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Targeting the binding interface on a shared receptor subunit of a cytokine family enables the inhibition of multiple member-cytokines with selectable target spectrum.

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Running Title: A Novel concept for designing multi-cytokine inhibitors

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Key words; Interleukin, Drug design, Cell proliferation, peptide interaction, Cytokine action, γ c-family cytokines, anti-cytokine therapy, HTLV-1 myelopathy,

Capsule

Background: Current therapeutic strategy cannot suppress multiple cytokines in human disease.

Results: We developed a new strategy to selectively block γ c-family cytokines.

Conclusion: Our novel strategy was proven effective both in vitro and in vivo.

Significance: This approach will fill in a gap in the existing therapeutic strategy.

SUMMARY (220 words)

The common gamma molecule (γc) is a shared signaling receptor subunit used by six γc -cytokines. These cytokines play crucial roles in the differentiation of mature immune system and are involved in many human diseases. Moreover, recent studies suggest that multiple γc -cytokines are pathogenically involved in a single disease, thus making the shared γc -molecule as a logical target for therapeutic intervention. However, the current therapeutic strategies seem to lack options to treat such cases, partly because of the lack of appropriate neutralizing antibodies recognizing the γc , and more importantly because of the inherent and practical limitations in the use of monoclonal antibodies. By targeting the binding interface of the γc and cytokines, we successfully designed peptides that can not only inhibit multiple γc -cytokines but with selectable target spectrum. Notably, the lead-peptide inhibited three γc -cytokines without affecting other three or non- γc cytokines. Biological and mutational analyses of our peptide provide new insights to our current understanding on the structural aspect of the binding of γc -cytokines the γc -molecule. Furthermore, we provide evidence that our peptide, when conjugated to polyethylene glycol to gain stability in vivo, efficiently block the action of one of the target cytokines in animal models. Collectively, our technology can be expandable to target various combinations of γc -cytokines and thereby will provide a novel strategy to the current anti-cytokine therapies against immune, inflammatory and malignant diseases.

INTRODUCTION

The four-helical bundle structure is a common feature shared by many cytokines (1). Another shared feature is the sharing of receptor and signaling components which account for the "Pleiotropy" and "Redundancy", the two important characteristics (2), of cytokines and provides structural basis for the family clustering of cytokines. Examples include the IL-6 family which uses the gp130 molecule, the γc -family (IL-2, -4, -7, -9, -15, and -21) using γc , and the IL-3 family

which uses the common β molecule. The γc -cytokines diversely control normal immune responses (3) as exemplified by defective immune functions of knockout mice or humans lacking individual γc -cytokines, receptor or signaling components (4-7). Moreover each γc -cytokine is connected with various immune and inflammatory disorders in humans. IL-2 has been implicated in inflammatory bowel diseases (IBD) (8-11). Likewise, IL-4 has been implicated in Asthma (12), IL-7 in multiple sclerosis, ulcerative colitis, and sarcoidosis (13), IL-9 in Allergic inflammation (14) and in Asthma (15). Over-expression of IL-15 in mice (16,17) and in humans (18) is associated with T/NK leukemia. IL-15 has been also strongly connected with Celiac disease (19-26). IL-21 (27) is a recent addition and involved in the differentiation of B and follicular helper T cells (28), which has been implicated in IBD/ Celiac disease, psoriasis, uveitis (29), atopic dermatitis, systemic lupus erythematosus (SLE), multiple sclerosis (MS) and type I diabetes (30,31).

In these cases, involvement of more than two cytokines from a family (A table is provided) has been implicated. Surprisingly, efficient treatment of these cases may prove challenging to the current anti-cytokine strategy that relies on monoclonal antibodies (one specific target) or signal inhibitors (32) (many targets but poor specificity). Because of the sigmoid nature of the cellular response to cytokines, a neutralization of only one factor in a combination of redundant factors would have negligible inhibitory effect when each factor is causing near-saturation effect. Thus we decided to design inhibitors that can target more than two γc -cytokines for clinical uses. To this end, we reasoned to target the interface of the cytokine and γc interaction by taking advantages of the recent advancement in the structural information on the binding of cytokines and receptors. We combined the natural sequences of γc -cytokines and synthesized composite peptides to be tested by biological assays. This design strategy not only allowed us to design inhibitors to target multiple- γc cytokines, but provided us a capacity to choose target cytokines by design. Here, we demonstrate that

our lead peptide (BNZ132-1) selectively inhibits IL-2, IL-9, and IL-15 of the γ c-cytokines but no other cytokines (a proof-of-concept). Additionally, we provide evidence for a translational merit of our concept using ex vivo cells from a human disease. Our concept may be expandable to eventually creating a library of compounds to target any human diseases that pathogenically involve multiple γ c-cytokines.

EXPERIMENTAL PROCEDURES

Cell Culture and Proliferation Assay - Cells were maintained in 10%FBS (fetal bovine serum, Gemini Bio, West Sacramento, CA)-RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 2mM glutamine, Penicillin and Streptomycin in a humidified 5% CO₂ incubator at 37°C. For cytokine proliferation assay, cells were plated at 0.2 million/ml (for cell lines, 2 million/ml for PBMCs) in 0.2 ml in 96-well plates in a triplicate manner for 20 h. One μ Ci ³H-thymidine (GE-Healthcare, Pittsburgh, PA) or 20 μ l WST-1 (Clontech, Mountain View, CA) reagent was added for 6 h before measurements. PBMCs were isolated from blood by the Ficoll-paque density gradient (GE-Healthcare), stimulated by 5 μ g/ml PHA-P (Sigma, St. Louis, MO), expanded by 1nM IL-2 (Peprotech, Rocky Hill, NJ) for 48 h before setting up a proliferation assay. In some occasions, T cells were negatively enriched from the expanded PBMCs using MACS (Miltenyi, Cambridge, MA). PT-18 and derivative clones (33) are maintained with 1nM of human IL-3 (supplemented as a conditioned medium of NIH3T3 fibroblasts transfected with the human IL-3 gene prepared in house).

Materials derived from Patients- Samples were obtained from HAM/TSP patients upon written and informed consent. The study was reviewed and approved by the institutional review board of the NINDS, NIH.

Peptide Synthesis and FITC-conjugation - Peptides were synthesized and FITC-conjugated by Bachem (Torrance CA). The control peptide is a 19 aa (Q-I-T-I-S-I-L-S-Q-I-N-R-V-F-H-E-K-F-I), a scrambled sequence of BNZ132-1. The F/P ratio (0.5) was determined based on OD₄₉₅ and OD₂₈₀.

Alanine substitution - A library of Alanine-substituted mutant peptides of BNZ132-1 were

synthesized by Mimotopes (Melbourne, Australia) (34). The working stocks were prepared in PBS (pH 6.5). The peptides were added to the CTLL-2 cells at 1 or 10 μ M (final concentrations) with 100pM IL-2 or IL-15. The proliferation was measured 24 hr thereafter. The Assays were performed in triplicate and in repeat of three times.

Neutralizing antibodies - Neutralizing antibodies against cytokines were purchased from the R & D Systems (Minneapolis, MN): Human IL-2; MAB202, mouse IL-4; MAB404, human IL-7; MAB207, mouse IL-9; MAB409, human IL-15; MAB247, mouse IL-21; AF594 (polyclonal Ab), mouse IL-3; MAB403, mouse GM-CSF; MAB415, mouse stem cell factor; MAB455, human FLT3-L; MAB308. Antibodies used for flow experiments (details in the supplemental information) were purchased from BioLegend (San Diego, CA) and eBioscience (San Diego, CA).

Immunoblotting - Cellular Proteins were extracted using the RIPA buffer (0.5 % Nonidet 40 /10mM Tris-Cl (pH8.0)/1mM EDTA/0.5mM EGTA/ 0.1% sodium deoxycholate/150mM NaCl/Proteinase inhibitor cocktail (Roche)). Samples were resolved on Tris-Glycine gels (8-16% gradient gel, Invitrogen) under reducing conditions, transferred to a PVDF membrane (Millipore, Billerica, MA) and blocked overnight in the Superblock solution (Pierce Scientific, Rockford, IL). Primary Ab was diluted at 1:2000 in 0.05%Tween20-PBS (T-PBS) for 1 h incubation at 37°C with the membrane. After 5 washes (5 min ea.) in T-PBS, the blot was incubated with horse radish peroxidase-conjugated secondary Abs against rabbit/mouse IgG (Cell Signaling Technologies, Danvers, MA, used at 1: 3, 000) for 1 h at room temperature, and visualized using chemiluminescence (ECL plus, Perkin) according to manufacturer's instructions. Sample loadings were verified by reprobing blots using anti-Vinculin (clone; hVin-1, Sigma) Ab.

Flow Cytometry - About 2x10⁵ cells were washed in 1%FBS-PBS (FlowCytometry Media, FCM) and re-suspended in 10 μ l Ab cocktail that contains appropriately (determined by prior titration experiments) diluted individual antibodies in FCM. Incubation was performed 45

min on ice, then pellets were washed twice in FCM. PMT of the FACSaria II (BD Biosciences, Washington DC) was calibrated using isotype control antibodies with matching fluorochromes. Antibodies were purchased from Biolegend (San Diego, CA). Data were analyzed using the FlowJo software (FlowJo, Ashland, OR).

Protein alignment - For the structural comparison of cytokines, the T-coffee algorithm was used (35).

PEG conjugation of BNZ132-1 - BNZ132-1 was synthesized with a specific linker (CGSGG), conjugated with Methoxy-PEG (Mr. 20, 40, and 60 kDa)-maleimide molecules and purified by a reverse phase HPLC. Conjugation was performed on the N- and C-termini of the peptide. Upon testing their inhibitory action using CTLL-2 cells¹, we decided to focus on the PEG40-conjugated BNZ132-1 (BNZ132-1-40, PEG40-linker- BNZ132-1). In vivo dose of BNZ132-1-40 was determined based on the estimated IC₅₀ values (~0.5 nM), to be at or around 0.8 mg/kg. Separate pharmacokinetics studies indicated that the PEG40-BNZ132-1 (BNZ132-1-40) shows 84.4 hr half life (NA, data not shown) in mice, in support that two injections per week would maintain effective biological concentrations necessary for effective inhibition of target cytokines.

