effector T cells in the presence of ex-vivo CRC cell suspensions, containing epithelial cells, lymphocytes, TAF and monocytes, is effective in inducing the expansion of infiltrating V\(\alpha\)2 T cells with potential antitumor activity. Along this line, we found that TAF isolated from four CRC specimens, characterized as CD105\(^+\) (as marker of mesenchymal cells) and FAP\(^+\) (not shown), express BTN3A1 (Fig. 7A; note the presence of vimentin and absence of cytokeratin in TAF lysate). When exposed to Zol, TAF were able to induce V\(\alpha\)2 T cell expansion (Fig. 7B, left panels: one representative case of TAF cultured with T lymphocytes isolated from two healthy donors: Fig. 7C, left histograms, black columns mean \pm SEM of four CRC-derived TAF:T co-cultures). Again, this V\(\alpha\)2 T cell population was mainly EM (Fig. 7C, right histograms, black columns) and could be activated in re-directed killing assay, the V\(\alpha\)2-enriched T cell cultures could be activated by the anti-CD3 and anti-V\(\alpha\)2 mAbs, but not by anti-CD8\(^+\) or unrelated mAbs (Fig. 6C); moreover, these cell populations showed a remarkable cytolytic activity against CRC cell lines (Fig. 6D, left and right panels). This data indicate that Zol added to ex-vivo tumor-derived cell suspensions, containing epithelial cells, lymphocytes, TAF and monocytes, is effective in inducing the expansion of infiltrating V\(\alpha\)2 T cells with potential antitumor activity.
Figure 4. Enhancement of BTN3A1 expression and expansion of antitumor Vδ2 T cells. SW620 (A, C, D) or DLD1 (B, C, D) cells were transfected with BTN3A1-containing plasmid and irradiated to avoid the overgrowth of untransfected cells; expression was evaluated on day 2, 5 and 7 by western blot using the anti-CD277 (A, B). (C) Wild type (WT, white columns) or BTN3A1-transfected (black columns) SW620 or DLD1 cells, untreated or treated with Zol (5 μM), as indicated were co-cultured with purified T lymphocytes; the percentage of Vδ2 T cells was evaluated by immunofluorescence with the specific anti-Vδ2 mAb and FACS analysis after 20 d of culture; results are expressed as percentage Vδ2 T lymphocytes and are the mean ± SEM from three transfection experiments with six different T cell donors for DLD1; one representative experiment with two T cell donors for SW620. (D) WT (white columns) or BTN3A1-transfected (black columns) SW620 or DLD1 cells, untreated or treated with Zol (5 μM) as indicated, were used as targets in a 4 h 51Cr release assay using as effectors IL-2-activated peripheral blood Vδ2 T cells at the E:T ratio of 10:1. Results are expressed as percentage specific lysis, calculated as described in Materials and Methods, and are the mean ± SEM from three experiments. *p < 0.01 vs. Zol-treated WT SW620.

Figure 7. Identification of BTN3A1-expressing CRC that can stimulate Vδ2 T lymphocytes. (A) CD277 expression by CRC cells when transfected with BTN3A1-containing plasmid is shown in Western blots. (B) Vδ2 T lymphocytes were killed by CRC cells transfected with BTN3A1 when using Zol treatment; the specificity of the killing effect was determined using a redirected killing assay. (C) Vδ2 T lymphocytes were able to kill CRC cells when the tumor microenvironment was exposed to Zol. (D) BTN3A1-expressing CRC could stimulate the expansion of Vδ2 T lymphocytes when co-cultured with T lymphocytes from healthy donors.

Finally, Vδ2 effector T cells showing antitumor activity can be derived from tumor cell suspensions also using monocytes, isolated from peripheral blood of healthy donors (we could not obtain enough monocytes from CRC cell suspensions), in the presence of Zol (Fig. S2A: two representative experiments; Fig. S2B: mean ± SD of five monocyte: CRC cultures, left histograms: percentage of Vδ2 T cells; right histograms: fold increase vs. the beginning of culture). The cytolytic activity was stimulated by anti-Vδ2 and anti-CD3 mAbs as re-directed killing assay (Fig. S2C) or was detected using CRC cells (Fig. S2D, HCT15: left histogram, CaCo2: right histogram). All these data would indicate that Zol can enable different cell types present in the CRC microenvironment (cancer cells, TAF and monocytes) to induce the expansion of antitumor effector γδ T lymphocytes.

