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The microenvironment differentially impairs passive and active immunotherapy in Chronic lymphocytic leukemia - Potential therapeutic synergism of CXCR4 antagonists

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The microenvironment differentially impairs passive and active immunotherapy in Chronic Lymphocytic Leukemia -

CXCR4 antagonists as potential adjuvants for monoclonal antibodies

Running title: Microenvironmental impact on immunotherapy in CLL

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Summary

Direct contact with stromal cells protects chronic lymphocytic leukemia (CLL) B cells from chemotherapy-induced apoptosis *in vitro*. Blockade of CXCR4 signaling antagonizes stroma-mediated interactions and restores CLL chemosensitivity. ***In vivo*, administration of CXCR4 antagonists effectively mobilizes hematopoetic progenitor cells**. Therefore, combinations of CXCR4 blockade with cytoreductive treatment with selective activity on CLL cells may avoid potential hematotoxicity. Hence, we tested CXCR4 antagonists in the context of passive and active immunotherapeutic approaches. We evaluated how efficiently rituximab, alemtuzumab, and cytotoxic T cells killed CLL cells co-cultured with stromal cells in the presence and absence of a CXCR4 antagonist. Stromal cell contact attenuated rituximab- and alemtuzumab-induced complement-dependent cytotoxicity (CDC) of CLL cells. Addition of CXCR4 antagonists abrogated the protective effect of stroma. In contrast, stromal cells did not impair antibody-dependent cell-mediated cytotoxicity (ADCC) and cytotoxicity induced by activated T cells. Destruction of microtubules in CLL target cells restored the protective effect of stroma coculture for CLL cells during NK cell attack by preventing mitochondrial relocalization towards the immunological synapse. Our data identify the combination of CXCR4 antagonists with passive - but not active - immunotherapy as a promising potential treatment concept in CLL.

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Introduction

B cell chronic lymphocytic leukemia (B-CLL) represents one of the most commonly diagnosed lymphoid malignancies in Western countries, and is characterized by continuous accumulation of mature, $CD5^+$ B cells. CLL remains incurable with cytoreductive therapy. The microenvironment in bone marrow and secondary lymphoid tissues favours survival and chemoresistance of CLL cells by upregulation of antiapoptotic molecules such as Mcl-1 and Bcl-xL (Caligaris-Cappio 2003, Chiorazzi*, et al* 2005). In contrast to the prolonged survival *in vivo*, CLL cells undergo rapid apoptosis in the absence of stroma *in vitro*. Mimicking the *in vivo* microenvironment by stromal cell coculture protects CLL cells from spontaneous and chemotherapy-induced apoptosis *in vitro* (Burger*, et al* 2005, Kurtova*, et al* 2009). This cell contact-mediated chemoresistance is described for other malignancies as well and is termed "cell adhesion-mediated drug resistance" (CAM-DR) (Damiano*, et al* 1999). Residual leukemic cells protected by stromal cells in the bone marrow or lymphatic tissue are thought to contribute to disease relapse after chemotherapy.

The homing of CLL cells towards the protective niches is mediated by the crosstalk between the chemokine CXCL12 and its receptor CXCR4. CXCR4 antagonists resensitize CLL cells to spontaneous and chemotherapy-induced apoptosis during stromal coculture *in vitro* (Burger*, et al* 1999, Burger*, et al* 2005). Combination therapy with CXCR4 antagonists is therefore predicted to enhance efficacy of conventional cytoreductive treatment in CLL (Burger and Kipps 2006). On the other hand, the CXCL12-CXCR4 interaction provides a physiological homing signal to the bone marrow for hematopoietic stem cells (HSCs). **Thus, administration of CXCR4 antagonists mobilizes HSCs to the periphery and may render them sensitive to concomitant chemotherapy.** To avoid this risk of stem cell toxicity, combination of CXCR4 antagonists with antineoplastic agents targeted to CLL cells would be conceptually advantageous.

Due to their exclusive action on cells of **leukocyte lineages without decreasing HSC viability (Lim*, et al* 2008),** passive and active immunotherapy represent promising treatment strategies in CLL (Faderl*, et al* 2005, Kater*, et al* 2007, Stanglmaier*, et al* 2004). The

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monoclonal anti-CD52 and anti-CD20 antibodies (mAb) alemtuzumab (ALT, Campath-1H) and rituximab (RIT) have been approved for passive immunotherapy of relapsed or refractory CLL. **Their precise mode of action is not fully understood. Monoclonal antibodies are capable to activate the classic complement pathway which results in pore formation and target cell lysis (Zipfel and Skerka 2009), known as complement dependent cytotoxicity (CDC). Furthermore, antibodies may bind to Fcγ receptors on various effector cells, such as natural killer (NK) cells, fixed macrophages, dendritic cells (DCs), neutrophils, and eosinophils (Nimmerjahn and Ravetch 2006). Efficient lysis of the obsoned cells by these effectors has been termed antibody-dependent cell-mediated cytotoxicity (ADCC). Additional direct apoptotic effects of, e.g. RIT are under controversial discussion (Lim*, et al* 2010).**

The graft-versus-leukemia effect observed after allogeneic hematopoietic stem cell transplantation is evidence for anti-leukemic activity of T cells against CLL (Giannopoulos and Schmitt 2006, Khouri*, et al* 1998). A potential CLL-associated T cell antigen is survivin, a member of the inhibitor of apoptosis (IAP) gene family (Schmidt*, et al* 2003). This *in vitro* study was conducted to determine whether stromal contact protects CLL cells against passive and active immunotherapy. We evaluated the *ex vivo* cytotoxic effect of RIT and ALT and the ability of survivin-specific and allogenic CTLs generated in mixed lymphocyte reactions to lyse primary CLL cells in presence and absence of the murine stromal cell line M2-10B4 with or without the CXCR4 antagonist TN14003.

Materials and methods

Patients and healthy donors

Peripheral blood samples were obtained from 32 patients at University Medical Center Freiburg (SI Table 1) satisfying criteria for B-CLL and 10 healthy volunteers (Cheson*, et al* 1996). Rai stage and treatment status were abstracted from clinical records. IgV_H mutation

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status, ZAP70 expression, and genomic aberrations were determined as described previously (Hamblin*, et al* 1999, Osterroth*, et al* 1999, Pfeifer*, et al* 2007, Rassenti*, et al* 2004). 19 Patients had not been treated, 13 patients had received low-dose chlorambucil and/or fludarabine, which had been discontinued at least 6 months prior to phlebotomy. Blood sampling had been approved by the local ethics committee and all patients gave informed consent.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll. Most CLL samples contained >85% CLL B cells as determined by flow cytometry with anti-CD19 (BioLegend, San Diego, CA), anti-CD5 (BD Pharmingen Heidelberg, Germany), and anti-CD23 (Beckman Coulter, Fullerton, CA) mAb. If purity was <85%, B cells were isolated by negative selection (B-cell isolation kit II; Miltenyi Biotech, Bergisch Gladbach, Germany). $CD8^+$ T cells were isolated from healthy donors by negative selection (CD8 Isolation Kit II; Miltenyi Biotech) to a purity of more than 85%.

Reagents and Cell lines

The **highly selective tetradeca-peptide** CXCR4 receptor antagonist TN14003 was a kind gift from N. Fujii, Kyoto, Japan **(Tamamura*, et al* 2001)**. The stromal cell line M2-10B4 (ATCC; Manassas, VA), maintained in RPMI 1640 containing 10% FCS was plated on 96 well plates at 2 x 10^5 cells/well, starved overnight, and incubated with CXCR4 30min prior to coculture with CLL cells where indicated. **To remove CLL cells from stromal cell coculture we performed gentle but persistent pipetting. Stromal cells were discriminated from CLL cells according to their distinct forward/sideward scatter properties in flow cytometry (Burger*, et al* 2005).**

Arabinosyl-2-fluoroadenine (F-ara-A) was purchased from Sigma-Aldrich (St. Louis, MO) **and used at 20µM final concentration.** ALT (Schering, Weimar, Germany) and RIT (Roche, Basel, Switzerland) were used at final concentrations of 20µg/ml and 10µg/ml, respectively. For complement-mediated lysis, 10% fresh human serum (HS) was added. **Z-VAD was purchased from Promega Inc and used at a final concentration of 20 µM.**

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Intracellular staining of Mcl-1

After 48 hour culture of CLL cells with or without stromal cell contact in the presence and absence of CXCR4 antagonists, cells were fixed and permeabilized according to manufacturer's protocol (Foxp3 staining kit, eBioscience, San Diego, CA), stained with a Mcl-1 antibody (Epitomics Inc., San Francisco, CA), and analyzed by flow cytometry.

Generation of effector CTLs

Effector CTLs generated in allogeneic mixed lymphocyte reactions (MLR) were stimulated similarly as described previously (Kato*, et al* 1998). CLL B cells were plated into 96-well plates at 1 x 10^5 cells/well (IMDM, 10% human serum). 1 x 10^5 healthy donor $CD8^+$ T cells/well were added and T cells were re-stimulated weekly with 2 x 10^4 CLL cells/well. For CD40 activation, CLL cells were stimulated for 48 h with 100 ng/ml sCD154 (Biosource, Camarillo, CA) before stimulation of T cells. Recombinant human IL-2 (R&D Systems, Minneapolis, MN) was added at 25 U/ml three days after each stimulation. After 1 to 4 stimulations, cytotoxicity of effector CTLs was assessed by flow cytometry or chromium (^{51}Cr) release assays. CLL cells were incubated with or without the CXCR4 blocking agent TN14003 for 30 min in a final concentration of 10 µg/ml prior to cultivation with the unlabelled stromal cell line M2-10B4 for 2 hours where indicated. Effector T cells were added to the respective culture for 4 hours. For generation of survivin-specific T cells, purified HLA-$A2^+$ healthy $CD8^+$ T cells were primarily stimulated with irradiated monocyte-derived, peptide-pulsed dendritic cells that were pulsed prior to stimulation with the native survivin-derived peptide (Sur9: ELTLGEFLKL) for 2 hours in the presence of ß2-microglobulin (3 µg/ml, Sigma Aldrich). T cells were restimulated weekly using CD40-activated B cells pulsed with the corresponding peptide. After 3 stimulations, cytotoxicity of CTLs against primary CLL cells was assessed in chromium ^{51}Cr release assay.