Mouse in vivo experiments - All mouse experiments have been conducted following a protocol approved by the IACUC of University of Maryland School of Medicine, and the mouse housing and injection part of the experiments were conducted at Bioqual Inc. (Rockville, MD).

Age matched (12 wk old) female congenic Thy 1.1-C57B6 mice (Jackson Lab) were used for the preclinical animal studies. T-reg expansion by IL-2 was induced in these mice by injecting 150 µg of recombinant human IL-2 (Peprotech, NJ) intraperitoneally (i.p.) on days 1 and 4. To observe the therapeutic, rather than prevention effects of our compounds, intervening compounds such as neutralizing anti-IL-2 antibody (R & D Systems, MAB 202, 100 µg per injection) or BNZ132-1-40 (1 mg/kg, ~ 25 µg per mouse) was injected intraperitoneally to expect slow release. Venous blood was collected on day 7 to purify PBMCs by the Ficoll-paque density separation. Cells were

stained by a mixture of antibodies (FITC-CD8; clone 53-6.7, PE-CD44; clone 1m7, PerCP/Cy5.5-CD4; clone RM4-5, APC-CD25; clone 3C7, Brilliant Violet 421-CD3; Clone 145-2C11) to identify major T subsets including the T-regs.

For the IL-15-leukemia study, an established cell line producing its own IL-15 (from IL-15 transgenic mice on the Thy1.2 C57B6 background, (17)) has been maintained in vitro in 10% FBS-RPMI without any exogenous cytokine in culture. One million cells were injected in 150µl sterile PBS intravenously (i.v.) via the tail-vein on day 0. Intervening compounds (anti-IL-15 Ab and BNZ132-1-40, Low at 1mg/kg, and high at 10 mg/kg) were injected on days 1, 4, 8, and 11. Tofacitinib was continuously administered using a subcutaneous osmotic pump (ALZET, Cupertino, CA) as described before (36). Body weight of the mice was monitored every week to detect signs of general distress. Over 20% of body weight loss, blood was collected from mice for flow analysis to detect the expansion of Thy 1.1+ leukemic cells. Antibodies used for flow cytometry were Brilliant Violet 421-Thy1.1 (CD90.1); clone OX-7 and Brilliant Violet 510-Thy1.2 (CD90.2); clone 53-2.1).

Results

Targeting the interface of the γ c and cytokine interaction - Based on data mining, we compiled a table of human diseases in which more than two members of the γ c-cytokines have been implicated (Table 1). We aimed at designing antagonists against γ c-cytokines that target multiple, but select members of this family. As a start, we chose IL-2 and IL-15. The D-helices (last of the four α -helices) of γ c-cytokines primarily interact with the γ c (37) and critical residues of IL-2 or IL-15 that interact with each receptor subunit (i.e., α , β , or γ c) have been identified (38). Consistently, the D-helices, among all 4 helices, show highest conservation across mammalian species (Table 2). The alignment of D-helices from human γ c-cytokines demonstrated a mildly conserved motif within this region (the γ c-box, Fig.1A). We also noticed an additional short motif (the IL-2/15-box, Fig.1B) involving IL-2 and IL-15 (IL-21 may be included, albeit weakly). These two form a subfamily in the

γ c-family due to the sharing of another receptor subunit IL-2/IL-15R β between them (39,40). Our finding may indicate that these D-helices of two cytokines spatially overlap more closely than with D-helices from other γ c-cytokines in their binding to the γ c.

Design of BNZ132-1, a specific inhibitor for the IL-2/IL-15 subfamily - Although our ultimate goal is to prepare a library of compounds that could cover all potential combinations of γ c-cytokines (Supplementary Table 1 for detail), we first attempted to design a common inhibitor for IL-2 and IL-15 to test our concept, because these two cytokines are structurally most related among γ c-cytokines (39,40). The 3D-structures of the D-helix of IL-2 and IL-15 are nearly super-imposable, but fine differences exist in their binding to γ c (38). We hypothesized that by leveraging differences in their primary structures, we could design an inhibitory peptide that equally blocks IL-2 and IL-15. Our design logic was as follows: 1; such peptide(s) should form a helical structure, 2; it should contain key aa responsible for the binding of target cytokines to γ c, 3; peptides with composite sequence derived nearly equally from IL-2 and IL-15 (minimum bias to either cytokine) should be tested. Of the 120 peptides (Supplementary Table 2) that were designed and screened by a computer docking simulation (Supplementary Figure 1), 39 showed appropriate docking. They were all synthesized in small scale and tested for inhibition on IL-2 and IL-15. BNZ132-1 (Fig. 1C) was finally chosen as the most potent inhibitor of IL-2 and IL-15 actions.

Direct binding of BNZ132-1 to the γ c (CD132) molecule - In order to prove that BNZ132-1 directly binds to the γ c molecule (CD132), we conducted a binding study. Human γ c-molecule was expressed on murine PT-18 cells by gene transfection (PT-18hu γ , Fig 2G, H). BNZ132-1 and a scrambled control peptide (See experimental procedures) were purchased as FITC (fluorescein isothiocyanate)-conjugated form and used in binding studies. Notably, PT-18 parental cells express marginal levels of mouse γ c (Tagaya et al. 1996, Fig 2E,F vs. H) that is almost undetectable by a commercial antibody (TUGm2). Similarly, FITC-

BNZ132-1 failed to show binding (Fig 2A) due to the low F/P ratio (0.5) of FITC-peptides, leaving only the transfected human γ c as the detectable target on PT-18 hu γ for FITC-peptides. Under these conditions, FITC-BNZ132-1 only show binding to PT-18hu γ cells (Fig 2B/C), but not with PT-18 parental cells (Fig 2A). FITC-Control peptides failed to show detectable binding to any cells used (Fig 2 A/B). To test the specificity of the binding, FITC-BNZ132-1 was incubated with 4-fold excess unlabeled BNZ132-1 before binding to target cells and the binding was nearly completely inhibited (Fig 2D) in a dose-dependent manner. These results collectively confirmed our original hypothesis that BNZ132-1 directly binds to the γ c subunit.

Inhibitory profile of BNZ132-1 to cytokines – Fig.3A shows the efficient inhibition of IL-2 and IL-15 by BNZ132-1, which is an affirmation of the screening process described above. To our knowledge, this is the first example of a single peptide equally blocking two cytokines from this family. We next tested if BNZ132-1 inhibits other γ c-cytokines using murine PT-18 cells (33,41). Fig. 3B demonstrates that BNZ132-1 does not inhibit IL-4 in PT-18 ($p=0.32$), but partially inhibits IL-9 ($p=0.001$), albeit not as robust as its inhibition on IL-2 or IL-15. A recent report mapped IL-9R α structurally close to IL-2/IL-15R β (42) among the private chains for γ c-cytokines, and this may account for the unexpected inhibition of IL-9 by BNZ132-1. Finally, we tested if the two remaining γ c-cytokines (IL-7 and -21) could be inhibited by BNZ132-1. We established a PT-18 subclone that responds to human IL-21 by transfecting human IL-21R α . Though hIL-21R α *PT-18 cells did not robustly proliferate to IL-21 (Fig.3B), IL-21 prevented their apoptotic death following the withdrawal of IL-3 from the culture (Fig.3C, $p=0.001$), and this process was not inhibited by BNZ132-1 ($p=0.59$, not significant). Next we tested if our peptide blocks the IL-7 function. Curiously, the human -mouse chimeric IL-7 receptor (h IL-7R α +m γ c) generated on PT-18 by gene transfection failed to confer response to human IL-7². Thus, we used ex vivo human peripheral T cells to address the question. As shown in Figure 3D,

human T cells demonstrated moderate proliferative response to 10 nM IL-7 (vs. No cytokine; $p=0.002$) which was not significantly inhibited by BNZ132-1 ($p=0.30$). Collectively, the inhibitory capacity of BNZ132-1 seems restricted to IL-2, -15, and -9, but excludes IL-4,-7 or -21.

Off-the-target effects of BNZ132-1 on non- γ c cytokines - Next, we tested several non- γ c cytokines to examine the potential off-the-target effects of our peptide. We again used PT-18 which natively responds to an array of non- γ c cytokines (33). As shown in Fig. 3E, BNZ132-1, even at an excess dose of 15 μ M, showed no inhibition on these non- γ c cytokines.

Dose-inhibition profile of BNZ132-1 - Next we estimated the affinity of BNZ132-1 binding to γ c by analyzing the dose-inhibition response. As the Cheng-Prusoff equation (43,44) dictates, IC_{50} is closely associated with the binding affinity of the antagonist to the cellular receptor. Fig. 3F-I demonstrate that BNZ132-1 inhibits IL-2, and -15 by 50% at or around 50-500 nM in human cells (NK92 cell line and ex vivo human T cells (Fig 4D). though doses for efficient biological inhibition varied between cell types including murine cells that are not shown here), which gives approximately 25~250nM of binding affinity of BNZ132-1 to target cells. The inhibition of IL-9 was less efficient as μ M concentration of peptide was needed for efficient blocking. These results, however, show some discrepancy from those obtained from direct binding assays (See discussion).