To confirm that Zol can work in vivo, we checked the expression of BTN3A1 at the tumor site. As shown in Fig. 8, BTN3A1 could be detected by PCR in CRC specimens (Fig. 8A, 10 CRC examined), and by western blot as protein in cell lysates obtained from the same CRC samples (Fig. 8B, BTN3A1 detected using either anti-CD277 mAb or anti-BTN3A1 antiserum). Immunohistochemistry (Fig. 8C, two representative cases out of 10 CRC) showed that BTN3A1 can be detected on either epithelial cells or TAF (transglutaminase, TGII or vimentin positive) in CRC, close to areas infiltrated by Vδ2 T lymphocytes (recognized by the anti-Vδ2 mAb BB3).
Discussion

There is general agreement on the role of γδ T lymphocytes-infiltrating solid tumors, including CRC, in the anticancer surveillance, so that treatments focused on γδ T cell-mediated immune responses are now considered as an attractive and promising therapeutic approach in oncology. Stimulating γδ T lymphocytes with PAg, through the engagement of the BTN3A1 molecule, leads to the generation of an efficient antitumor immune response. In particular, N-PBs that induce IPP accumulation, have been used in different clinical trials.

In this paper, we show that (i) the N-BP Zol, besides sensitizing CRC cells to γδ T cell-mediated cytotoxicity, stimulates the expansion of Vδ2 T cells with EM phenotype and antitumor cytotoxic activity; (ii) this effect is partially related to BTN3A1 expression and in particular by its cellular re-distribution; (iii) BTN3A1 is detected at the tumor site, both on epithelial and stromal cells, in the areas infiltrated by Vδ2 T lymphocytes; (iv) Zol is effective in stimulating antitumor effector Vδ2 T cells from ex-vivo CRC cell suspensions; and (v) both CRC cells and TAF can be primed by Zol to trigger Vδ2 T cells.

First, we found that different CRC cell lines exposed to Zol as a source of IPP, could successfully promote the expansion of γδ T lymphocytes able to kill both the stimulating tumor cells and other CRC cell lines. The phenotype of such lymphocyte population, that was mostly naive (co-expression of CD45RA and CD27) at the beginning of the co-culture, was that of EM cells, based on the lack of CD45RA and CD27 expression.

However, non-stimulating CRC cell lines were unable to trigger γδ T cell expansion, even when pre-treated with high doses of Zol; furthermore, IPP production in response to Zol was lower in the three non-stimulating cell lines tested and they did not enhance IPP production in response to high doses of Zol, with the exception of DLD1. Thus, we further investigated any possible difference in the expression of proteins of the butyrophilin family, mainly BTN3A1 that is an essential molecule for...
the recognition of PAg by \( \gamma \delta \) T lymphocytes, due to its B30.2-binding domain.\textsuperscript{25-31}

The amount of BTN3A1 mRNA evaluated by Q-RT-PCR analysis is heterogeneous in stimulating and in low-stimulating CRC cell lines (not shown); the expression of the protein assayed with the anti-CD277 mAb, recognizing the immunoglobulin-like extracellular domain, was not clearly detectable in the CRC cell lines, regardless of their ability to promote \( \gamma \delta \) T cell expansion. When BTN3A1 expression was assayed with an anti-BTN3A1 antiserum recognizing the cytoplasmic tail of the molecule, at least one of the two major reported bands for BTN3A1\textsuperscript{7,32,33} was detected in most CRC cell lines, regardless of their stimulating efficiency. This could explain the finding that also low-stimulating CRC, once treated with Zol, can be killed by \( \gamma \delta \) T lymphocytes. On the other hand, overexpression of BTN3A1 obtained by transfection in two CRC cell lines (SW620 and DLD1) could only partially enhance their stimulating activity or their sensitivity to \( \gamma \delta \) T cell killing upon Zol treatment. It has to be noted that no significant differences in the transcription of the other members of BTN3A family, i.e., BTN3A2 and BTN3A3, was found in the different CRC cell lines (not shown); however, BTNL2, known to downregulate the effect of BTN3A1,\textsuperscript{37} was poorly expressed in most of the stimulating cell lines.