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Immunoblotting

Immunoblotting was performed as previously described (Buchner*, et al* 2009). Primary antibodies included pAkt^{S473}, Akt (Cell Signaling Technology), Mcl-1 (Epitomics, San Francisco, CA), or β-actin (Sigma-Aldrich), secondary antibodies were horseradish peroxidase-conjugated from Cell Signaling Technology. Densitometric analysis was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

CLL cell treatment and viability assessment

B-CLL cells from frozen stocks thawed prior to experiments were incubated for 48 hours with F-ara-A or with RIT and ALT for 4 h (ADCC) and 12h (CDC), respectively. Cells were activated with IL-2 (500 U/ml) overnight and used at 30:1 (PBMC:CLL). To distinguish between PBMC and CLL cells, the latter were stained with CFSE. Cell viability was determined by propidium iodide (PI) (Sigma Aldrich) for ADCC. Otherwise, Annexin V-FITC (BD Bioscience) and PI was used. CLL cells were removed from stromal cell coculture by gentle but persistent pipetting.

Chromium (^{51}Cr) release assay

Chromium release assay was performed as previously described (Zirlik*, et al* 2006). In brief, survivin-specific CTLs or effector T cells from allogeneic MLR were incubated with ^{51}Cr-labeled target cells. CTL activity was titrated at different effector-to-target ratios (30:1, 10:1, 3:1) for 4 hours and ^{51}Cr was measured in supernatants.

Cell polarization

For confocal analysis of cell polarization in target cells, CLL cells were stained with Mitotracker Red CM-H_2XROS (Invitrogen, Carlsbad, CA) and treated for 30min with 10µM colchicine or control. After 3 washing steps, cells were preincubated with alemtuzumab (20µg/ml) before magnetically isolated, IL-2 activated NK cells labeled with Celltracker Green

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were added with a ratio of 1:1 onto adhesion slides (Marienfeld GmbH, Lauda-Königshofen, Germany), cells were fixed with 2% formaldehyde, mounted using DAPI containing mounting medium (Vector Laboratories, Burlingame, CA) and analyzed using a Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Statistical analysis was performed using student`s t-test or Wilcoxon test using the Graphpad Prism Software.

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Results

*Stromal cells protect CLL cells from chemotherapy-induced apoptosis via CXCL12 - CXCR4 interaction**, Akt activation,*** *and upregulation of Mcl-1*

To confirm **the previously reported** protective effect of the microenvironment on the viability of CLL cells *in vitro,* we assessed apoptosis of CLL cells in the presence and absence of the CXCL12-secreting stromal cell line M2-10B4 (Burger*, et al* 2005). After 48 hours under standard culture conditions, only **50±7%** of CLL cells remained viable. Coculture of CLL cells with stromal cells significantly enhanced viability to **81±3%** (n=16, p<0.01). The CXCR4 antagonist TN14003 reversed this protective effect **(Supporting Information (SI) Fig. 1A)**, while it did not affect CLL cell survival in the absence of stroma (shown in Fig. 1A). **Similar results were obtained for the CXCR4 antagonist AMD3100 (SI Fig 1B).**

Fludarabine is the most effective single chemotherapeutic agent in CLL. *In vitro,* treatment of CLL cells with 20µM F-ara-A, the dephosphorylated, active fludarabine derivative, reduced absolute viability of CLL cells from 27±3% to 9±3% after 48hrs (SI Fig. **2**). To compare pro-apoptotic F-ara-A effects under various culture conditions, we used the relative viability by defining the respective untreated control (without stroma, with stroma, with stroma and

TN14003 - as indicated) as 100% (Burger*, et al* 1999) **accounting for variabilities in spontaneous apoptosis rates in different patients' samples (Kurtova*, et al* 2009).** Coculture with stromal cells significantly enhanced the relative viability after F-ara-A treatment from 35±9% to 59±8% after 48hrs (Fig. 1**A**, n=8, p<0.05). The protective effect of stromal cells on chemotherapy-induced apoptosis was mainly prevented by the CXCR4 receptor antagonist TN14003 (Fig. 1**A**, p<0.05), **in line with previous findings (Burger*, et al* 2005). The viability of stromal cells and their capacity to secrete CXCL12 was not influenced by 48 h F-ara-A treatment (SI Fig. 3A and B).**

Mechanistically, Mcl-1 protein expression was significantly increased in primary CLL cells after coculture with M2-10B4 cells, an effect abrogated by the CXCR4 antagonist TN14003 (Fig. 1B). Addition of the caspase inhibitior Z-VAD did not affect Mcl-1 expression after treatment with the CXCR4 inhibitor. Therefore, the increased apoptosis induction and emerging caspase activation does not account for the Mcl-1 downregulation.

In line with previous reports, we found increased activation of Akt upon stromal cell coculture. Again, this effect could be blocked by CXCR4 inhibition (Fig. 1C). In contrast, the expression of the other proapoptotic mediators Bid and Bim was not regulated and Bad was not expressed at all (data not shown). These data suggest that stromal cell-induced apoptosis resistance is largely mediated by Mcl-1 upregulation (Balakrishnan*, et al* 2009).

CXCR4 antagonists increase the efficacy of CDC but not ADCC of alemtuzumab and rituximab by attenuating the protective effects of stromal cells

To investigate whether stromal cells protect CLL cells in the setting of passive immunotherapy, we examined the *in vitro* activity of ALT and RIT in the presence of human complement or activated natural killer (NK) cells from healthy PBMCs on CLL cells alone and in co-culture with stromal cells. Both antibodies significantly reduced viability of CLL cells by

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CDC and ADCC compared to IgG control as determined by %viability in flow cytometry (SI Fig. 4A, C and D). **Similar results for CDC were obtained when total viable cell count was determined (SI Fig. 4B).** In accordance with previously published data, ALT mediated CDC *in vitro* more efficiently than RIT (Voso*, et al* 2002). Coculture with stromal cells inhibited CDC of CLL cells for both antibodies. The CXCR4 antagonist TN14003 abrogated this protective effect (Fig. 2A and B). **Again, similar results were obtained when total viable cell count was determined (SI Fig. 5A and B). The viability and the capacity to secrete CXCL12 of stromal cells were not influenced by the treatment of ALT and RIT (SI Fig. 3C and D).**

We next evaluated the influence of the microenvironment on ALT- and RIT-mediated ADCC of primary CLL cell using activated NK cells from healthy PBMCs as effector cells. **PBMCs from healthy donors were used since NK from CLL patients are severely impaired (Gorgun*, et al* 2005, Katrinakis*, et al* 1996, Kay and Zarling 1987, Kay and Zarling 1984, Ramsay*, et al* 2008) and autologous NK cells from patients with CLL show inferior rituximab antibody-dependent cellular cytotoxicity than allogeneic NK cells (Weitzman*, et al* 2009).** In contrast to CDC, we detected no increase in CLL cell viability upon coculture with stromal cells (Fig. 2C and D). This effect was independent of various effector-to-target ratios (Fig. 2C and D; E:T ratio 30:1, and SI Fig. 6A and B; E:T ratio 10:1). Taken together, this suggests that the microenvironment exerts no protective effect against cellular effector mechanisms.

CTL-mediated killing of allogeneic CLL cells is not impaired by stromal cell contact

To examine CTL-mediated apoptosis, allogeneic CTL were incubated with target cells in the presence and absence of stromal cells and CXCR4 antagonists. To exclude differences in T cell quality as cause for variable effector function, $CD8^+$ T cells from one donor were stimulated against a total of 19 different CLL samples in parallel. **T cells from a healthy donor were used as CLL-derived T-cell function is impaired (Gorgun*, et al* 2005,**

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Ramsay*, et al* 2008). Overall, CLL cells had inefficient allostimulatory capacity, since we observed measurable killing of CLL cells in only 13 of 19 cases (Fig. 3A). CTL-mediated apoptosis was CLL-cell specific and dose-dependent (Fig. 3B and C). At an effector-to-target ratio of 30:1, the measurable median target cell killing was 9% (range 4-36%, n=13). No differences in cytotoxicity were observed according to prognostic factors (data not shown). Coculture of CLL cells with the stromal cell line or with the stromal cell line and TN14003 did not modulate apoptosis induced by allogeneic CTLs (Fig. 4A+B). Allogeneic CTLs were cytotoxic only for the allogeneic CLL cells against which they were originally primed, as these cells did not manifest any cytotoxicity of autologous PBMCs (Fig. 4B). However, even under conditions of prestimulation with CD40L, stromal cells did not limit CTL-induced apoptosis of CLL cells (data not shown).

To analyse possible effects of stromal contact on T cell-mediated cytotoxicity independent of the variable and rather weak allostimulatory capacity of CLL cells, we validated our results with survivin-specific, HLA-A2-restricted healthy T cells. Survivin-specific T cells effectively lysed HLA-A2^{+} survivin^{+} CLL cells but not HLA-A2^{-} survivin^{-} CLL cells in a dose-dependent manner (Fig. 4C). In line with a previous report (Schmidt*, et al* 2003), survivin was expressed in malignant B cells from a HLA-A2 positive patient with CLL as determined by quantitative real-time PCR (data not shown). Stromal cell contact did not significantly reduce antigen-specific cytotoxicity, nor did the addition of the CXCR4 antagonist increase cytotoxicity, indicating that T cell-mediated cytotoxicity is indeed independent of stromal cell contact (Fig. 4C).

Target cell polarisation is required for efficient cytotoxicity in stroma context

To functionally explain the observed differences in stroma protection capacity in terms of cell-mediated or complement-mediated apoptosis, we added the toxin colchicine to the cytotoxicity assay. Colchicine destroys microtubule formation and therefore abrogates target cell polarisation and mitochondrial relocalization (Goping*, et al* 2008). Incubation with the

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toxin did not influence the survival of CLL or stromal cells in the observed time frame (Fig. 5A, SI Fig. 3E). NK cell activity was limited during toxin treatment (data not shown) but was maintained when the target cells were preincubated and repeatedly washed prior to addition of the activated PBMCs. In line with our previous finding (Fig. 2 C+D) we observed no protective effect of stroma in ADCC-mediated cytotoxicity, suggesting that effector cells may overcome stromal cell mediated protective effects. **However, inhibition of microtubule formation by colchicine treatment resulted in a significant -albeit minor- protection of CLL cells only in stroma context (Fig. 5 A, p<0.01; SI Fig.7 for absolute viability). Morphologically,** colchicine treatment prevented mitochondrial accumulation towards the immunological synapse as assessed by confocal microscopy (Fig. 5B).