Efficient inhibition by BNZ132-1 of the combined cytokines sharing redundant function - So far, we have shown that BNZ132-1 inhibits not only one or all γ c-cytokines, but three of these cytokines (IL-2, -9 and -15). We next asked if BNZ132-1 can effectively block the combined effect of two γ c-cytokines (i.e., IL-2 and IL-15). To this end, we conducted a series of experiments using human ex vivo T cells to determine basic conditions such as the saturating cytokine concentration and antibody concentrations necessary to achieve full inhibition of target cytokines (Fig 4A~C). Notably, even a 50:50 mixture of IL-2 and IL-15 cannot be inhibited by

50% using either anti-IL-2 or anti-IL-15 antibody (Ab) alone if the combined cytokines make near-saturating response (Fig 4B, 100pM + 100 pM). Naturally, each antibody could more efficiently block the mixture if the single and combined effects of cytokines are on the linear phase of the response (50 pM + 50 pM). This can be explained by two factors; 1) the gradient of the response-dose curve at the semi-linear phase is not equal to 1 (Fig 4A, 25pM; 10%, 50 pM ; 45%, 100 pM; 75%), 2) at near saturation dose, doubling the cytokine concentration (e.g. from 100 to 100+100 combination could only block ~20% of the response) only increases the biological response from 75% to 95%, therefore an Ab to either factor). These demonstrations suggest that a single Ab treatment may fail to show discernible effects in vivo against a mixture of factors even if the target cytokine represented near 50% of the biological activity in the mixture. BNZ132-1 *per se* blocked the T-cell proliferation as efficiently as the combined antibodies at two doses of the stimulation, demonstrating the unique and efficient therapeutic potential of our compound for multi-cytokine diseases.

A translational merit for BNZ132-1 in a human disease -We and others previously reported that freshly isolated T-lymphocytes from HAM-TSP patients spontaneously proliferate *ex vivo* without external activations (45,46) and that this proliferation is mediated by the formation of an autocrine loop involving at least IL-2 and IL-15 as a consequence of transcriptional trans-activation by Tax of HTLV-1 (46,47). Above, we have shown that one Ab against each cytokine would have limited inhibitory capacity to the overall cellular responses to a combination of functionally-redundant cytokines. Similar result was observed in HAM-TSP cases (46). As shown in Figure 4E, BNZ132-1 indeed inhibited HAM-TSP T cells as effective as the combined anti-IL-2 and -IL-15 antibodies, which underscores two major arguments of our study; 1. There are human diseases that pathogenically involve more than two members of a cytokine family. 2. BNZ132-1 (and similar peptides from our concept) would be a superior inhibitor to a single Ab for such circumstances.

Inhibition of the signaling events by BNZ132-1 is specific for and limited to ones generated by the target cytokine - Cellular proliferation often represents a combination of distinct signaling pathways, and thus inhibiting one branch of the signaling could stop the proliferation, while keeping other branches remain activated and leaving non-proliferative activation machineries of the cell in operation. Therefore, we tested if BNZ132-1 comprehensively silenced multiple signaling cascades triggered by its target cytokines. Fig. 5 shows the inhibition by BNZ132-1 of the tyrosine-phosphorylation of key molecules representing major branches of the γ c-cytokine signaling; i.e., Jak-STAT, PI3kinase-Akt, and MAP-kinase axes. A complete inhibition by BNZ132-1 of these signaling cascades by human IL-15 (A; in PT-18 β (33)), mouse IL-9 (B; in PT-18) and human IL-2 (C; in human Peripheral T cells) has been observed. Cellular proliferation shown in Figure 3 paralleled the pattern of inhibition. IL-4, a non-target cytokine for BNZ132-1, utilizes STAT6 (Fig 5B), although a marginal phosphorylation of STAT5 is known to happen (Fig 5B), but BNZ132-1 did not inhibit this action of IL-4 whereas similar phosphorylation events caused by IL-9 in the same cell were completely blocked, strongly illustrating its specificity (no off-the-target effects even on a γ c-cytokine outside of the target spectrum). This observation further implies that BNZ 132-1 may have less severe side-effects than small signal inhibitors (see Discussion).

Dissection of the function-structure aspects of the BNZ132-1 peptide- To investigate the structure-function relationship of BNZ132-1, we next conducted the Alanine scanning mutation analysis (48) on BNZ132-1. As shown in Figure 6A and B, substitution of each residue resulted in various perturbations of the cytokine inhibition by BNZ 132-1. We used an excess dose of mutants (10 μ M) to ascertain saturated effect of them. Summary of our observations: 1; No substitution augmented the inhibitory capacity of the peptide beyond that of the original peptide. 2; IL-2 competition by BNZ132-1 is more susceptible to Ala single mutation than the IL-15 competition. 3; the N-terminus seems more critical for the inhibitory function as substitutions of the first 7 aa

led to a profound loss of inhibition for both IL-2 and IL-15, albeit these substitutions more specifically affect the inhibition on IL-15. 4); the C-terminus seems slightly dispensable for the inhibitory function on IL-15. Curiously IL-2 inhibition seems to require this region. 5; the Gln13 appear to have special importance, which is consistent with a structural demonstration that Gln13 is a conserved residue because it is crucial for the binding of many γ c-cytokines to γ c (38). In general, replacement of structurally important amino acids (i.e., those involved in the physical contact with the γ c) with Ala shows detrimental effects on the inhibitory activity of BNZ132-1, suggesting that the binding of BNZ132-1 to the γ c is mediated by a similar mechanism as has been demonstrated with IL-2 or IL-15 in a previous publication (38). Additional mutational and structure analyses are underway to decipher the unique nature of BNZ132-1 more in depth as a novel inhibitor of multiple γ c-cytokines.

Potent in vivo inhibitory action of BNZ132-1 on the action of IL-2, one of the target cytokines of this peptide - To test the potential of BNZ132-1 for clinical application, we conducted a few animal studies. Continued presence of human IL-2 in mice, by transgenesis or cytokine administration, is known to cause a temporal expansion of CD4⁺CD25⁺regulatory T cells (T-regs) as reported by us and others (49,50). We thus challenged C57B6 mice with recombinant human IL-2 and tested if the subsequent expansion of T-regs would be blocked by BNZ132-1. To elongate the in vivo half-life of BNZ132-1, we conjugated the peptide to polyethylene glycol (PEG, 40kDa average molecular weight) at the N-terminus of the peptide (see methodology section for detail). As shown in Fig. 7A, the PEG conjugation hardly changed the inhibitory nature of BNZ132-1. Human recombinant IL-2 was administered (intraperitoneal, i.p.) into C57BL6 mice on days 1 and 4 (150 μ g in 150 μ l sterile PBS per injection) into 3 groups of mice (n=5). One additional group (n=3) was injected with PBS alone as a negative control. Out of the IL-2-challenged 3 groups, one group received no intervention (received only PBS injection), whereas another group received intervention by BNZ132-1-40 (5 mg/kg) and the

last group received an intervention by neutralizing anti-IL-2 antibody (R & D Systems) as shown in Fig 7B. Blood was collected on day 7 and was analyzed by polychromatic flow cytometry to trace the changes of T-regs in the CD4 T cell compartment. The experimental design is illustrated in Fig. 7B. In Fig 7C, a representative flow cytometry pattern indicating the temporal expansion of T-regs by IL-2, and the concurrent inhibition of the expansion by BNZ132-1-40. Notably, BNZ132-1-40 was as efficient as anti-IL-2 antibody by this assessment. Fig 7D shows the statistical comparison of the results obtained from individual group.

BNZ132-1 potently prevented mice from IL-15-mediated experimental leukemia – To further assess the therapeutic effect of BNZ132-1 in vivo, we have used a unique IL-15-mediated leukemic model for human LGL leukemia that we have established (17,51). The leukemic cells were established from IL-15 transgenic mice (51), producing human IL-15 and sustain their in vitro and in vivo growth (17). The cells quickly manifest secondary leukemia upon injection into syngeneic C57B6 normal mice (17). We have already demonstrated that interventions to IL-15 action (i.e., by neutralizing anti-IL-15 (17) or by Tofacitinib, a Jak3 inhibitor (36)) prevents the IL-15-dependent expansion of leukemic cells in host mice. As shown in Fig 8A, BNZ132-1-40 completely protected mice (n=5) from leukemic death caused by the injection of IL-15-leukemic cells (one million cells injected i.v. on day 0). In contrast, untreated mice died within 30 days (n=3). Neutralizing anti-IL-15 antibody, the positive control for the experiment, protected mice as well as we previously reported (17). Tofacitinib, which never perfectly protected host mice from leukemic death in our hands (36) only showed life-elongating effects. The experiment was designed in such a way that we could distinguish injected cells and host cells by a congenic marker Thy 1 (see methodology section for detail). This allowed us to track if the injected leukemic cells (Thy 1.2⁺) expanded in the host (Thy 1.1⁺) environment. Fig.8B clearly shows the lack of even traces of Thy 1.2⁺ cells in BNZ132-1-40 treated mice whereas untreated mice or Tofacitinib-treated mice near death showed massive to moderate expansion of Thy1.2⁺

leukemic cells in their blood. Collectively, these two preclinical animal studies strongly suggest that the BNZ132-1 peptide, when appropriated modified to ensure in vivo stability, would efficiently block the pathogenic action of its target cytokines (i.e., IL-2 and IL-15).

Discussion

Recent studies demonstrate increasing examples of human diseases in which more than one cytokines are involved (Table 1). We would like to emphasize that the current anti-cytokine strategy relying on a specific monoclonal Ab lacks the capacity to cope with such situations as shown in Fig 4A. To address this challenge, we set the following goals; 1) to design cytokine inhibitor peptides that target multiple related cytokines, 2) such inhibitors should not block all γ c-cytokines, but should provide selectable target spectrum so we can tailor each of them to treat a particular disease. Using the γ c-family as a model, we have constructed a logic to accomplish our goals as the lead peptide, BNZ132-1, showed potent inhibitory action on chosen members of the γ c-family (i.e., IL-2, -9 and -15), but not on other γ c- or non- γ c-cytokines. Not only that, we designed two additional peptides with different target specificities (supplementary Table 1). We also demonstrated a novel translational potential of our peptide for a human disease (HAM-TSP) which is currently without established treatment regimen (52). We are also pursuing the structural implications of our observations by extending the studies to the structural aspect of the binding between γ c and various γ c-cytokines. We hope that our approach could fill in the existing gap in the current anti-cytokine therapeutic strategy.