Based on these data, the role of BTN3A1 molecule does not appear to be directly related to the amount of protein expressed; rather, the different distribution of BTN3A1, in particular its cell membrane localization or cytoskeletal association, may be responsible for the activity of the molecule.\textsuperscript{32} It has been recently reported that a PAg-induced conformational change in BTN3A1 leads to its recognition by V\textsubscript{\gamma9}V\textsubscript{\delta2} TCR.\textsuperscript{33} In agreement with this finding, we found that Zol treatment induces a re-distribution of BTN3A1 in TAF and, to a lesser extent in CRC cells, increasing its binding to the cell membrane and to the cytoskeleton, thus explaining the efficiency in stimulating \( \gamma \delta \) T cell expansion and effector function. Nevertheless, CRC cells should also display a functional farnesyl diphosphate synthase to produce IPP and deliver an efficient activating signal to \( \gamma \delta \) T lymphocytes.

An interesting observation is that Zol added to \textit{ex-vivo} tumor-derived cell suspensions, containing epithelial cells, lymphocytes, TAF and monocytes, is effective in inducing the growth of \( \gamma \delta \) T cells showing cytolytic activity against CRC target cells. Thus, whatever the mechanism of BTN3A1 responsible for Zol-induced IPP accumulation, using this drug it is possible to induce the expansion of infiltrating \( \gamma \delta \) T cells with potential antitumor activity. Along this line, in the presence of

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**Figure 6.** Differentiation of \( \gamma \delta \) T cells from \textit{ex-vivo} CRC cell suspensions. (A) Cell suspensions from CRC were stained with specific anti-CD3, anti-\( \gamma \delta \)2, anti-EPCAM, anti-CD14 and anti-CD105 mAbs followed by aAPC-conjugated anti-isotype antiserum and analyzed by flow cytometry. Percentages of positive cells were calculated and results expressed as mean ± SD (n = 10). (B) CRC cell suspensions were cultured in the presence or absence of Zol (5 \( \mu \)M) and IL2 for 20 d, double stained with the specific PE-anti-CD3 and APC-anti-\( \gamma \delta \)2 mAbs and the percentage of positive cells was calculated. Left plots: one representative experiment out of eight. Central histogram: percentage of \( \gamma \delta \)2 T cells obtained from CRC cultures without (white) or with Zol (5 \( \mu \)M, black); mean ± SEM of eight experiments. Right histogram: results analyzed as fold increase (percentage of \( \gamma \delta \)2 T cells on day 20 vs. day 0, white bar IL2 alone, black bar 5 \( \mu \)M Zol). Mean ± SEM (n = 8). (C and D) \( \gamma \delta \)2 T cells obtained from 20 d of CRC cultures, with Zol, were used in re-directed killing assay against the P815 cell line in the presence of the anti-CD8, anti-\( \gamma \delta \)2, anti-CD3 mAbs or an unrelated mAb matched for the isotype (CTR) (C) at the E:T ratio of 5:1, or in a 4 h \( ^{3} \text{H} \) release cytolysis assay against the HCT15 or CaCo2 cell lines (D, left and right histograms) at the E:T ratio of 10:1, 5:1 and 2:1. One representative experiment of three is shown. Mean ± SEM of sample duplicate.
Zol, both TAF and tissue-derived CRC cells can induce the expansion of V\(\delta\)2 effector T lymphocytes that can kill CRC cells, starting from a T cell population isolated from peripheral blood. Also, V\(\delta\)2 cytotoxic effector T cells can be derived from tumor cell suspensions using Zol-treated peripheral blood monocytes.