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Discussion

Interactions between CLL cells and the microenvironment provide prolonged cell survival and growth advantage and confer drug resistance to CLL cells (Caligaris-Cappio 2003, Chiorazzi, *et al* 2005). *In vitro*, the inhibition of CXCL12-CXCR4 interactions restores susceptibility of CLL cells in stromal contact to spontaneous and drug-induced apoptosis, suggesting synergistic effects of combined therapy of CXCR4 antagonists and chemotherapy *in vivo*. **Although recent studies suggest safety and efficacy of combining cytotoxic chemotherapy with CXCR4 antagonists in acute myeloid leukemia (AML) (Uy GL 2008), this combination may provoke toxicity towards mobilized healthy hematopoietic progenitors that are normally protected in the marrow microenvironment.** Therefore, we evaluated the effect of stromal coculture in the presence and absence of CXCR4 antagonists in more specific passive and active immunotherapeutic approaches targeting CLL cells but not HSCs.

Indeed, stromal cell contact reduced complement-mediated cytotoxicity of alemtuzumab and rituximab. The addition of the CXCR4 antagonist TN14003 reversed the protective effect of

stromal cells resulting in decreased viability of CLL cells identifying CXCR4 antagonists as potential adjuvants for selective therapeutic intervention. Promising results in preclinical tumor models indicate that CXCR4 antagonists may have anti-tumor activity in patients with various malignancies without serious side effects (Khan*, et al* 2007, Stone*, et al* 2007, Voso*, et al* 2002, Wendt*, et al* 2008, Yoon*, et al* 2007).

Molecular mechanisms of the antiapoptotic function of stromal cell contact remain largely unknown. Although up-regulated in CLL, Bcl-2 does not seem to be critical for protection of CLL cells in stroma contact (Balakrishnan*, et al* 2009). We and others observed upregulation of the anti-apoptotic Bcl-2 family member Mcl-1 upon stromal cell contact (Balakrishnan*, et al* 2009). The potential role of Mcl-1 as an oncogene in lymphoid malignancies was confirmed in transgenic mouse models in which animals with deregulated expression of Mcl-1 eventually developed widely disseminated B-cell lymphoma **(Zhou*, et al* 1998). Interestingly, in CLL, stromal cell contact also upregulates Akt activation (Edelmann, et al 2008), an effect abrogated by CXCR4 antagonists (Fig. 1C).** Since active Akt also increases Mcl-1 half-live (Maurer*, et al* 2006), the Akt/Mcl-1 pathway might indeed mediate antiapoptotic signals during stromal cell support. **Accordingly, the inhibition of this pathway may explain the efficacy of CXCR4 antagonists in this setting.** In line with our observations, Mcl-1 has been reported to mediate apoptosis resistance to fludarabine and rituximab *in vivo (Awan, et al 2009, Johnston, et al 2004, Pepper, et al 2008, Zhou, et al 1998)*, and downregulation of Mcl-1 by siRNA enhances RIT-mediated apoptosis *in vitro* (Hussain*, et al* 2007).

A strong correlation of high-affinity $F_C\gamma R$ polymorphisms with clinical response to rituximab in follicular lymphoma (FL) (Cartron*, et al* 2002) provides evidence for an important role of ADCC in clinical lymphoma responses**. In CLL, however, this correlation does not exist which argues against a dominant role of ADCC in CLL. Although mainly natural killer (NK) cells are established ADCC effector cells, other cells such as monocytes and**

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macrophages may contribute to ADCC (Dalle*, et al* 2008, Lefebvre*, et al* 2006). These cell types were not analyzed in our study.

With respect to active immunotherapy, B-CLL cells *per se* are inefficient antigen-presenting cells (APCs), largely due to an inadequate co-stimulatory capacity (Dazzi*, et al* 1995, Wolos and Davey 1979) and the induction of an anergic phenotype in T cells by CLL cells. The latter phenomenon is not only induced in autologous but also in allogeneic T cells, resulting in down-modulation of genes important for proliferation and effector function (Gorgun*, et al* 2005). Recently, T cells of CLL patients and also healthy T cells after direct CLL cell contact were shown to have impaired ability to form immunological synapses and subsequent polarisation events (Ramsay*, et al* 2008). In agreement with these findings we observed efficient lysis of CLL cells in only 13 of 19 cases and overall poor cytotoxic T cell responses. Effective cellular immune therapy, such as vaccines (Hus*, et al* 2008) or genetically engineered T cell transfer (Hollyman*, et al* 2009) will have to overcome factors responsible for the disease-related immune deficiency of patients with CLL and the capacity of leukemia cells to effect cellular immune tolerance. Recent work has focused on strategies to overcome these limitations and encouraging biologic effects and clinical responses have been observed (Biagi*, et al* 2005, Hus*, et al* 2005, Kater*, et al* 2004a, Kater*, et al* 2004b, Mayr*, et al* 2005, Zirlik*, et al* 2006).

Unlike in the setting of chemotherapy- and CDC-induced apoptosis, CLL-stromal cell interactions did not impair T cell-mediated cytotoxicity **suggesting that** the microenvironment does not limit the efficacy of active immunotherapy in B-CLL. This conclusion could be drawn from the unspecific setting in MLRs as well as from the specific setting using survivin-specific T cells. **Of note, no change in stroma cell viability was observed after addition of any of the components, including activated NK and allospecific T cells (data not shown). In view of our data showing no influence on the susceptibility of B-CLL cells to ADCC and allogeneic CTL attack by stroma, it might be**

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speculated that the mode of action to induce apoptosis is critical. Allo-responsive CTLs are able to lyse resting and CD40-stimulated B-CLL cells with equal efficiency, although CD40 stimulation results in upregulation of anti-apoptotic molecules, including Mcl-1 (Chu*, et al* 2000, Kater*, et al* 2004a, Kitada*, et al* 1999). In line with our findings, Mcl-1 downregulation has been shown to sensitize CLL cells against CDC but not ADCC mediated cytotoxicity of rituximab (Hussain*, et al* 2007). Directed exocytosis of lytic molecules such as granzymes toward the target cell is one of the major mechanisms used by CTLs an NK cells. By mitochondrial relocalization towards the site of granzyme B entry during CTL attack, activated T cells are able to overcome upregulation of Bcl-2, usually blocking Granzyme B (Goping*, et al* 2008). This suggests that cell polarization by immunological synapse formation may overcome antiapoptotic events mediated by stroma. **For CLL, disruption of the microtubulus network restored to some extent the stroma protective effect, although the biological significance remains to be determined.** A direct effect of microtubule disruption is unlikely as it would also affect ADCC efficacy without stroma. **A potential explanation for this effect may be the missing mitochondria relocation towards the immunological synapse observed after colchicine treatment.**

However, the fact that cell-mediated immunotherapy, such as active vaccinations strategies, T cell transfer or "graft vs. leukemia"-effect after allogeneic hematopoietic stem cell transplantation, is not limited **by contact to stromal cells** supports the application of this therapeutic strategy. **Therefore, we propose this approach to be further investigated *in vivo*.**

In conclusion, this study demonstrates that adhesion-mediated drug resistance protects CLL cells against monoclonal antibody treatment, an effect abrogated by CXCR4 antagonists. Therefore, combined therapy with CXCR4 antagonists may represent a promising strategy to enhance the efficacy of ALT and RIT therapy in CLL. In addition, we show that CLL-stromal cell interactions do not impair T cell-mediated cytotoxicity on CLL cells.

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Figure legends

Figure 1: Stromal cells protect CLL cells from chemotherapy-induced apoptosis via CXCR4 - CXCL12 interactions and pAkt-dependent Mcl-1 upregulation. (A) Viability of CLL cells cultured with or without stromal cells and treated with 20µM F-ara-A or the combination of F-ara-A and 10µg/ml TN14003 was determined by annexin V/PI staining. Relative CLL cell viability was determined by defining the respective control as 100% (as indicated with the control bar) after 48hrs treatment. Data represent means±SEM (n=8). (B) Means (± SEM) of mean fluorescent intensity (MFI by flow cytometry) of Mcl-1 expression in CLL cells cultured with or without stroma in the presence or absence of the CXCR4 antagonist TN14003 are displayed on the left (n=12). A representative experiment shows MFI of Mcl-1 expression in primary CLL cells cultured with or without stromal cells on the right. Below, a representative Mcl-1 immunoblot of CLL cells is shown in the presence and absence of stroma, with and without addition of TN14003 and/or the caspase inhibitor Z-VAD (20 µM). (C) Immunoblot shows pAkt and Akt of a representative CLL sample after 15min with or without coculture of stroma in the presence and absence of the CXCR4 antagonist TN14003.

Figure 2: CXCR4 antagonists increase the efficacy of ALT and RIT complement induced cytotoxicity by attenuating the protective effects of stromal cells. (A+B) CLL cells (n=7) cultured with or without stromal cells in the presence or absence of the CXCR4 antagonist TN14003 treated with 20µg/ml ALT (A) or 10µg/ml RIT (B) for 12hrs. Relative viabilities were determined by staining with Annexin V and PI and presented as means±SEM. (C+D) Relative viability of CLL cells (n=12) cultured with or without stromal cells in the presence or absence of the CXCR4 antagonist TN14003 treated with 20µg/ml ALT (C) or 10µg/ml RIT (D) for 4hrs in the presence of IL-2-activated PBMC at a ratio 30:1. IgG control of the respective culture setting (w/o stroma, w/stroma, or stroma+TN14003) was defined as 100% relative viability. Using annexin V and PI staining, CLL relative cell viability was determined and presented as means±SEM.