The functional redundancy among γ c-cytokines is largely due to the sharing of the γ c-subunit (53). Thus, we rationalized to target the binding interface of cytokines and γ c for inhibiting multiple γ c-cytokines. Based on the structural studies on the γ c-cytokine system (37,38), we designed candidate peptides through series of *in silico* and *in vitro* experiments (supplementary Figure 1, supplementary table 2) and synthesized a few 'finalists' including BNZ132-1. The selective

target spectrum of BNZ132-1, as opposed to blocking all γ c-cytokines, may appear counterintuitive, because it targets the shared binding interface. However, the binding of γ c-cytokines and γ c has been known only resolved for select members (IL-2, 4, and -15)(38,54). We hypothesized that binding interfaces of each γ c-cytokine on γ c overlaps but are not identical, allowing BNZ132-1 only to compete with select targets. Computer-simulated docking pre-screening of candidate peptides showed that 1/3 of them displayed a competitive binding geometry with IL-2 or IL-15, but even those ones never fully displaced cytokines from their binding pockets on γ c. A full-length γ c-cytokine interacts with multiple receptor subunits which are assembled in a step-wise manner (53). It is plausible that the position of the D-helix in a full length γ c-cytokine is influenced by the private chain(s), while that of BNZ132-1 is not due to the lack of other helices (A,B,C). These may account for the selective target spectrum of BNZ132-1. Mutational analyses provided additional insights in support of our working hypothesis mentioned herein, because the mutation of some residues of BNZ132-1 differentially affected its inhibitory ability on IL-2 and on IL-15. It is interesting that mutations of a few residues that have been known critical for the binding of D-helices of IL-2 and IL-15 to the γ c crippled the biological inhibitory effects of BNZ132-1, which seemed in disagreement with the *in silico* observations described above that the binding geometry of BNZ132-1 showed slight difference from that of natural cytokines. We believe that these residues are also critical to support the α -helical structure of the peptide. To address these issues, we are currently resolving the co-crystal structure of BNZ132-1 and γ c to test the hypothesis.

The awareness that one Ab can be disappointingly ineffective in blocking the action of a combination of redundant cytokines (Fig 4A/B) alarmed us to propose a reevaluation of previous clinical trials on Asthma. Initially, linkage studies suggested the involvement of IL-4 and 9 in this disorder (12,15). However, consequent clinical trials targeted each cytokine separately and

produced unsatisfactory outcomes (55,56). As IL-4 and IL-9 are functionally overlapping γ c-cytokines, this may not be too surprising in light of our demonstration (Fig 4). Perhaps targeting both cytokines could have generated more successful outcome. However, clinical therapy involving more than two antibodies, while scientifically sound, will be extremely costly. We have a third peptide that targets IL-4 and IL-9 (Table S1), an interesting candidate for treating Asthma. One important lesson from these observations would be that the lack of effective inhibition when only one Ab was employed does not exclude the target from the pathogenic entities in the disorder of interest. This prompts us to propose that strategies enabling simultaneous blocking of redundant cytokines should be seriously pursued.

Another clinical strategy to target multiple cytokines involves the use of single inhibitors. An inhibitor to the Jak3 kinase has been developed (CP690,550, a.k.a. Tofacitinib) (42) and approved by the FDA for treating ulcerative colitis, Rheumatoid Arthritis and GvHD upon kidney-transplant (57). However, a long-term use of Tofacitinib may be problematic as it could cause immunodeficiency due to the inhibition of IL-7 (4,7). Tofacitinib also inhibits other Jak kinases (Jak1 and 2) (58) and causes anemia (59) as Jak2 is essential for erythropoiesis. In contrast, BNZ132-1 has sharp target spectrum (Fig 3) and acts only in the extracellular compartment (Fig 5). Thus, our concept may provide safe and versatile means that are alternative to the current strategy.

The two preclinical experiments represented by Fig. 7 and 8 provided encouraging results that BNZ132-1 could be a strong candidate for clinical treatment of cytokine-mediated disorders. These results were of another assurance as we observed two contrasting indications for the affinity of BNZ132-1 to the γ c. While binding assay shown in Fig 2 indicates μ M range of binding affinity, the biological inhibition shown in Fig 3 and 4 suggests affinity ranging from 25 to 250 nM. We only provide our working hypothesis that the binding of BNZ132-1 and γ c-cytokines to the γ c molecule may be stabilized by the presence of private receptor subunits. Nonetheless, preclinical studies may suggest that indeed BNZ132-1 seems effectively

blocking target cytokines in the range of 100~500 nM *in vivo*. We have learned through parallel toxicology studies that BNZ132-1-40 shows negligible toxicity *in vivo* on hematopoietic cells even at a high dose of 10 mg/kg in mice and in cynomolgus monkeys³.

The γ c-family is a mathematical group of 6 members. The unique combination of 6 members generates 63 subsets (${}^6C_6+{}^6C_5+{}^6C_4+{}^6C_3+{}^6C_2+{}^6C_1 = 63$) (Table S1) and each one of these may represent a unique human disease. Eventually, it may be possible to complete a library of compounds (peptides, antibodies and small chemicals) to cover all these subsets. Such library would empower us to treat any human diseases that pathogenically involve unique combinations of γ c-cytokines.

We limited our study on the γ c-family cytokines and one may wonder why not including factors from other family. While this may be a future area to cover, we feel that intra-family redundancy is more relevant than inter-family cooperation of cytokines because factors from different families show less overlapping signaling pathways and thus contributes different cellular outcomes than to a single biological consequence.

In summary, we have developed a new technology that enables targeting more than two γ c-cytokines with selectable target spectrum. We have hereby demonstrated the *in vitro* proof-of-concept. Successful *in vivo* preclinical studies would be a next major milestone leading to clinical applications. This technology clearly has advantages over the single monoclonal Ab

therapy or ones that involve signal inhibitors such as Tofacitinib.

The lead BNZ132-1 may be a good candidate to treat HAM-TSP, an orphan disease without existing treatment. In an accompanying paper (Massoud et al. Submitted), we explored the therapeutic potential of BNZ132-1 on HAM-TSP with extensive analyses of the effective inhibition of *ex vivo* activation/proliferation of T cells from HAM-TSP patients by this peptide. In addition, we are testing if BNZ132-1 could be applied to treating IBD. The BNZ132-1 peptide may have its own limitation. In some cases of refractory Celiac disease, IL-15 and more recently IL-21 have been shown to be pathogenic factors (19-21,23,26). On the other hands, IL-2 may inhibit the progression of the disease by activating T-regs (53). Thus, BNZ132-1 is not a perfect fit for this disease, and what we need is a multi-inhibitor targeting IL-15 and -21, but not IL-2. We have developed BNZ132-2 (Table S1) to tailor this need. These examples would emphasize the conceptual justification for our long-term goal of constructing a library of multi-cytokine inhibitors with different target specificities as explained in Table S1. Testing these novel peptides in appropriate animal models would advance our understanding on the complex cytokine milieu that are associated with many human disease. Finally, our research could provide safe and novel options for treating many more human necrotic conditions such as autoimmunity, inflammatory diseases and cancer.

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The BNZ-gamma peptides (including BNZ132-1,2,3) and the multi-cytokine targeting concept with selectable target spectrum are covered by a US patent 08455449 (May 2013, to YT and NA) and related intellectual properties belong to the BIONIZ Inc. (Irvine, CA).

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Conflict of Interest;

AB is an employee of BIONIZ Inc. NA is the founder of BIONIZ. BIONIZ holds the patent for the technology behind this work. This work was supported in part by a research funding from BIONIZ to TN, FC and YT. BIONIZ Inc. holds the US patent (mentioned above) for the concept and peptides used in this paper. We also declare that these financial involvements of BIONIZ did not undermine the scientific objectivity and integrity of the presented work.

Author Contributions;

TN and AB; *These two authors contributed equally to this work and therefore are co-first authors; TN determined the inhibitory properties of BNZ132-1 including the target spectrum, and effects on intracellular signaling. AB determined the binding of BNZ132-1, participated in the rational design and screening of BNZ132-1 and the alanine-scanning mutation analysis.

TN, AB, FC, RvB, RM, SJ, NA and YT designed the experiments, participated in the study discussion, provided scientific inputs to the manuscript.

TN, AB, FC, RvB, RM and YT conducted the experiments.

TN, AB, NA, and YT wrote the manuscript.

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[Footnotes]

Unpublished observations;

1. Y. Tagaya and R. Massoud
2. Y. Tagaya
3. N. Azimi and Y. Tagaya
4. A. Basheer

[Figure legends]

Figure 1. Conserved primary structures of the D-helices from γ c-cytokines.

A. Alignment of the six human γ c-cytokines.

Amino acid sequences of the D-helices from human γ c-cytokines (Table 2) have been aligned using the T-coffee algorithm (35). A region with moderate conservation (identical aa or conservative substitutions) was named as the γ c-Box. Color codes represent how many human γ c-cytokines share the indicated aa.

B. Alignment of human IL-2 and IL-15.

Between IL-2 and IL-15, another conserved region was identified and named 'the IL-2/15 box'.

"*" identical between IL-2 and -15, ":" conservative substitutions

C. Design of the BNZ 132-1 peptide.

Based on a logic described for supplementary Figure 1, 120 candidate peptides (Table S2) were designed and run through a series of computer simulations (Figure S1). The resultant 39 peptides were examined by biological assays (examples shown in Figure 3) to choose a final peptide shown in C.

Color codes: Green; common to IL-2/15, Red; from the IL-15 sequence. Blue; from the IL-2 sequence.

Figure 2. Binding of the FITC-BNZ132-1 peptide to the γ c molecule.

A. No binding of FITC-BNZ132-1 to the parental PT-18 cell line that lacks high expression of mouse γ c. As shown in Figure S2, PT-18 cells express marginal levels of mouse γ c-molecule (33) which is insufficient to be detected by the FITC-BNZ132-1 binding due to the low F/P ratio of this conjugate. No binding was observed by the FITC-control peptide.