All these data indicate that Zol can enable different cell types present in the CRC microenvironment (cancer cells,
TAF and monocytes) to induce the expansion of antitumor γδ T lymphocytes, so that N-BPs like Zol can conceivably be proposed in therapeutic schemes of CRC. Of note, BTN3A1 could be detected by PCR in CRC specimens and by western blot as protein in cell lysates obtained from the same CRC samples. Immunohistochemistry showed that BTN3A1 can be detected on either epithelial cells or TAF in CRC, close to areas infiltrated by Vδ2 T lymphocytes. This observation confirms that Zol can work as antitumor immune-stimulator in vivo; moreover, immunohistochemical analysis of BTN3A1 expression at the tumor site, together with the evaluation of BTN1L2, can help to select potentially responders to Zol treatment among CRC patients.

Figure 8. BTN3A1 expression in CRC tissue specimens. BTN3A1 expression was evaluated in CRC tissue sections by Q-RT-PCR (A), western blot (B) or immunohistochemistry (C). (A) RNA was extracted from tissue sections of 10 CRC specimens, reverse transcribed and Q-RT-PCR for BTN3A1 performed. Results are expressed as 1/ΔCt normalized to 18s. (B) Immunoblots of lysates obtained from the indicated CRC tissue specimens, as described in Materials and Methods, with the anti-CD277 mAb (upper blot) or with a rabbit polyclonal anti-BTN3A1 antiserum (lower blot). β-actin is shown as a loading control. (C) Immunohistochemistry of two representative cases out of the 10 indicated in panels A and B, performed as described in Materials and Methods, with the indicated antibodies: polyclonal rabbit anti-BTN3A1 antiserum (arrows), anti-Vδ2 mAb (BB3, arrows in the inset), polyclonal rabbit anti-TGII antiserum, anti-vimentin mAb and a matched isotype-unrelated antibody as negative control (goat anti-rabbit antiserum in the inset of the upper CTR). Slides were counterstained with hematoxylin, coverslipped with Eukitt and analyzed under a Leica DM MB2 microscope with a charged-coupled device camera (Olympus DP70) at a 40× enlargement, as indicated.
Methods

CRC patients’ tissue specimens

Tissue samples were obtained from 10 CRC patients undergoing therapeutic intervention at the Unit of Oncological Surgery, IRCCS-AOU San Martino-IST, Genoa, provided informed consent (the study was approved by the institutional and regional ethical committee, PR163REG2014). Samples were used for (i) preparation of cell suspensions for phenotypic and functional assays; (ii) RNA extraction for Q-RT-PCR; (iii) preparation of cell lysates for immunoblotting; and (iv) immunohistochemistry.

Isolation of CRC cell suspension, primary tumor-associated fibroblasts and epithelial cells

CRC specimens were minced by scissors, transferred into 15 mL conical tube and digested with 2 mg/mL collagenase type I and II (Sigma-Aldrich, Darmstadt, Germany) in RPMI 1640 (Gibco, Monza, Italy) for 90 min at 37°C. Residual tissue debris were removed by soft centrifugation (300 rpm, 1 min), cells were pelleted (1,800 rpm, 10 min) and passed through a 100-um cell strainer (Euroclone, Milan, Italy). Cell suspensions were then purified by density gradient centrifugation (Lympholyte, Cederlane, Burlington, Ontario, Canada) and phenotypically characterized (CD3, CD14, Vimentin, Monitoring, CA, USA) by flow cytometry (see below). A portion of cell suspension was plated in RPMI 1640 10% fetal calf serum (FCS, Sigma), after 16 h non-adherent cells were removed and adherent cells were switched to MEM alpha (Euroclone) complete medium to obtain TAF primary cultures; after two in vitro passages, cells were tested for CD105 and fibroblast activation protein (FAP) expression and vimentin content and four TAF (TAF16-01, TAF16-02, TAF16-03, TAF16-03) were frozen for further experiments. The CRC15-045 and CRC13-011 epithelial cell lines were derived from a stage IIA and a stage IVB (UICC 2009) CRC, respectively, showing a strong peri- and intra-tumoral infiltration of lymphocytes. After collagenase digestion, intact crypts collected from the pellet of residual tissue debris were plated in DMEM/F12 with Hepes buffer (Euroclone), containing B27 supplement, EGF (5 ng/mL) and DTT 10 mM (Sigma). The primary cell cultures initially developed as organoids, which formed small, loosely-adherent colonies after the first trypsin digestion, expressing EPCAM (not shown) and cytokeratin.