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Figure 3: CTL-mediated killing of allogeneic CLL cells. (A) Killing of CLL cells of 19 CLL patients induced by effector T cells generated in allogeneic MLR with spontaneous levels of apoptosis of B-CLL cells subtracted as determined by flow cytometry (CFSE/PI staining) at an effector-to-target ratio of 30:1 (M=mutated IgV_H; UM=unmutated IgV_H). (B) Flow cytometry analysis of cytolytic activity of effector T-cells from allogeneic MLR against CLL cells by staining with CFSE/PI presented as effector-to-target ratios 3:1 and 30:1. (C) Dose-dependent killing of CLL cells induced by effector T cells with spontaneous levels of apoptosis of B-CLL cells subtracted as determined by flow cytometry (CFSE/PI staining) represent means±SEM of triplicates.

Figure 4: CLL - stromal cell interactions do not impair T cell mediated cytotoxicity. (A) Viability of CLL cells (n=11) in the presence or absence of stromal coculture after incubation with T cells generated in allogeneic mixed lymphocyte reaction as assessed by flow cytometry ($CFSE^+/PI^-$). Untreated control without addition of T cells of the respective culture setting was defined as 100% viability. Data represent means±SEM. (B) Cytolytic activity of effector CTLs generated in allogeneic MLR as assessed by flow cytometry against CLL cells in presence of the stromal cell line M210B4 with or without the CXCR4 inhibitor TN14003 and against autologous PMBCs at three different effector-to-target ratios. Displayed are means±SEM of PI^+-values of samples from 3 representative patients. (C) Killing by allogeneic CTLs generated against a native (Sur9: ELTLGEFLKL) survivin-derived peptide of HLA-A2-positive CLL cells cultured with or without stromal cells in the presence or absence of the CXCR4 antagonist TN14003 and HLA-A2-negative CLL cells at 3 different effector-target ratios.

Figure 5: Prevention of mitochondrial relocalization in target cells restores protective stromal effect. (A) $CFSE^+$-CLL cells were pretreated with 10µM colchicine after incubation with and without stroma before addition of ALT or IgG control and IL-2-activated PBMCs.

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After 2hrs, viabililty was measured by %CFSE$^+$/PI$^-$ and the relative viability calculated by defining the respective control culture as 100 % relative viability. Data are presented as means±SEM (n=7). (B) Confocal images of colchicine- or control-pretreated CLL cells stained for mitochondria (red) in direct contact to IL-2-activated NK cells (green) during alemtuzumab treatment.

Supporting information Figure Legends:

SI Fig. 1: (A) Viability of CLL cells cultured with or without stromal cells in the presence or absence of the CXCR4 antagonist TN14003 determined by annexin V/PI staining. Displayed are the mean percentage viability values (± SEM) of leukemic cells assessed by staining with Annexin V and PI (n=16). (B) Direct comparison of the CXCR4 antagonist TN14003 and AMD1300 (10 µM, Sigma Aldrich) revealed equal effects. Both antagonists significantly reduced stromal cell induced survival ($p<0.05$, n=5, respectively).

SI Fig. 2: Absolute viability of CLL cells defined as Annexin$^-$/PI$^-$, cultivated with or without stromal cells and treated with 20 µM F-ara-A or the combination of F-ara-A and/or 10 µg/ml TN14003. Data are presented as mean ± SEM values (n=8).

SI Fig. 3: Standard MTT assay and CXCL12 ELISA (BD Bioscience) were performed after incubation of stromal cells with 20 µM F-ara-A (A+B), IgG, rituximab, or alemtuzumab (C+D) for the same time frames as during CLL treatment. (E) Incubation with 10 µM colchicine for 30 min did not alter stromal cell viability as determined by MTT assay.

SI Fig. 4: (A) Relative viability of CLL cells cultured with ALT (20 µg/ml) or RIT (10 µg/ml) for 12 hours in the presence of 10% fresh human serum (n=7) for complement mediated cytotoxicity determined by Annexin V/PI staining. (B) Viable cell count of CLL cells treated with IgG (20 µg/ml), ALT (20 µg/ml) or RIT (10 µg/ml) for 12 hours in the presence of 10%

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fresh human serum (n=7) for complement mediated cytotoxicity determined by Guava viacount. (C+D) Relative viability of CLL cells cultured with ALT (20 µg/ml) or RIT (10 µg/ml) for 4 h with IL-2 activated PBMC (n=8) for ADCC at a effector-to-target ratio of 30:1 (C) and 10:1 (D), IgG control was defined as 100% viability. Data are presented as mean ± SEM values.

SI Fig. 5: Viable cell count of CLL cells treated with rituximab (A) or alemtuzumab (B) in the presence of fresh human stroma, measured using the Guava viacount technology for the indicated culture conditions (n=7). Data are presented as mean ± SEM values.

SI Fig. 6: (A+B) Relative viability of CLL cells (n=8) cultured with or without stromal cells in the presence or absence of the CXCR4 antagonist TN14003 treated with 20 µg/ml ALT (A) or 10 µg/ml RIT (B) for 4 hours in the presence of IL-2 activated PBMC at a ratio 10:1. IgG control of the respective culture setting (w/o stroma, w/ stroma, or stroma+TN14003) was defined as 100% relative viability. Data are presented as mean ± SEM values.

SI Fig. 7: $CFSE^+$ CLL cells were pretreated with 10 µM colchicine after incubation with and without stroma before IL-2 activated PBMCs were added. After 2 hours, viabililty was measured by $\%CFSE^+/PI^-$. Data are presented as mean ± SEM values.

Supporting information Table 1: Clinical characteristics of CLL patients.

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Figure 1

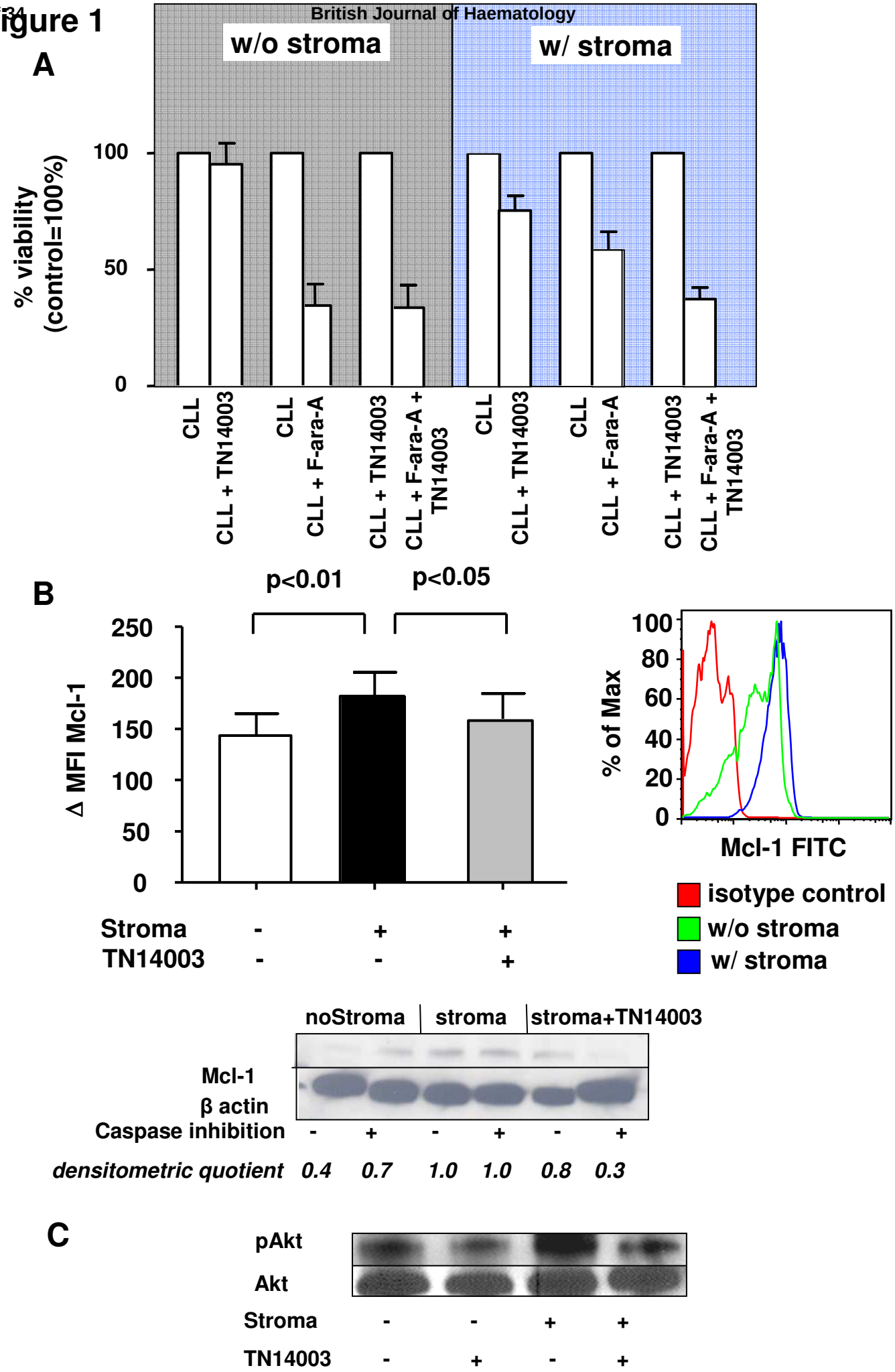

A
w/o stroma
w/ stroma
% viability (control=100%)
100
50
0
CLL
CLL + TN14003
CLL + F-ara-A
CLL + F-ara-A + TN14003
p<0.01
p<0.05
B
Δ MFI Mcl-1
250
200
150
100
50
0
Stroma - + +
TN14003 - - +
% of Max
100
80
60
40
20
0
Mcl-1 FITC
isotype control
w/o stroma
w/ stroma
noStroma
stroma
stroma+TN14003
Mcl-1
β actin
Caspase inhibition - + - + - +
densitometric quotient 0.4 0.7 1.0 1.0 0.8 0.3
C
pAkt
Akt
Stroma - - + +
TN14003 - + - +