B. Binding of FITC-BNZ132-1, but not that of FITC-control peptide to PT-18huy.

PT-18hu γ is a subclone of PT-18 that expresses extremely high levels of human γ c (CD132) due to gene transfection. Only FITC-BNZ132-1, but not FITC-Control peptide, shows binding to these cells.

C. Dose-dependent binding of FITC-BNZ132-1 to PT-18hu γ .

To test the specificity of the FITC-BNZ132-1 binding, various concentrations of FITC-BNZ132-1 was incubated with target cells, showing the dose-dependent nature of the binding.

D. Inhibition of FITC-BNZ132-1 binding by the unlabeled BNZ132-1 peptide.

To further test the specificity of the binding of BNZ132-1, a competition with cold (unlabeled) ligand was performed. The results show dose-dependent inhibition of the binding of FITC-BNZ132-1 by the unlabeled peptide.

Figure 3. Inhibitory spectrum of BNZ132-1 over γ c- and non- γ c-cytokines.

A. Inhibition of IL-2 and IL-15 by BNZ132-1; CTLL-2 proliferation assay

CTLL-2 has been incubated with 1nM of human IL-2 or human IL-15 (PeproTech), in the presence of indicated concentrations (μ M) of BNZ132-1. Neutralizing antibodies against each cytokine (R & D systems, 5 μ g/ml) were included as controls. After 20 h, the cellular proliferation was determined by the WST-1 reagent. A 19-mer control peptide was used as a negative control at 5 μ M (Abbreviated as 'Ctrl pep'; throughout the Figures).

B. Inhibition of IL-9, but not of IL-4 by BNZ132-1; PT-18 proliferation assay

PT-18 β (subclone of PT-18 transfected with human IL-2/IL-15R β (CD122) (33)) or PT-18h21R α (subclone transfected with human IL-21R α) cells were incubated with 1 nM of mouse IL-4, mouse IL-9, or mouse IL-21 in the presence or absence of the indicated doses (μ M) of BNZ132-1. Neutralizing antibodies to each cytokine (5 μ g/ml, except anti-mouse IL-21 Ab at 15 μ g/ml) and a control peptide (5 μ M) were included as negative controls. WST-1 assay was conducted as described above.

C. No inhibition of IL-21 by BNZ132-1; PT-18 h21R α survival assay.

PT-18h21R α cells do not proliferate robustly to human IL-21 (Fig 3B). Both parental and hIL-21R α positive PT-18 cells were washed and incubated without IL-3 for 12 h before the assay which would cause apoptotic death within 24 hr due to death by neglect. 1nM of human IL-21 (PeproTech) was added in the presence or absence of 5 μ M BNZ132-1 or control peptide for 24 hr and cellular apoptosis was determined by the staining of PE-Annexin V (BD Biosciences). Only hIL-21R α transfected, but not the parental cells, showed survival by human IL-21 ($p=0.001$) which was not blocked by BNZ132-1 ($p=0.48$, non-significant).

D. No inhibition of IL-7 by BNZ132-1; human PBMC proliferation assay.

Ex vivo T cells were prepared from human PBMCs as described in the text. IL-2 was withdrawn from the culture 12 h prior to the assay. Cells were incubated with 10 nM human IL-7 (Gemini) for 24 h in the presence or absence of indicated amount (μ M) of BNZ132-1. Human IL-2 or IL-15 (1nM ea.) was used as positive controls. As negative controls, neutralizing antibodies (5 μ g/ml) against each cytokine and a control peptide (5 μ M) were tested. WST-1 proliferation assay was conducted as described above.

E. No inhibition of non- γ c cytokines by BNZ132-1; PT-18 proliferation assay.

PT-18 β cells were depleted of IL-3 for 12 h prior to the assay. Indicated cytokines (IL-15/Flt3-ligand from human, others from mouse sequences, each at 1nM – see Fig S3 for the responses of PT-18 to them) were added to the fasted PT-18 β for 20h with or without 15 μ M BNZ132-1, and then pulsed with 3 H-thymidine (GE-Health). Neutralizing antibodies (5 μ g/ml, except anti-mSCF Ab at 20 μ g/ml) to each cytokine and a control peptide were included as controls. The Y-axis represents cpm values measured by a β -counter.

F, G, H. Dose-dependent inhibition of human IL-2, IL-15 and IL-9 in NK92 cells by BNZ132-1.

NK 92 cells were cultured with human IL-2. Cells were withdrawn of IL-2 for overnight and 2×10^5 cells (in 200 μ l volume) were incubated with the indicated concentrations of cytokines and BNZ132-1 for 24 h then 20 μ l WST-1 reagent (Clontech) was added for 6 hr to measure A_{450} .

I. Dose-dependent co-inhibition of human IL-2 and IL-15 in human Peripheral T cells by BNZ132-1. Human PBMCs were prepared as described above and enriched by a MACS (Miltenyi) negative selection. Purified T cells were cultured without IL-2 overnight, and then 4×10^5 cells (in 200 μ l culture) were incubated with cytokines and BNZ132-1 at the indicated concentrations for 24 h, then with WST-1 reagent (Clontech) for 12 hr before OD_{450} measurements.

Experiments were conducted in triplicate and results represent one of at least three independent occasions (applies to all experiments in Fig 3).

Figure 4. Effective inhibition of a combined function of γ c-cytokines by BNZ132-1.

A. Dose response of human ex vivo T cells to recombinant human IL-2 or IL-15. Human ex vivo peripheral T cells were prepared as described in the methodology section. Four hundred thousand cells (in 200 μ l culture) were incubated with the cytokines, antibodies, and BNZ132-1 at the indicated concentrations for 24 h, and cellular proliferation was determined using the WST-1 reagent (Clontech). The Y-axis represents OD_{450} values. The graph indicates the EC_{50} concentrations of each cytokine (approximately 50 pM).

B. Dose-inhibition response of anti IL-2 and anti IL-15 antibodies used in this study. Approximately 1 μ g/ml inhibition of each antibody was sufficient to suppress the saturating effect of each target cytokines on ex vivo human T cells, with no cross-reactivity to the other cytokine at a higher 5 μ g/ml (Right panel).

C. Antibody titration and dose-inhibition relationship of single and combined Abs on IL-2, IL-15 or a combination of both. Each of anti-IL-2 or anti-IL-15 antibodies had marginal effects (25~30%) in inhibiting the equimolar mixture of IL-2 and IL-15 (100 pM each, to achieve saturating response of T cells as a combination) at high concentrations (5 and 10 μ g/ml). When each cytokine was combined at near the ED_{50} concentration, the inhibition by the single Ab was discernibly better (40~45%). The numbers on the X-axis for the mixture of antibodies indicate those of each antibody (i.e., 1.25 = 1.25 μ g/ml anti IL-2 + 1.25 μ g/ml anti IL-15). The mixture of Abs reached near 100% inhibition irrespective of the concentration of mixed cytokines.

D. Combinatorial effects of IL-2 and IL-15 to human ex vivo T cells were effectively inhibited by BNZ132-1. Show is the dose-inhibition curve of combined IL-2+IL-15 effects on ex vivo human T cells by BNZ132-1. Notably, the inhibition by BNZ132-1 is similar to those by anti-IL-2+anti-IL-15 antibodies shown in Fig 4C. E. Translational potential of BNZ132-1 for HAM-TSP. PBMCs of HAM-TSP patients were tested in a spontaneous proliferation assay as previously described (60). The cellular proliferation was measured by the thymidine incorporation assay. The Y-axis represents cpm values. Doses on the X-axis; BNZ132-1, 3 and 0.3 μ M. Antibodies against IL-2 or IL-15 (1 and 10 μ g/ml of each, R & D Systems). The p values; between no treatment and BNZ132-1(0.3): 0.018, between no treatment and BNZ132-1 (3): 0.0018, between BNZ132-1 (3) and Anti IL-2+IL-15 (10): 0.29

The assay was performed in triplicate and the figure represents a typical result out of 5 different HAM-TSP patients.

Figure 5. Comprehensive inhibition by BN132-1 of signaling events caused by target γ c-cytokines.

A. Comprehensive inhibition of IL-15 signaling in PT-18 β by BNZ132-1. Cells had been withdrawn of IL-3 for 12 h and then stimulated with 1 nM human IL-15 with or without 0.5 μ M BNZ132-1 for 15 min

before extracting cellular proteins. Phosphorylation of targets was detected using commercial antibodies (Cell Signaling), followed by ECL. Lanes: 1. no stimulation, 2. IL-15(1nM), 3. IL-15 (1nM) + BNZ132-1 (0.5 μ M)

B. Signal transduction of IL-4 in PT-18 was not inhibited whereas the IL-9 signal was inhibited by BNZ132-

1. Cells were fasted of IL-3 for 12 h then stimulated with 1nM mouse IL-4 or mouse IL-9 in the presence or absence of excess dose of BNZ132-1 (5 μ M) for 15 min before extracting cellular proteins.

Phosphorylation of the indicated proteins was detected using phosphospecific antibodies (Cell signaling), followed by ECL. IL-4 is the only γ c-cytokine that induces the phosphorylation of STAT6. As shown before, only IL-4, but not IL-9, induced the tyrosine-phosphorylation of STAT6. Conversely, IL-4 showed only marginal phosphorylation of STAT5 whereas IL-9 induced a strong STAT5 phosphorylation. Notably, only IL-9 action was inhibited by BNZ132-1. Lanes: 1. no stimulation, 2. IL-4, 3. IL-4 + BNZ132-1, 4. IL-9, 5. IL-9 + BNZ132-1

C. IL-2 signal in human PBMC was blocked by BNZ132-1. Human ex vivo T cells were prepared as described in the text. After 24 h of IL-2 depletion, cells were stimulated by 1nM IL-2 with or without BNZ132-1 (0.5 or 0.1 μ M) for 20 min before lysis. Tyrosine-phosphorylation of targets was detected by antibodies (Cell Signaling), followed by ECL. Lanes: 1. no stimulation, 2. IL-2 stimulation, 3. IL-2 + BNZ132-1 (0.5 μ M), 4. IL-2+BNZ132-1 (0.1 μ M)

In all blots, anti-Vinculin Ab (Sigma) was used to validate equal protein loadings.