Isolation of T cells and monocytes and co-cultures

Peripheral blood mononuclear cells (PBMC) were obtained after Ficoll-Hypaque density centrifugation of blood samples derived from healthy donors. Highly purified (> 98% pure as assessed by flow cytometry upon CD3 staining, see below) T cells were obtained from PBMC using the RosetteSep T cell enrichment Kit (StemCell Technologies, Vancouver, Canada). Monocytes (Mo) were isolated from PBMC using the anti-CD14 mAb (MEM18, IgG1, Exbio, Prague, CZ) and EasySep custom Kit (StemCell Technologies, Inc.) according to manufacturer’s instructions, with recovery of > 97% CD14 positive cells.36 The human CRC cell lines HT29, HCT15, HCT116, SW48, SW480, SW620, Colo741, Colo205, Colo320, CaCo2, LS180, WiDr, LoVo and DLD1 were obtained from the Biological Bank and Cell Factory of the IRCCS AOU San Martino IST (Genoa, Italy) and maintained in RPMI 1640 medium supplemented with 10% FCS and 1% L-glutamine (Gibco). T cells were cultured in RPMI 1640 complete medium with or without irradiated CRC cells or TAF, or CRC15-045 cell line, at a ratio of 1:10, or Mo (Mo/T ratios from 1:10 to 1:80), untreated or exposed for 24 h to zoledronic acid (Zol 5 μM, kindly provided as sodium salt by Novartis Pharma, Basel, Switzerland, MTA 37518); the dose was selected on the basis of the effectiveness of γδ T cells proliferation from PBMC cultures and absence of toxic effects according to the literature and our previous data.49-51 In some experiments, the CRC cell lines LS180, DLD1, HCT15 and SW620 were pre-treated for 4 h with 100 μM or 50 μM Zol, then washed and used for stimulation experiments.34 In other experiments, 5 μM Zol was added to the cell suspensions obtained from CRC patients’ specimens. On the third day, IL-2 (4 ng/mL) was added and every 2 d complete medium supplemented with IL-2 was changed. T cells were recovered at 14 (not shown) or 20 d and ViTs T cell expansion was assessed by FACS analysis using the anti-γδ mAb BB3 (see below).

Immunofluorescence and cytfluorimetric analyses

Immunofluorescence was performed as described.36 For the identification of γδ T cell subpopulations, we used the anti-γδ mAb BB3 (IgG1).36 BTN3A1 expression was checked with the anti-CD277 mAb (clone 20.1, IgG1, Affimetrix ebioscience, Hatfield, UK). Tissue-derived or cultured cell populations were also characterized with the anti-CD27 or the anti-CD45 mAb, purchased from BD Biosciences Europe (Milan, Italy), the anti-CD14 mAb (MEM18, IgG1), the anti-CD3 mAb (UCHT-1, IgG1, Ancell, Bayport, MN55003, USA), the anti-CD105 (from the producing hybridoma purchased from the American Type Culture Collection, ATCC, Manassas, VA, USA), the anti-EPCAM mAb (ab98003, IgG1, Abcam, MA, USA) or the anti-FAP mAb (F11-C2, IgG1, ebioscience, San Diego, CA, USA). Negative controls were stained with APC-labeled isotype-matched irrelevant mAbs. Samples were analyzed by CyAn ADP flow cytometer (Beckman Coulter Inc., Brea, CA), gated on viable cells and/or on lymphocytes (based on FSC and SSC parameters). Results are expressed as log of mean fluorescence intensity (MFI, arbitrary units, a.u.) or percentage of positive cells.