Figure 2

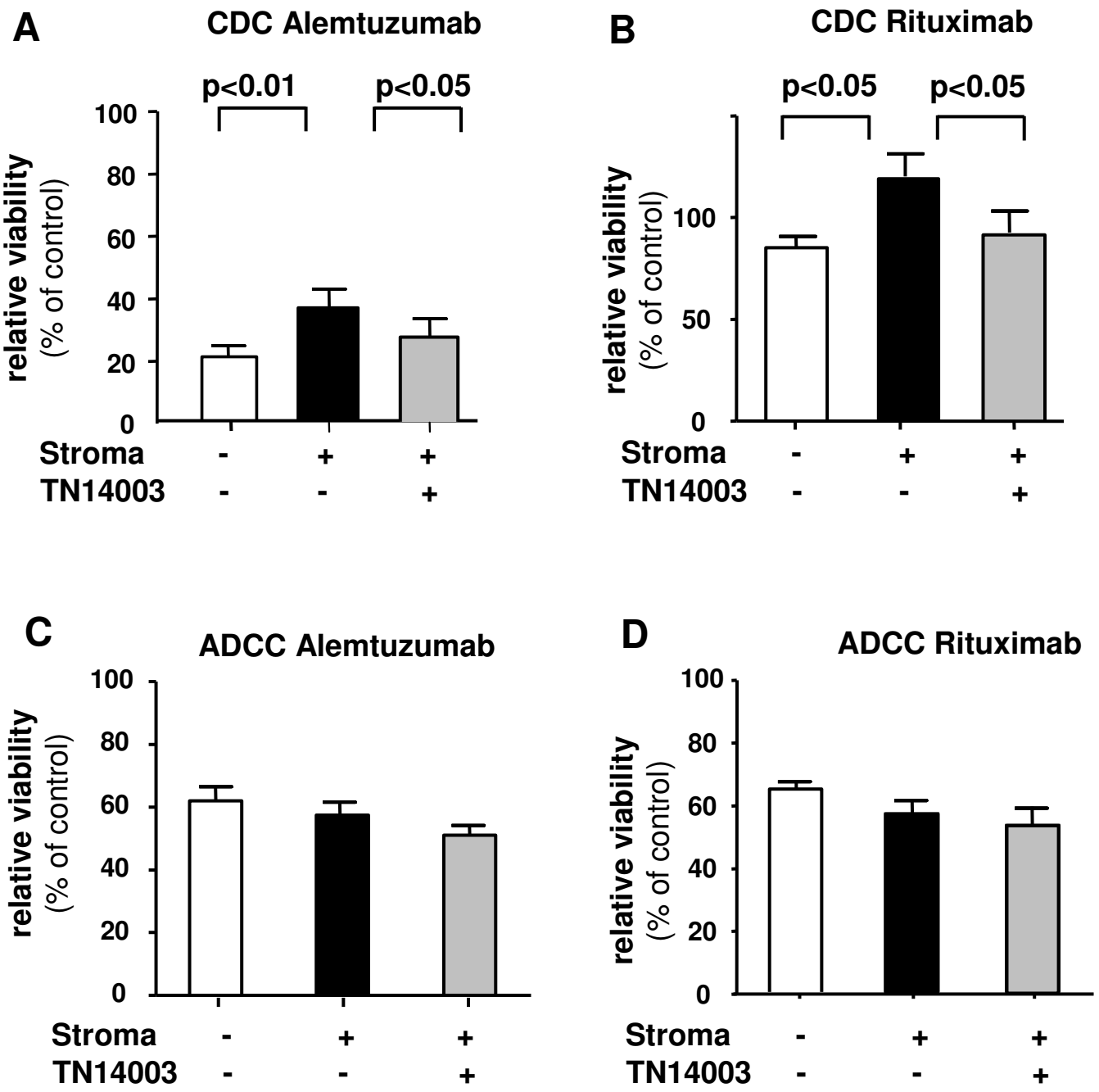

A
CDC Alemtuzumab
p<0.01
p<0.05
relative viability
(% of control)
100
80
60
40
20
0
Stroma - + +
TN14003 - - +
B
CDC Rituximab
p<0.05
p<0.05
relative viability
(% of control)
100
50
0
Stroma - + +
TN14003 - - +
C
ADCC Alemtuzumab
relative viability
(% of control)
100
80
60
40
20
0
Stroma - + +
TN14003 - - +
D
ADCC Rituximab
relative viability
(% of control)
100
80
60
40
20
0
Stroma - + +
TN14003 - - +

Figure 3

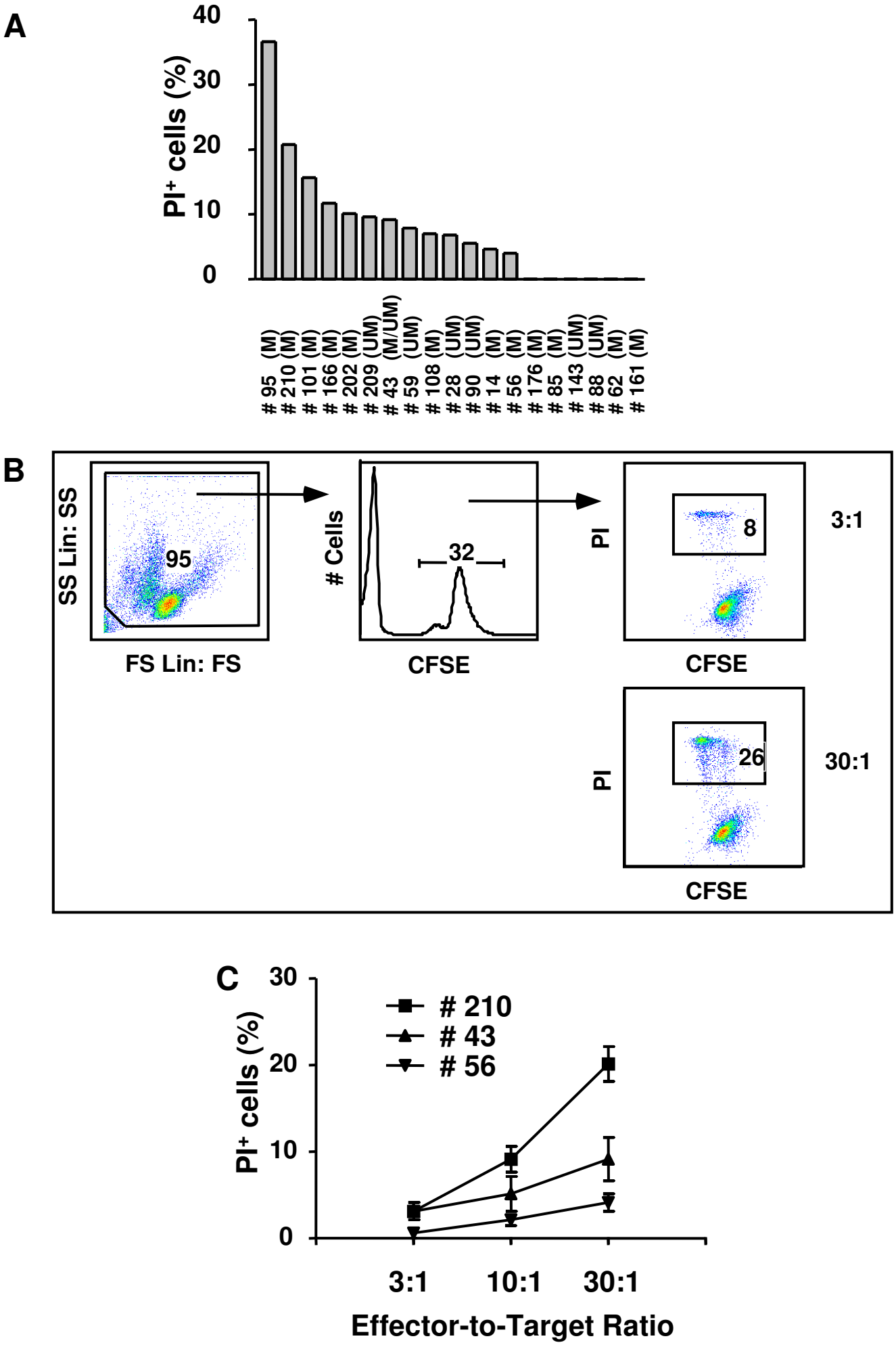

A
PI+ cells (%)
40
30
20
10
0
95 (M)
210 (M)
101 (M)
166 (M)
202 (M)
209 (UM)
43 (M/UM)
59 (UM)
108 (M)
28 (UM)
90 (UM)
14 (M)
56 (M)
176 (M)
85 (M)
143 (UM)
88 (UM)
62 (M)
161 (M)
B
SS Lin: SS
95
FS Lin: FS
Cells
32
CFSE
PI
8
3:1
CFSE
PI
26
30:1
CFSE
C
PI+ cells (%)
30
20
10
0
210
43
56
3:1
10:1
30:1
Effector-to-Target Ratio