Figure 6. Mutational analysis to evaluate the functional importance of each of the 19 aa of BNZ132-1: Alanine scanning.

Alanine substituted peptides were synthesized in a small scale and were tested using CTLL-2 cell assay if they inhibit the proliferative activity of IL-2 and IL-15, as described in the text. A"X" (X-axis) denotes that the "X"th aa was replaced by Alanine.

The Y-axis of the graph represents the inhibitory activity of the mutated peptide relative to the original BNZ132-1. One hundred % indicates that the mutant is as potent as BNZ132-1 and zero % indicates that the peptide has lost the inhibitory function. In some cases, the peptides became agonistic (stimulating proliferation, rather than inhibiting it) after the mutation, which are represented by negative numbers in the graph. Results represent 3 independent experiments.

Figure 7. Effective in vivo inhibition of IL-2-mediated T-reg expansion in mice by BNZ132-1-40.

A. Intact biological characteristics of BNZ132-1 following PEG40 conjugation. BNZ132-1 peptide was conjugated to PEG40 at the N-terminus of the peptide as described in the methodology section, in order to prolong the half life in vivo. To test that the conjugation did not affected the inhibitory action of the peptide on target cytokines, a proliferation assay was conducted using IL-2 and CTLL-2 cell line. Y-axis represents the OD450 reading (cellular proliferative response) after the addition of WST-1 reagent. At 100 ~1000 nM, the peptide and the PEG40-conjugated form (BNZ132-1-40) showed similar inhibitory profile on the action of IL-2. Similar results were obtained with IL-15 and IL-9 actions⁴. Control peptide (a 19-mer scrambled peptide shown above) showed no inhibition even at a higher dose of 5 μ M.

B. Design of the cytokine injection study. Recombinant human IL-2 (Peprotech) was injected (i.p.) to induce T-regs in mice. Intervention was performed by injecting relevant compounds (neutralizing anti-IL-2 antibody or BNZ132-1-40, i.p.) on days 2 and 5. Venous blood was collected on day 7 to purify PBMCs for subsequent flow cytometry analyses.

C. Representative flow cytometry data. Following cytokine injections and interventions by anti-cytokine compounds, mouse PBMCs were analyzed by polychromatic flow cytometry. T cells were gated by anti-

CD3 staining and the percentages of CD25⁺CD4⁺ (T-regs), CD25⁻CD4⁺ (conventional CD4), CD4⁻CD25⁻ (CD8 T cells) were measured as shown.

D. Statistical evaluation of the T-reg data. Obtained percentages of T-regs in the CD4 compartment have been processed by a Medcalc software (MedCalc bvba, Ostend, Belgium) and resultant average and 95% confidence ranges are grouped by treatment and shown. p values by the Student t-test; pre-treatment vs. PBS - 0.075 (no), PBS vs. IL-2 - 0.0016 (**~***), IL-2 vs. IL-2+BNZ132-1-40 - 0.001 (***), IL-2 + anti-IL-2 Ab - 0.001 (***), IL-2+BNZ132-1-40 vs. IL-2+anti IL-2 Ab - 0.72(no)

Figure 8. Protection of mice from IL-15 mediated CD8 T leukemia.

A. Survival of mice treated with BNZ132-1-40.

B. Lack of the expansion of Thy1.2⁺ leukemic cells in surviving mice.

We have previously reported the occurrence of fatal CD8 T cell leukemia in our IL-15 transgenic mice (17,36,51). We established a mouse leukemic cell line (K2) which produces human IL-15 and sustains perpetual growth into fatal secondary CD8 T cell leukemia when injected into syngeneic (C57B6) mice. One million K2 cells were injected (i.v.) into congenic Thy1.1⁺ C57B6 mice. Without any treatment, mice died of leukemia from 20 to 30 days after injection (shown in the Kaplan-Meier survival plot in A). Blood collected from morbid mice show massive expansion of Thy 1.2⁺ K2 leukemic cells in Thy 1.1⁺ host mice (Fig 8B). As we reported previously (17,36), this leukemic death was completely prevented by repeated injections of neutralizing anti-IL-15 antibody (R & D system, MAB247, 150 µg per injection, i.p. on days 1, 4, 8, and 11). Tofacitinib (Sigma-Aldrich, MO) which was continuously administered using subcutaneous osmotic pump (ALZET) only prolonged the survival but did not prevent the death of mice as we previously described (36). BNZ132-1-40 was administered i.p. on days 1, 4, 8 and 11 (Low dose at 1 mg/kg and high dose at 10 mg/kg) and completely protected mice from leukemic death (8A). No propagation of Thy 1.2⁺ K2 leukemic cells was observed in the PBMCs from BNZ132-1-40 treated mice (8B).

Table 1. Human diseases associated with multiple γc-cytokines

The table was compiled through literature survey.

Representative references for each disease listed;

HAM-TSP: (43,58) Celiac Disease/IBD : (19-21,26) Uveitis: (29) Asthma: (14,15, 55,56)

Multiple Sclerosis: (61-63) Rheumatoid Arthritis: (64-67)

Table 2 Conservation of D-helices among γc-cytokines across mammalian species.

Left; Homology/similarity of IL-2 at the amino acid level were compared among mammalian species for each of the four helices (A~D).

Right: Amino acid sequence of 6 human γc-cytokines were compared (similarity levels, && denotes no significant homology was observed) at each of the four helices (A~D).

The similarity /homology scores were determined by the T-coffee algorithm (35)

Table 1

List of Human Diseases involving multiple γ c-cytokines

Disease	Cytokines
HAM-TSP	IL-2, IL-15, (IL-9?)
Celiac Disease/ IBD (inflammatory bowel disease)	(IL-2), IL-15, IL-21
Uveitis	IL-2, IL-21
Asthma / COPD (chronic obstructive pulmonary disease)	IL-4, IL-9 IL-5, IL-13
MS (multiple sclerosis)	IL-2, IL-9, IL-15, IL-21
RA (Rheumatoid Arthritis)	IL-7, IL-15, IL-21 IL-6

Table 2

Helix A	Human	Primate	Mouse	Helix C	Human	Primate	Mouse
Primate	68.1/100			Primate	29.1/100		
Mouse	27.8/67	27.8/67		Mouse	(-) /16.7	(-) /21.3	
Bovine	24.8/80	24.8/80	21.4/78	Bovine	18.9/75	19.7/62	(-)/14.3
Helix B	Human	Primate	Mouse	Helix D	Human	Primate	Mouse
Primate	71/100			Primate	67.7 /100		
Mouse	32/77	32/77		Mouse	50.3/78	50.3/78	
Bovine	46.9/95	46.9/84	16.8/60	Bovine	52/88	52/88	37.7/71

IL-2 homology matrix

Helix A	IL-2	IL-4	IL-7	IL-9	IL-15	Helix C	IL-2	IL-4	IL-7	IL-9	IL-15
IL-4	10					IL-4	12.5				
IL-7	15	**				IL-7	9.1	12.5			
IL-9	14.3	15.4	14.3			IL-9	18.8	20.8	**		
IL-15	10	15.8	5.3	15.6		IL-15	27.7	4.2	16.7	13.3	
IL-21	18.2	9.1	13.6	13.6	13.6	IL-21	33.3	12	8.3	33.3	15.4

Helix B	IL-2	IL-4	IL-7	IL-9	IL-15	Helix D	IL-2	IL-4	IL-7	IL-9	IL-15
IL-4	13.6					IL-4	27.3				
IL-7	17.4	45				IL-7	20	20.6			
IL-9	13.6	17.7	15			IL-9	15	22.7	23.5		
IL-15	20	5	9.5	15		IL-15	22.2	11.1	20.6	20	
IL-21	18.2	22.2	10	11.8	20.8	IL-21	28.6	18.2	11.8	23.8	28

human cytokine homology matrix

Figure 1

A

```

IL-15  -----ECEEIKEFLQSFVHIVQMFI--NTS
IL-2    -----TIVEFLNRWITFCQSII--STLT
IL-21   -----PLEFLERFKSLLQKMIHQHLS
IL-4     -----NQSTLENFLERLKTIMREKYSKCSS
IL-7     LEENKSLKEQKKL-NDLCFLKRLLQEIKTCW-NKIL
IL-9     -----NALTFLESLLEFLQKEKMRGMR
  
```



The γ -Box

■ :6
 ■ :5
 ■ :4
 ■ :3
 ■ :2
 ■ :1

B

```

IL-15      IKEFLQSFVHIVQMFINT-S
IL-2       IVEFLNRWITFCQSIISTLT
           *  ***:  ::  :  *  : *  *  :
  