Cytotoxicity assay

Cytolytic activity of γδ T cells was analyzed against the various CRC cell lines at an E:T ratio of 10:1 to 2.5:1, in V-bottomed microwells, in a 4-h 51Cr-release assay as described.15,16 Some samples were set up after exposure of the target cell lines to Zol at 5 μM concentration for 24 h. In some samples, the effector cells were exposed to saturating amounts (5 μg/mL) of the anti-Vδ2 mAb at the onset of the cytotoxicity assay; an unrelated mAb, matched for the isotype (BD PharMingen, BD Italy, Milan, Italy), was used as control. Other experiments were performed using as target cells the BTN3A1-transfected SW620 or DLD1 cell lines (see below), either untreated or Zol-treated. Reverse cytotoxicity was performed using the anti-CD3 or the anti-Vδ2 or the anti-CD8 (Leu2a, IgG1, BD PharMingen) mAbs, all at 2 μg/mL, and the FcyR positive murine P815 cell line. 100 μL of SN were measured in a γ-counter and the percentage of 51Cr-specific release was calculated as: experimental
release (counts) - spontaneous release (counts)/maximum release (counts) - spontaneous release (counts). Maximum and spontaneous release were calculated as described. 36

**cDNA reverse transcription and quantitative real-time PCR (Q-RT-PCR)**

RNA was extracted either from cultured CRC cell lines or CRC tissue samples or cell suspensions. Paraflin-embedded sections (8-µm thick) of the CRC patients were fixed on PEN membrane glass slides (MDS Analytical Technologies GmbH, Ismaning, Germany), dried at room temperature under a chemical safety hood for 5 min, dipped in xylene for 10 min twice for each sample, followed by a 3-step immersion in 100%/95%/75% ethanol solution. After washing in DEPC RNase-free water and staining (HistogeneActurus Italia srl, Milan, Italy), samples were dipped in 75%/95%/100% ethanol solution for 30 sec each passage followed by xylene for 5 min. Tissue sections were then dried at room temperature. RNA was extracted with the Paradise™ Reagent System (Acturus Bioscience) after incubation with proteinase K for 4 to 6 h at 56°C. A DNase treatment step was included. RNA was diluted in 50-µL elution buffer, according to the manufacturer’s protocol and quantitated by NanoDrop Spectrophotometer (ND-1000 Cellbio, Euroclone) and by Qubit TM fluorometer (Invitrogen, Life Technologies Italia, Monza, Italy) using the Quant-it TM Assay Kit (Invitrogen). cDNA synthesis was performed with random hexamers by the use of the High Capacity Archive Kit (Applied Biosystems, Life Technologies). To verify quantitative RT-PCR efficiency, decreasing amounts (50 ng, 10 ng and 0.1 ng) of normal RNA were used for CT titration. For CRC cell lines, RNA was extracted with TriPure (Roche diagnostic, Milan, Italy). cDNA synthesis was performed with random primers. Primers and probes for BTN3A1 and BTN12, were purchased by Applied Biosystem (Life Technologies Europe, Monza, Italy). Quantitative real-time PCR (Q-RT-PCR) was performed on the 7900HT FastRT-PCR system (Applied Biosystem) with the fluorescent Taqman method and normalized to 18s (Applied Biosystem). After subtracting the threshold cycle (C\textsubscript{T}) value for 18s from the C\textsubscript{T} values of target genes, results were expressed as 2^{-\Delta\textsubscript{CT}} or ΔC\textsubscript{T} ratio vs. 18s. 42