Figure 4

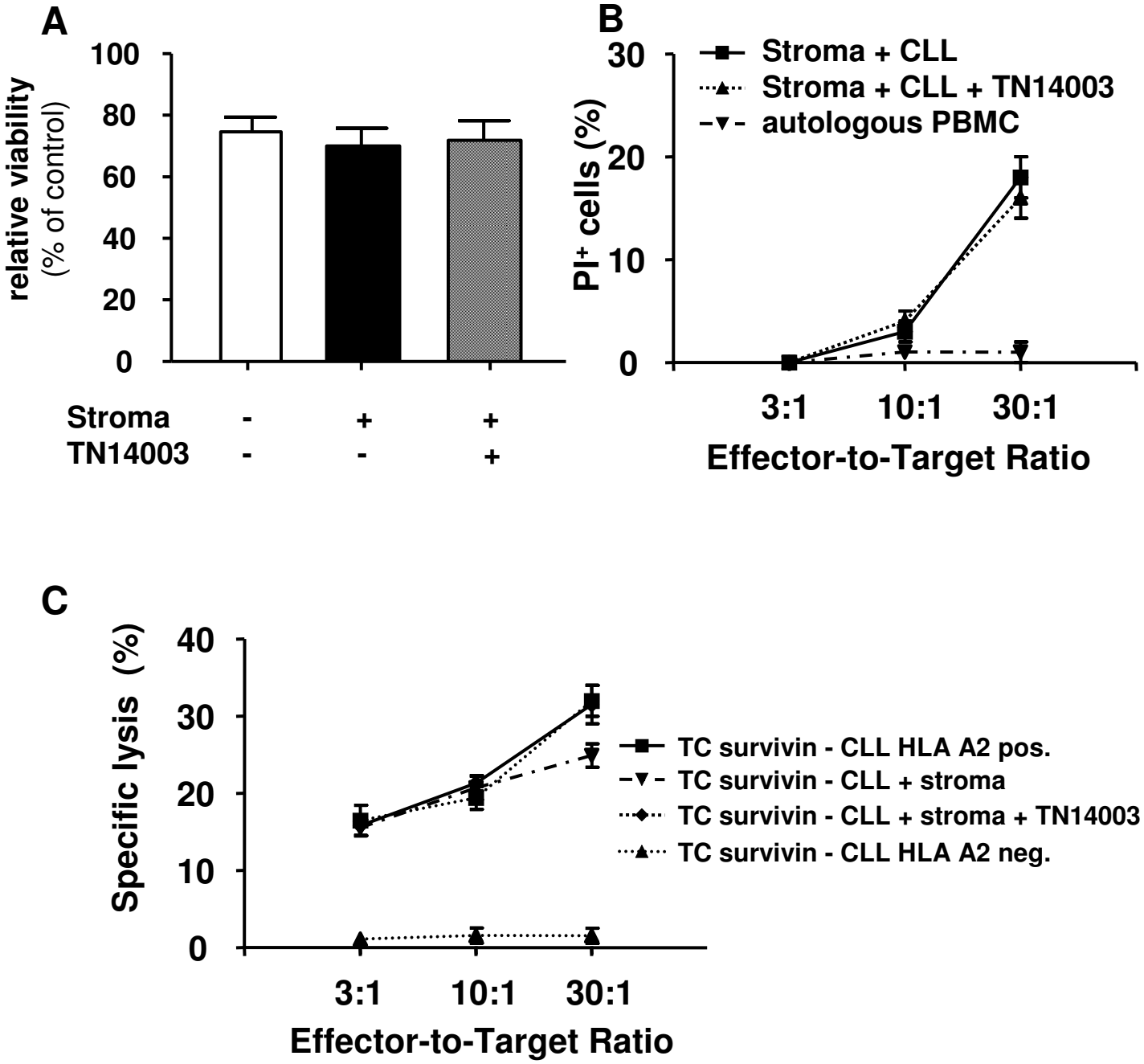
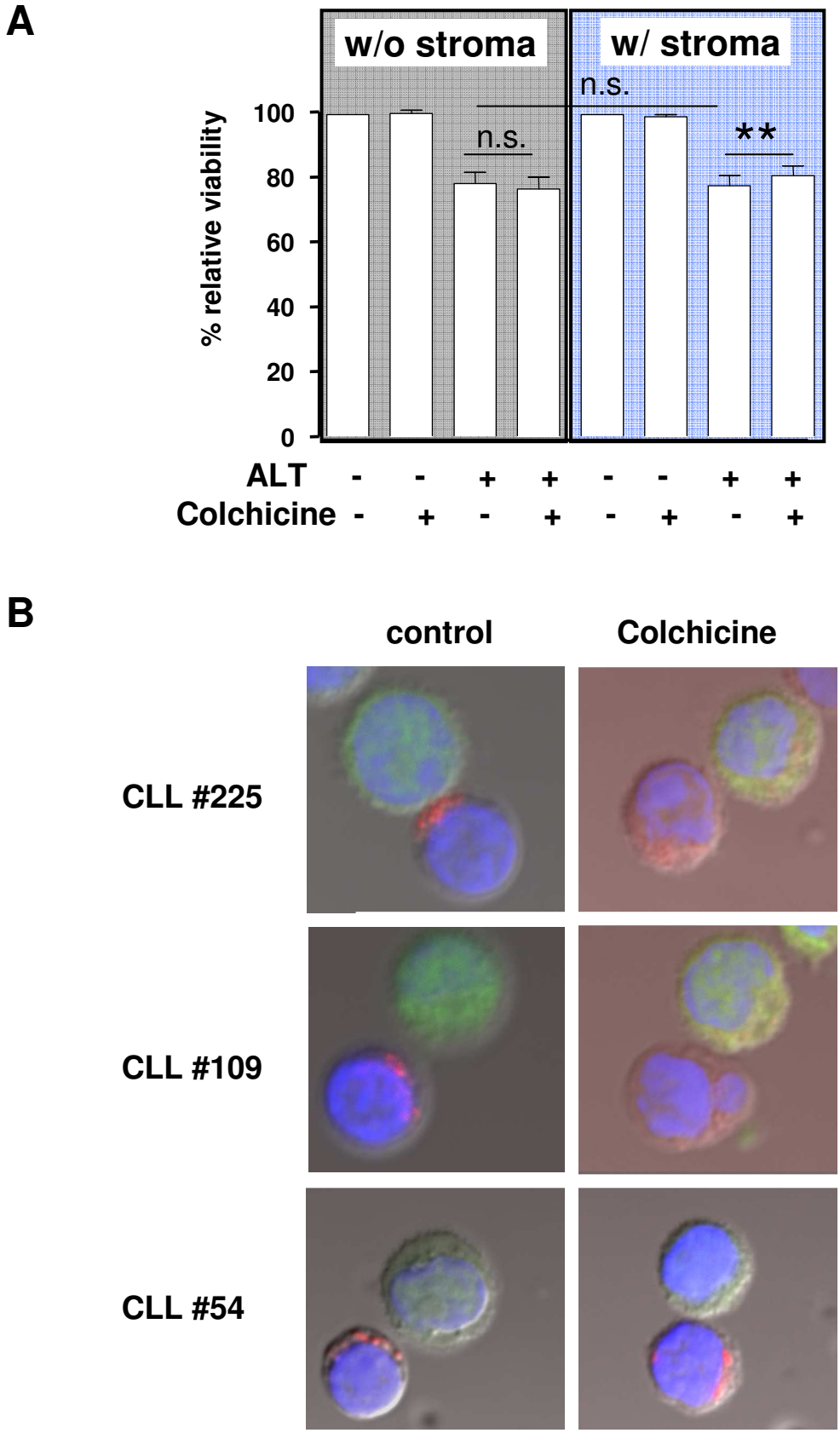
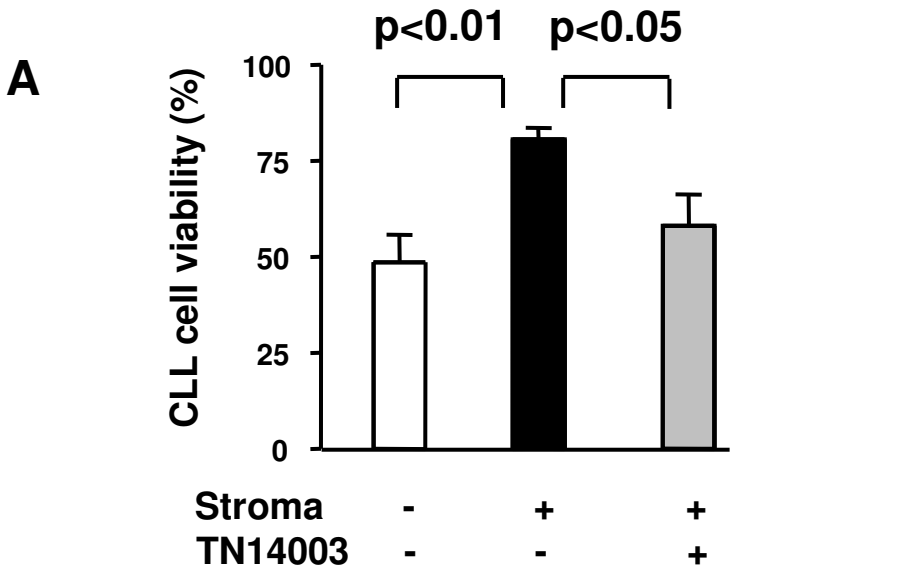
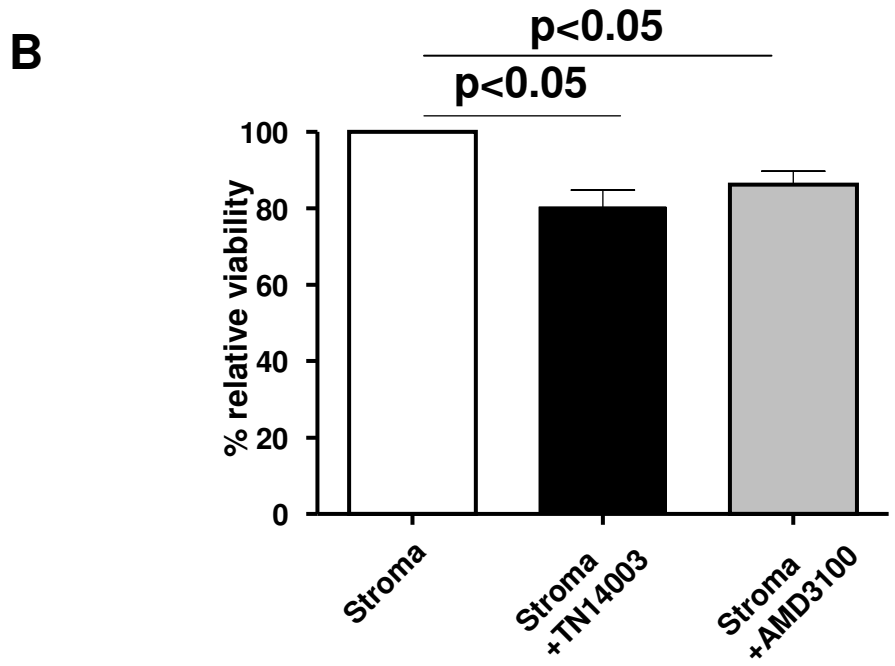