```



The IL-2/15 box

* - Identical

: - Side chain properties conserved

C

I-K-E-F-L-Q*-R*-F*-I*-H*-I*-V*-Q-S-I*-I-N-T-S

■ : AA common to IL-2/IL-15

■ : From the IL-15 sequence

■ : From the IL-2 sequence

* : Chemical property conserved

Figure 2

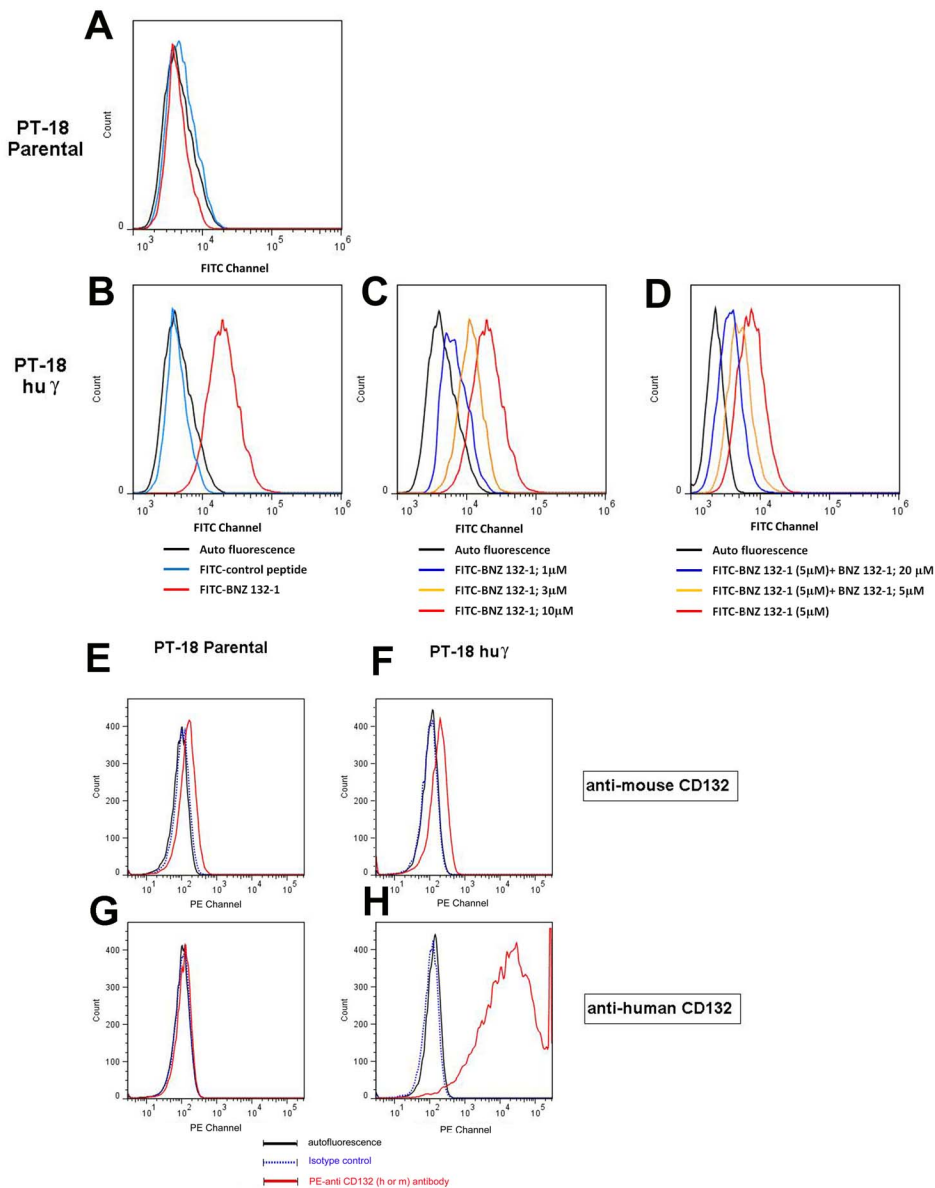


Figure 3

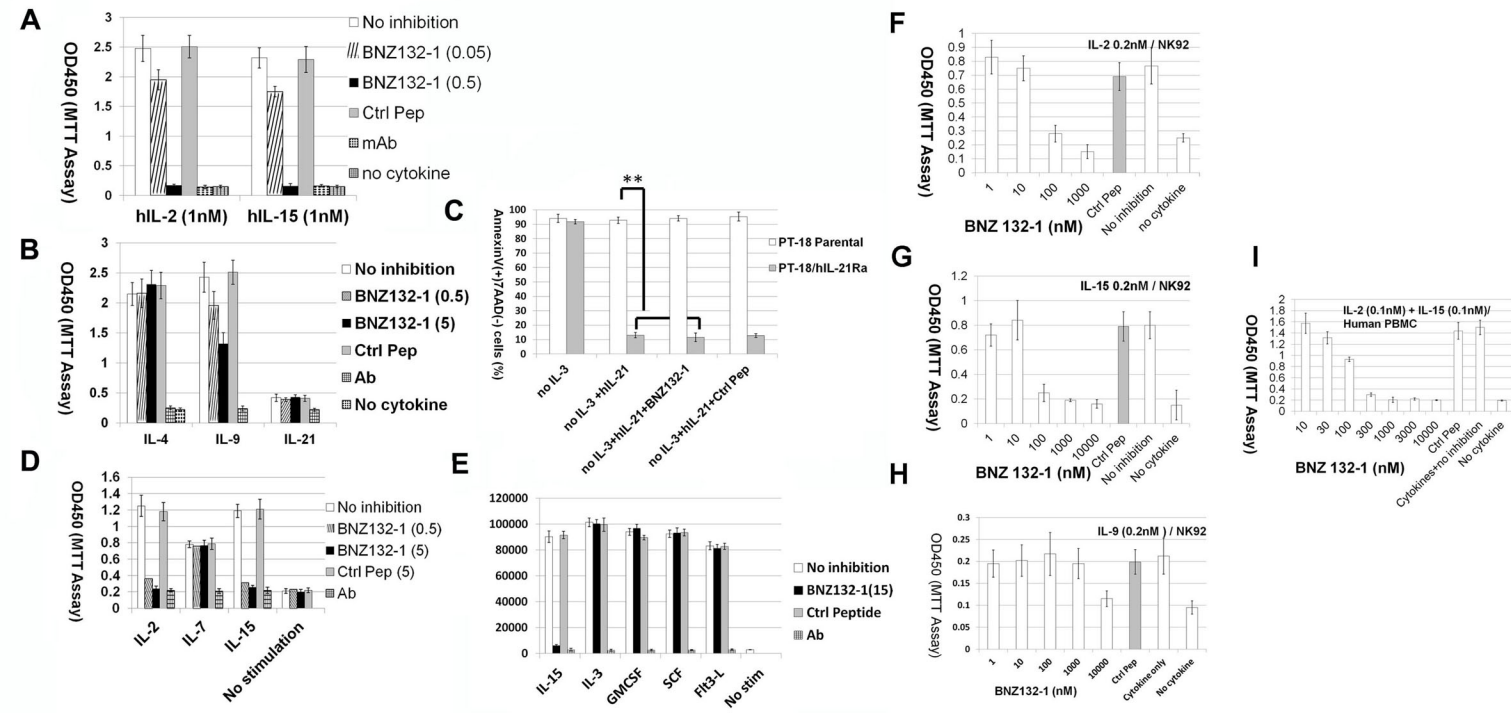


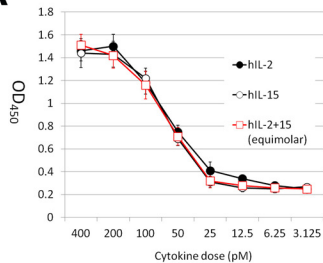
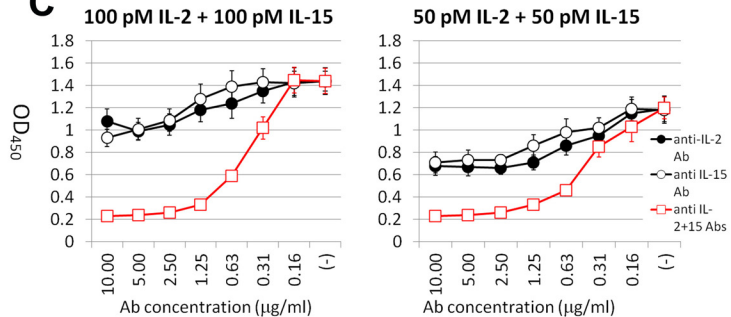
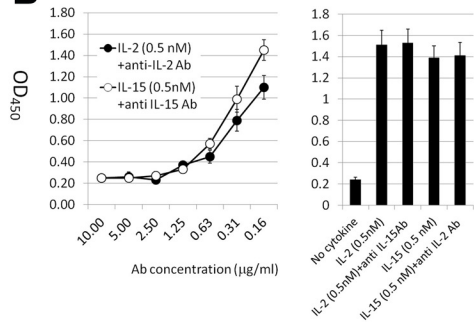
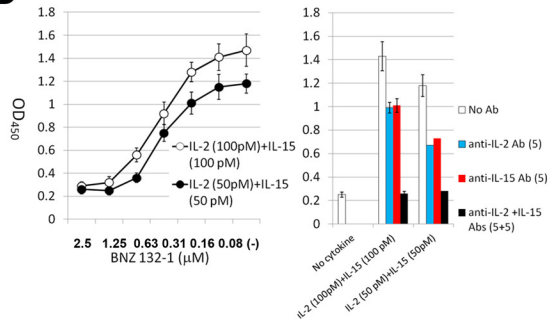
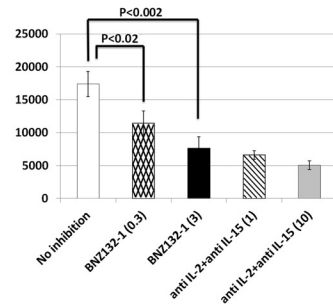
Figure 4**A****C****B****D****E**