**BTN3A1 transfection and western blot**

The pLX304-BTN3A1 plasmid (HsCD00443486, DNASU, Arizona State University) was used to overexpress BTN3A1 protein. DLD1 or SW620 cells were transfected in serum-free Opti-MEM medium (Gibco, Life Technology) with Lipofectamine 2000 (Invitrogen, Life Technology) following the manufacturer’s instructions. Protein expression by western blot was analyzed from day 2 up to day 7 after transfection. 42 CRC cell lines were harvested and lysed with ice-cold RIPA buffer containing protease and phosphatase inhibitors. CRC tissues frozen at −80°C in 80-µL RIPA buffer (with 1mM OV, 1mM DTT and 1:100 protease inhibitors cocktail, Sigma, P8340) were thawed on ice and minced with sharp scissors adding 100 µL of fresh RIPA buffer to each sample. After 90 min incubation on ice, samples were potterized and centrifuged (16,000 rpm, 4°C; Eppendorf 5417-R centrifuge). Supernatants were collected and protein content quantified by the DC protein assay (BioRad Italia, Milan, Italy). Equal amounts of protein (35 µg/lane) were loaded under reducing or non-reducing conditions on precast 8–16% gradient gels (Thermo Fisher Scientific, Waltham, MA, USA) and then transferred to PVDF membranes (GE Healthcare, Little Chalfont, UK). After blocking, membranes were probed overnight at 4°C with the mouse monoclonal anti-CD277 (BT3.1, 20.1, Affymetrix eBioscience, recognizing the Ig-like domain of the molecule) or the rabbit polyclonal anti-BTN3A1 (NPBI-90750, Novus Biologicals, recognizing the C-terminal domain partially shared by BTN3A1 and BTN3A3) diluted according to the manufacturer’s instructions. Some samples were probed with an anti-epithelial keratin 8/18 (IgG1, Cell Signaling Technology, EuroClone, Milan, Italy) or an anti-vimentin (clone V9, IgG1, Sigma) mAb. Subcellular fractionation was performed with the Q proteome cell compartment kit (Qiagen, Milan, Italy). 32 Enrichment of marker proteins in subcellular fractions was probed with the following antibodies: lamin B (Cell Signaling Technology), GAPDH-HRP (Novus Biologicals), β-tubulin (Tub2.1, Sigma) and vimentin (clone V9, Sigma). After washing, membranes were incubated for 1 h at room temperature with the relevant horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology), and proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Anti-β-actin HRP-conjugated antibody (Cell Signaling) was used as a loading control.

**Immunohistochemistry**

Paraffin-embedded samples from 10 CRC patients were analyzed for the expression in situ of BTN3A1, V82, vimentin and TGI. Immunohistochemistry was performed on 6-µm-thin sections, deparaffinized in xylene, and treated with Peroxoblok (Novex, Life Technologies) to quench endogenous peroxidase, followed by Ultra Blok reagent (Ultravision Detection System, Thermo Scientific BioOptica, Milan Italy). The following antibodies were added: polyclonal rabbit anti-BTN3A1 antiserum (1:100, Novus Biologicals), anti-V82 mAb (BB3, 2 µg/mL), anti-CID4 mAb (1:200, Santa Cruz Biotechnology), anti-vimentin clone V9 (1:200, Sigma), polyclonal rabbit anti-TGII antiserum (1:100, Thermo Scientific) and an isotopic-unrelated antibody was used as negative control (Dako Cytomation). Biotinylated goat anti-rabbit or goat anti-mouse antiserum (Bio-Optica) was then added, followed by HRP-conjugated avidin (Thermo Scientific) and the reaction developed using 3,3′-diaminobenzidine (DAB) as chromogen. Then, the slides were counterstained with hematoxylin, coverslipped with Eukitt (Bio Optica), and analyzed under a Leica DM MB2 microscope equipped with a charged coupled device camera (Olympus DP70 with a 20× or 40× objective).

**HPLC negative ion electrospray ionization TOF-MS**

IPP production by the CRC cell lines SW620, LS180, DLD1, HCT15 and Colo320, either untreated or exposed to Zol, either as continuous treatment with 5 µM for 24 h, or as pulse treatment of 50 µM and 100 µM for 4 h, as described in Supplementary Materials and Methods, was performed according to...
Jauhiainen et al. with modifications, as detailed in supporting information (Supplementary Materials and Methods).

The amount of IPP/DMAPP, expressed as pmol/mg protein, was evaluated by calculating the peak area of the extracted ion current (EIC m/z 244.99[M-H]-), referred to a standard curve of IPP (range 0.1–15 μM) in control cell extracts. Total protein content was determined with the DC Protein Assay (BioRad). Data are shown as IPP pmol/mg of total protein extracted by ACN/total protein content in cell lysates after ACN extraction.

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