A
relative viability
(% of control)
100
80
60
40
20
0
Stroma - + +
TN14003 - - +
B
PI+ cells (%)
30
20
10
0
Stroma + CLL
Stroma + CLL + TN14003
autologous PBMC
3:1
10:1
30:1
Effector-to-Target Ratio
C
Specific lysis (%)
40
30
20
10
0
TC survivin - CLL HLA A2 pos.
TC survivin - CLL + stroma
TC survivin - CLL + stroma + TN14003
TC survivin - CLL HLA A2 neg.
3:1
10:1
30:1
Effector-to-Target Ratio

Figure 5

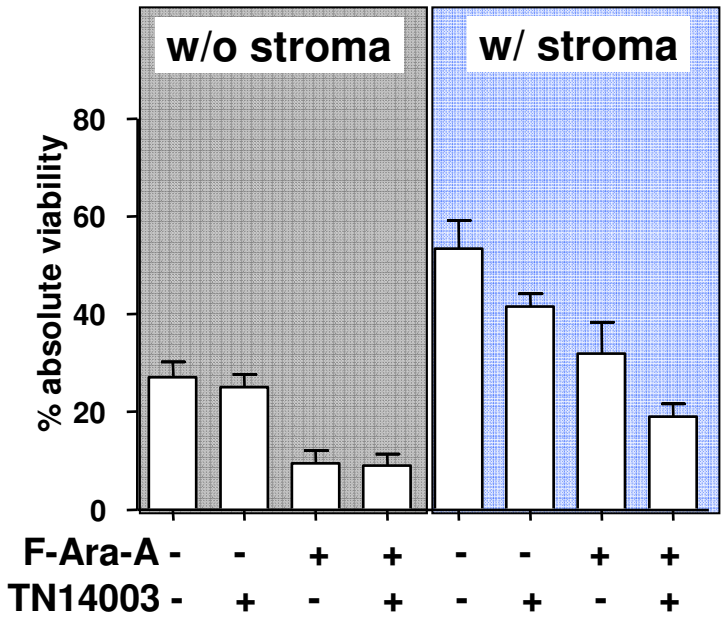

A
w/o stroma
w/ stroma
n.s.
n.s.
**
% relative viability
100
80
60
40
20
0
ALT - - + + - - + +
Colchicine - + - + - + - +
B
control
Colchicine
CLL #225
CLL #109
CLL #54