Figure 5

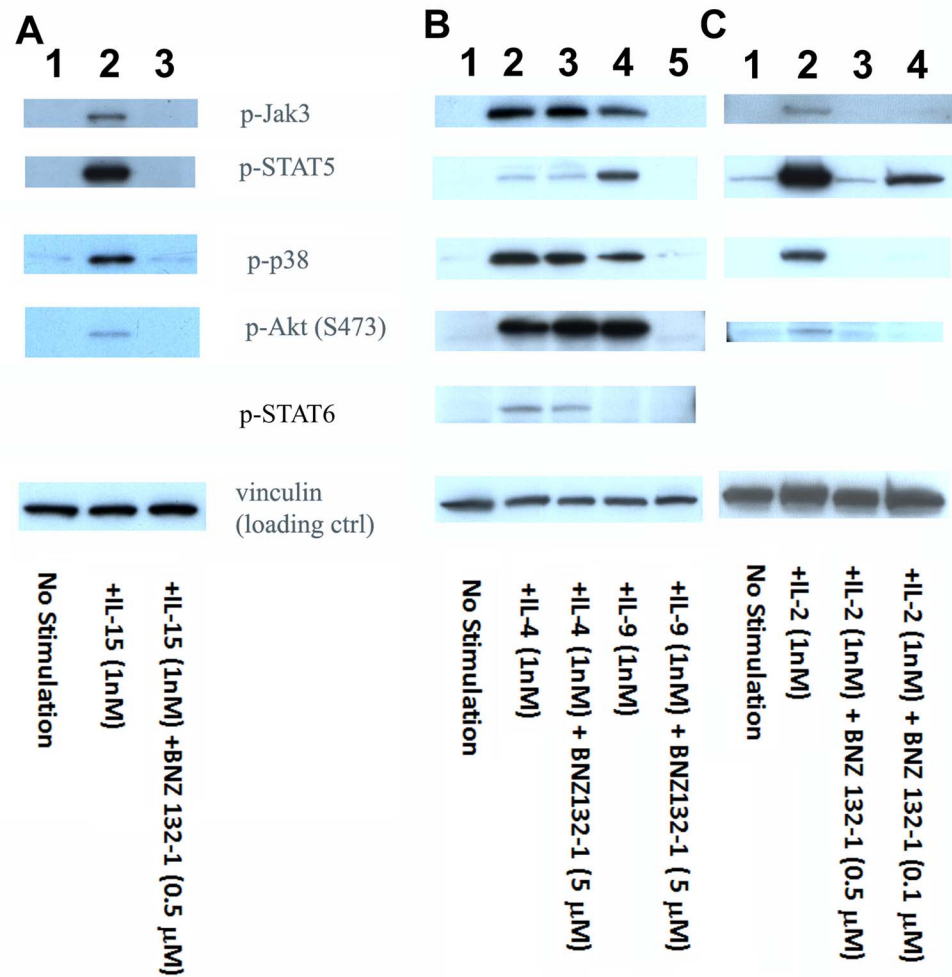
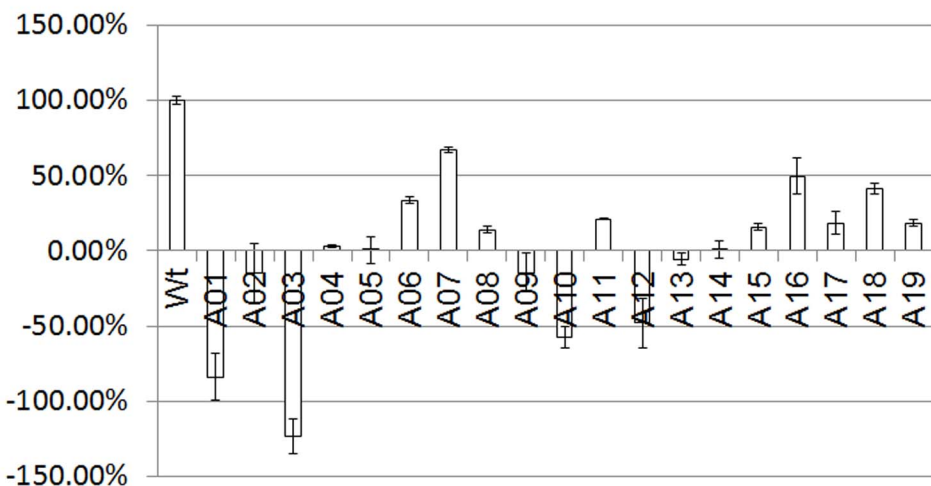


Figure 6

IL-2 (100 pM) + BNZ132-1 (10 μ M)



IL-15 (100 pM)+BNZ132-1 (10 μ M)

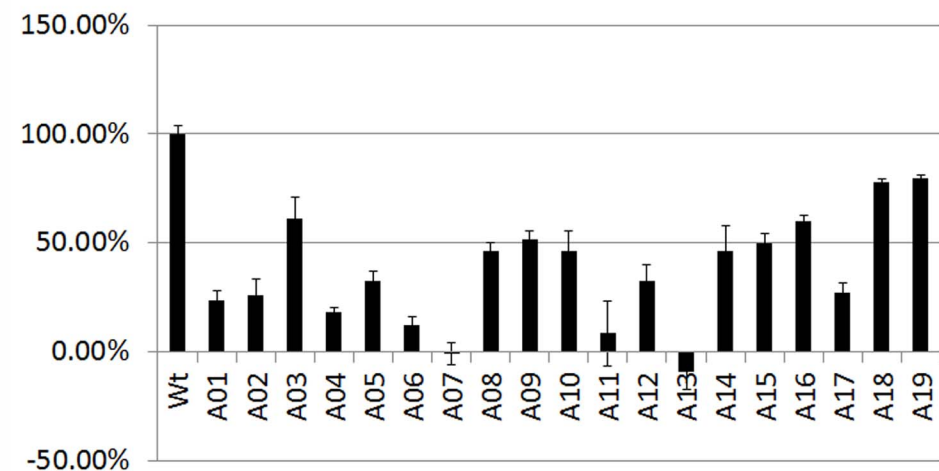
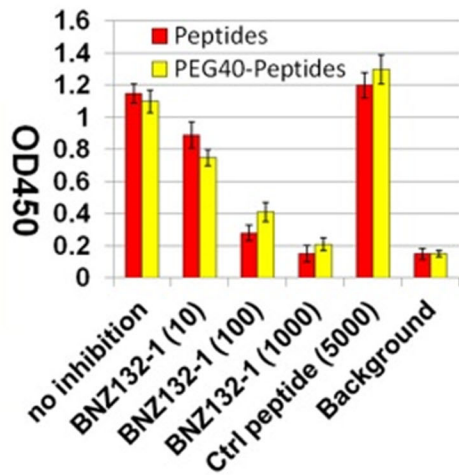
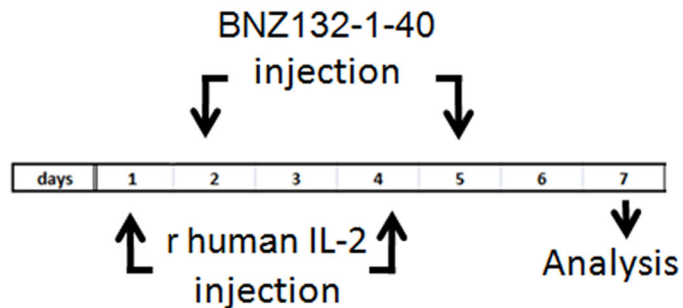


Figure 7

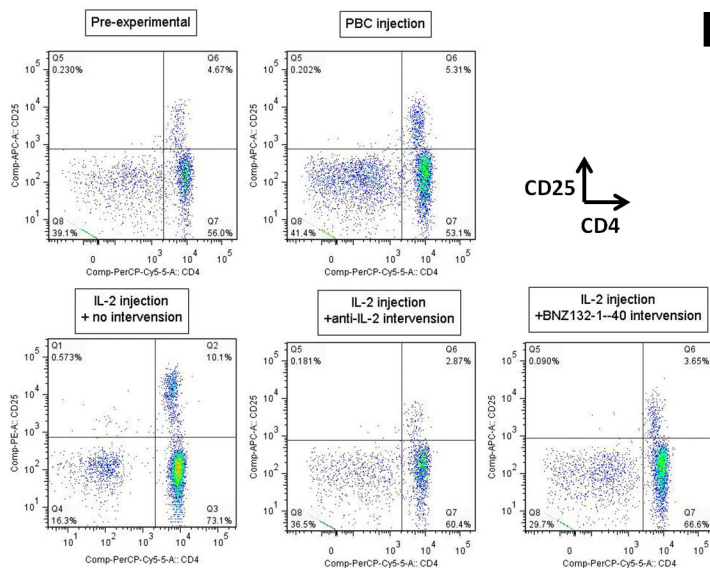
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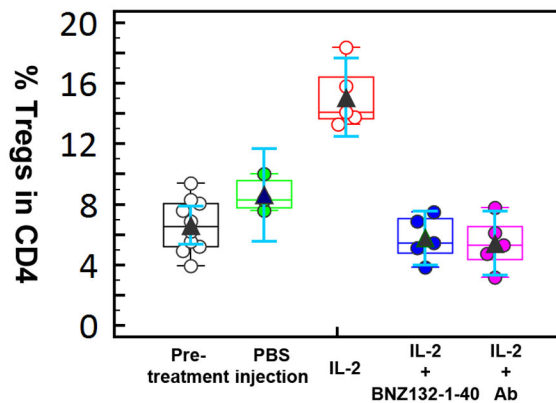
B



C



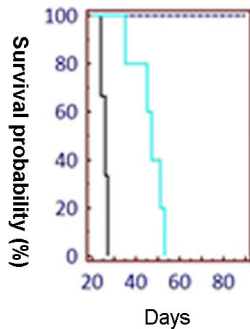
D



CD3 gated

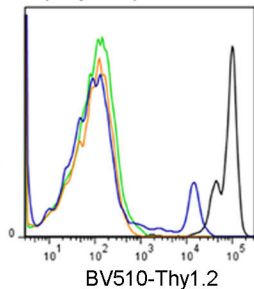
Figure 8

A



B

Propagation of leukemic cells (Thy 1.2) in host mice



Treatments

- PBS (control)
- - - anti-IL-15 Ab
- - - BNZ 132-1-40 (H,L)
- Tofacitinib

- No treatment
- BNZ132-1-40 (High)
- BNZ132-1-40 (Low)
- Tofacitinib

Cell Biology:

Targeting the binding interface on a shared receptor subunit of a cytokine family enables the inhibition of multiple member-cytokines with selectable target spectrum.

Toshie Nata, Asjad Basheer, Fiorenza Cocchi,
Richard Van Besien, Raya Massoud, Steven
Jacobson, Nazli Azimi and Yutaka Tagaya
J. Biol. Chem. published online July 16, 2015

CELL BIOLOGY

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