SI Figure 1

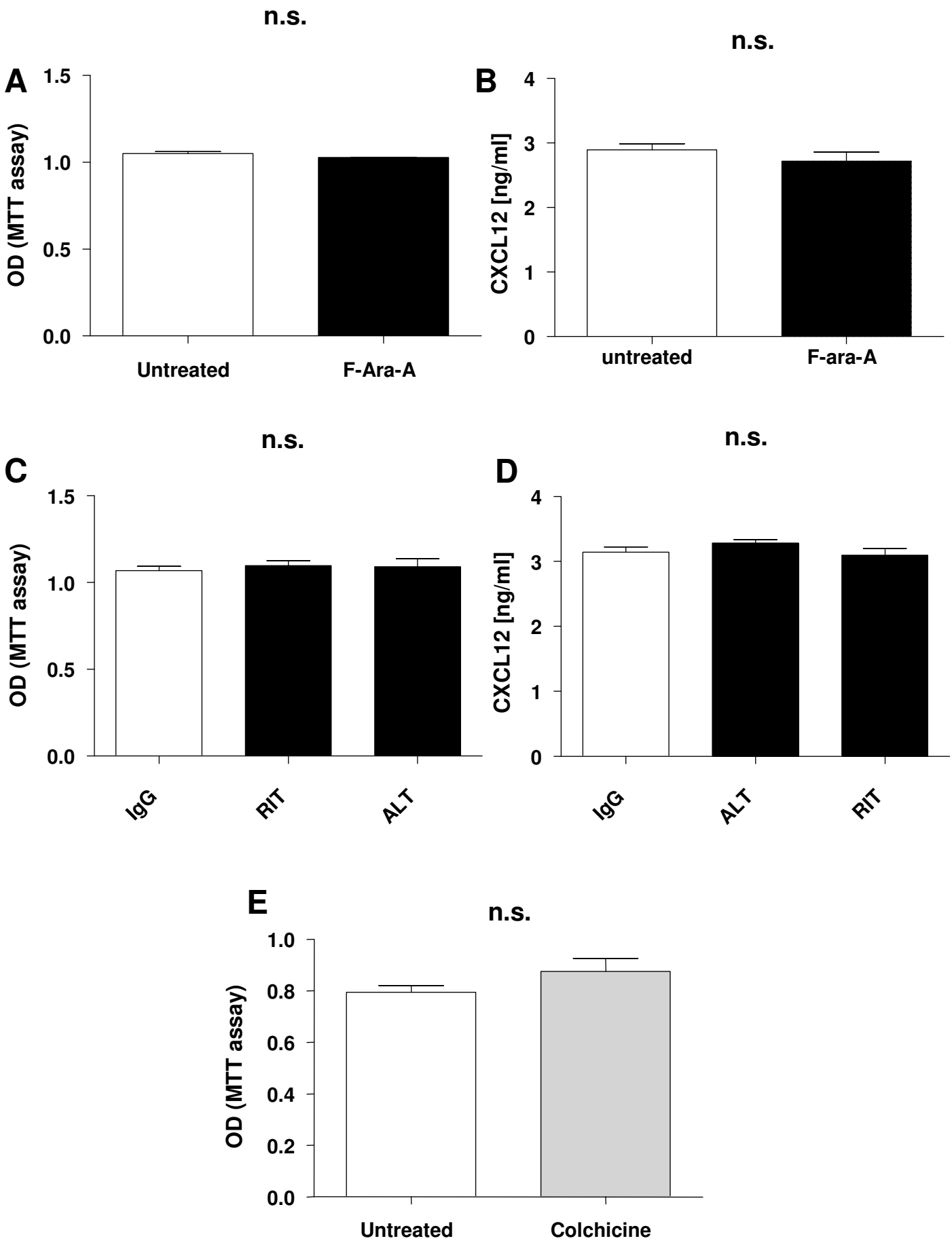




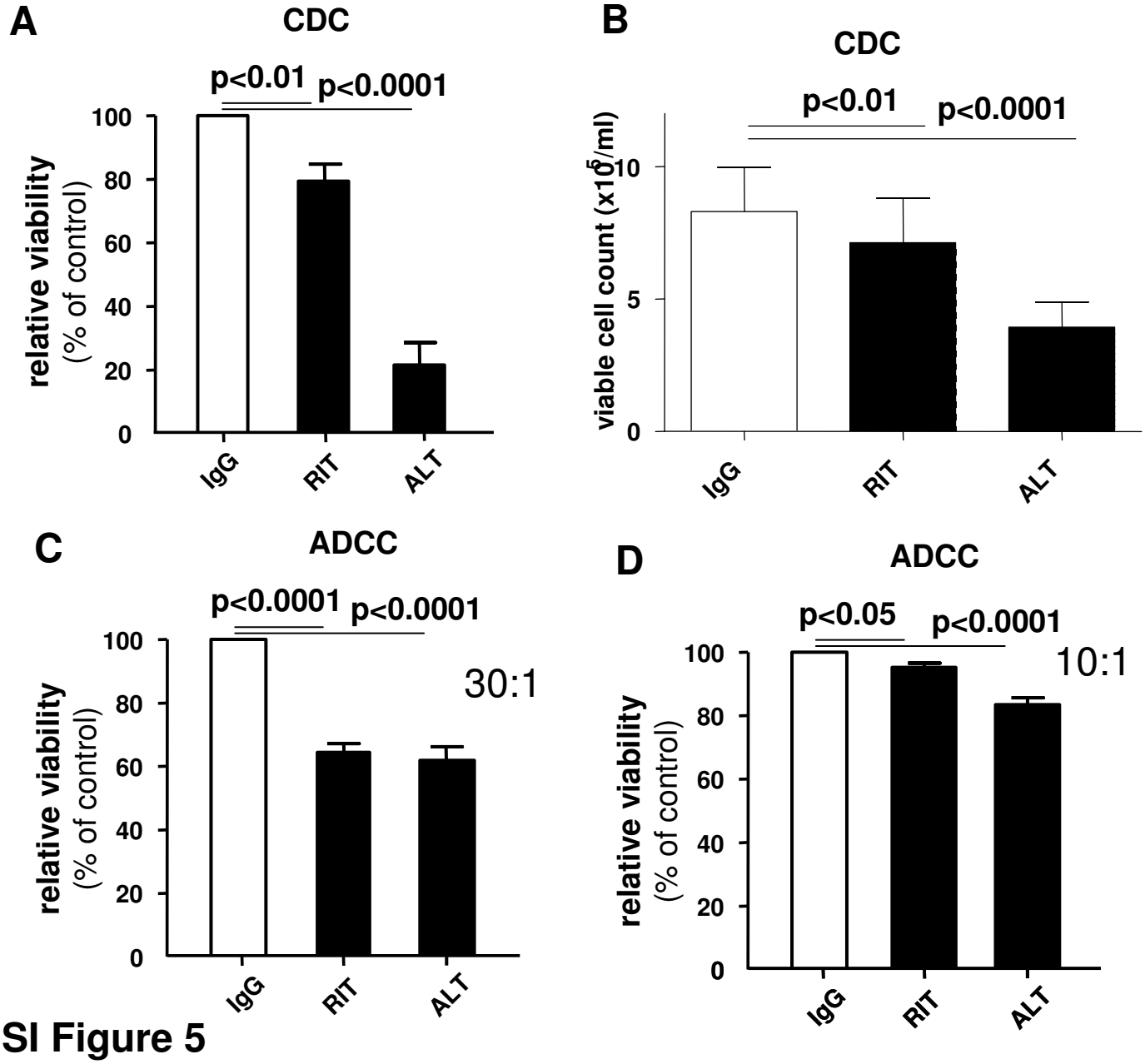
SI Figure 2



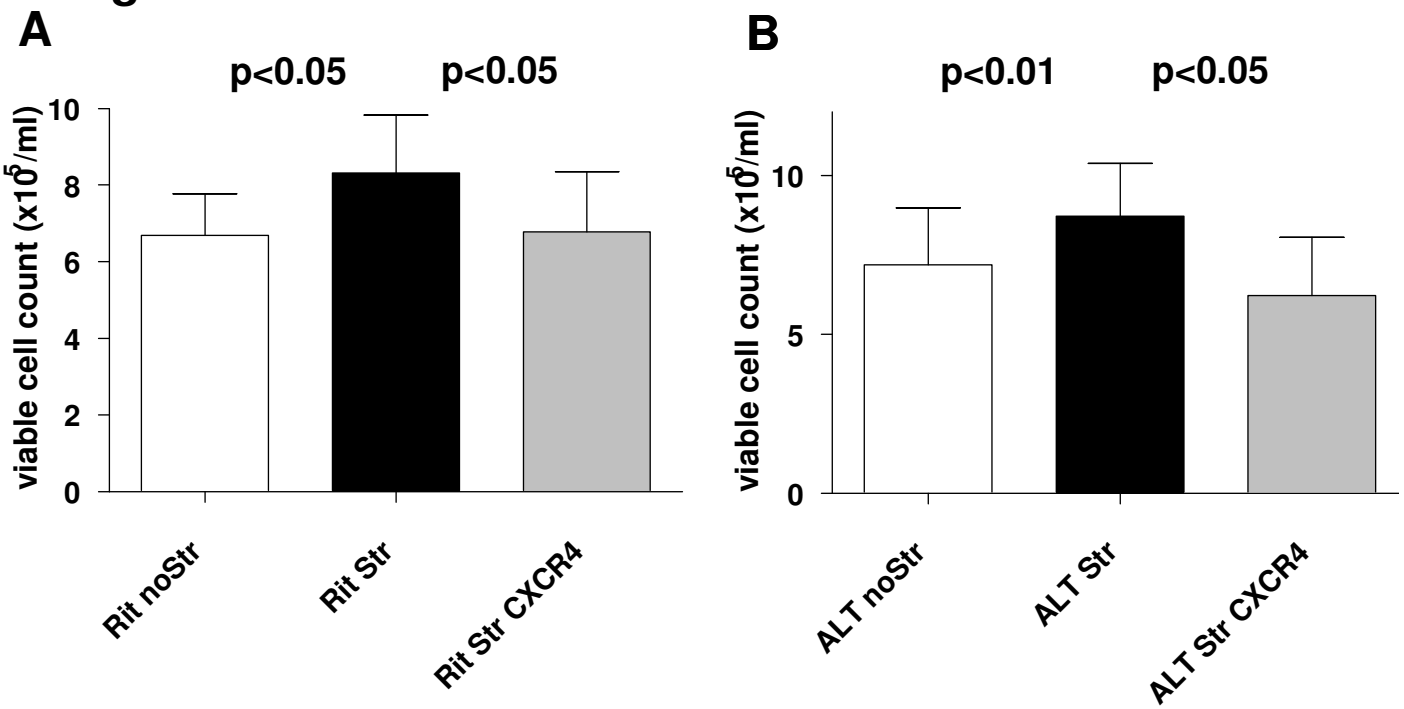
SI Figure 3


A
n.s.
OD (MTT assay)
1.5
1.0
0.5
0.0
Untreated
F-Ara-A
B
n.s.
CXCL12 [ng/ml]
4
3
2
1
0
untreated
F-ara-A
C
n.s.
OD (MTT assay)
1.5
1.0
0.5
0.0
IgG
RIT
ALT
D
n.s.
CXCL12 [ng/ml]
4
3
2
1
0
IgG
ALT
RIT
E
n.s.
OD (MTT assay)
1.0
0.8
0.6
0.4
0.2
0.0
Untreated
Colchicine

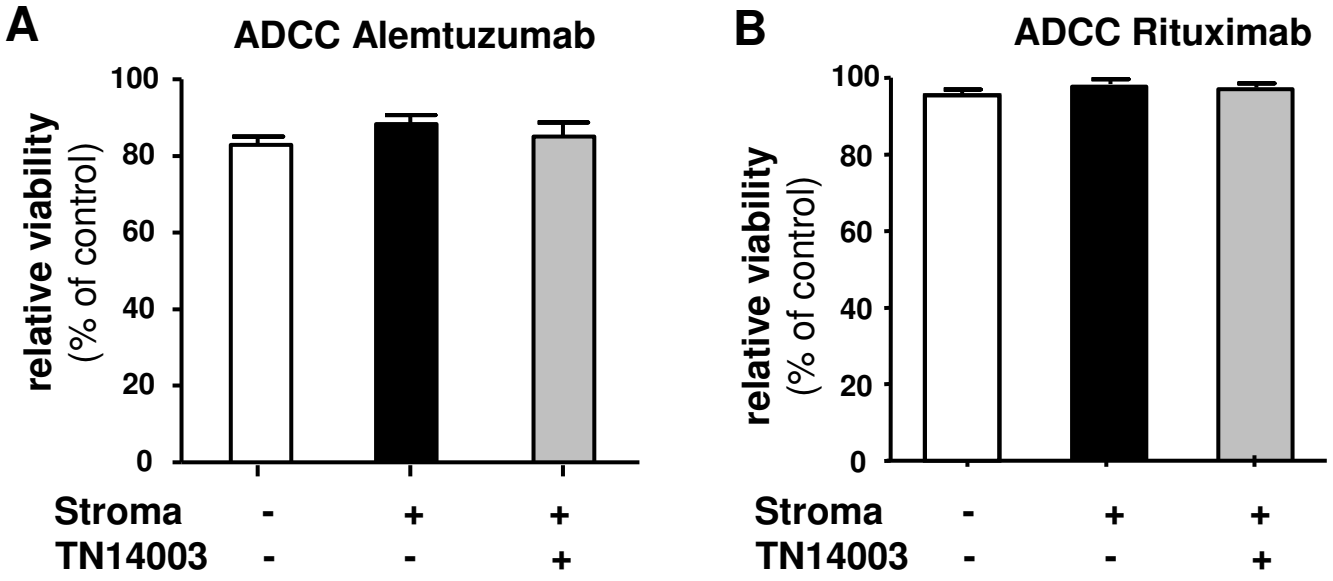
SI Figure 4



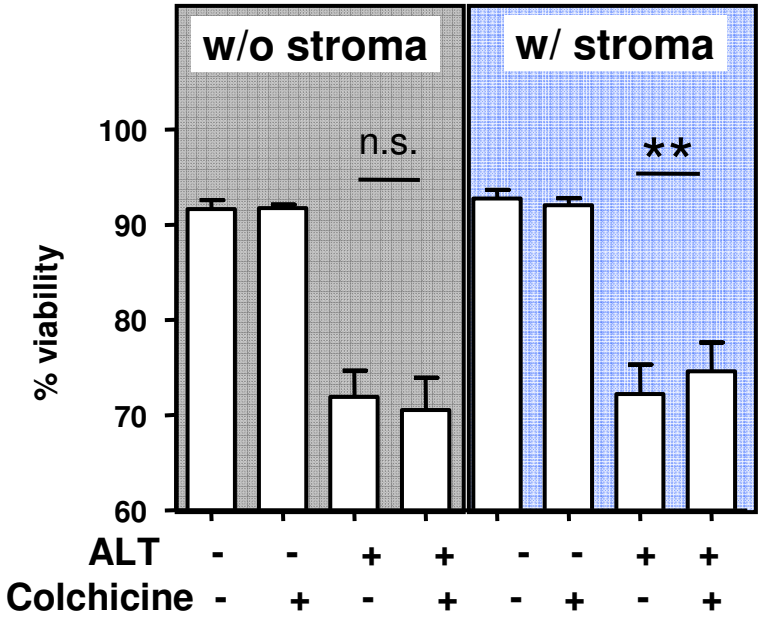
SI Figure 5



SI Figure 6



SI Figure 7



CLL #	Age	Sex	Rai	Binet	IgV_H	% mut.	ZAP70	Gen. Ab.	Prior therapy (at least 6 month ago)
95	69	M	2	B	M	2.1	positive	Del13q14	Chlorambucil
210	68	F	1	A	M	6.1	negative	n.d.	None
101	67	M	4	C	M	3.1	negative	Del13q14	Chlorambucil
166	85	F	0	A	M	6.8	negative	Del13q14	None
202	69	M	4	C	n.d.		negative	Del13q14	Fludarabine
209	66	M	0	A	UM	0	positive	n.d.	None
43	57	M	2	B	M / UM	7.2 / 0.7	negative	Trisomy 12	None
59	56	M	4	C	UM	0	positive	Del17p13	Fludarabine
108	68	M	0	A	n.d.		negative	n.d.	None
28	66	F	1	A	UM	0	positive	None	Chlorambucil
90	74	M	3	B	UM	0	n.d.	Del13q14	Chlorambucil
14	71	M	0	A	M	4.1	negative	Del13q14 homozygot	None
56	67	F	1	A	M	7.5	negative	Del13q14	None
176	71	F	0	A	n.d.		negative	Del13q14	None
85	73	M	2	A	M	10.8	negative	Del13q14	None
143	69	F	1	B	UM	0	n.d.	Trisomy 12	Chlorambucil
88	66	M	1	B	UM	0	n.d.	Trisomy 12	Chlorambucil
62	68	F	2	A	M	3.8	negative	Trisomy 3	None
161	71	M	0	A	M	6.8	n.d.	Del13q14 biallelic	none
140	55	M	2	A	M	4,8	n.d.	Del13q14	none
146	69	M	0	A	M	8,9	negativ	Del13q14	none
77	58	M	1	A	n.d.		n.d.		none
23	67	M	2	B	UM	0	n.d.	Del13q14, Del17p13	none
69, 183	78	M	3	C	UM	1,3	positiv	none	none
91	45	M	0	A	M	3,4	n.d.	Del13q14	none
107	74	F	2	B	M	3,1	negativ	keine	Chlorambucil
100	73	F	2	A	M	8,2	negativ	Del13q14	Chlorambucil, Fludarabin
45	59	M	2	A	M	6,9	negativ	Del13q14	none
191	69	F	0	A	M	9,9	n.d.	n.d.	none
109	70	M	2	A	UM	0	n.d.	Del13q14	Fludarabin, Bendamustin
225	60	F	4	C	M		n.d.	Del 13q14	Chlorambucil
54	48	M	2	B	UM	0	pos	Del18p19	Fludarabin