Differential effects of BTK inhibitors ibrutinib and zanubrutinib on NK-cell effector function in patients with mantle cell lymphoma

Ibrutinib, the first clinically approved Bruton's tyrosine kinase (BTK) inhibitor, is an effective and widely used therapy for chronic lymphocytic leukemia (CLL).[1] The significant homology between BTK and IL-2-inducible kinase (ITK) makes ITK an off-target kinase inhibited by ibrutinib.[2] This may lead to unintended consequences, as ITK regulates T-cell development and effector function of natural killer (NK) cells.[3] Indeed, ibrutinib has been shown to inhibit healthy donor NK-cell cytotoxic activity *in vitro*[4-6] but, at the same time, potentiated NK-cell function in xenograft mouse models of B-cell lymphoma.[7] Given these contrasting experimental findings, it remains unknown whether patient NK-cell effector function is adversely affected during ibrutinib therapy.

A major obstacle to investigating NK-cell repertoire and function in patients with CLL is their typically high B-cell tumor burden, which makes phenotypic and functional analyses of circulating NK cells challenging. To overcome this obstacle, we investigated patients with mantle cell lymphoma (MCL) rather than CLL. MCL is an aggressive disease with median survival of 3-6 years. We recently reported the results of a clinical trial in which ibrutinib was used to treat patients with MCL,[8] while a second clinical trial assessing the safety and optimal dose of a new generation BTK inhibitor, zanubrutinib (BGB-3111), in B-cell malignancies including MCL has been completed (*Tam et al., 2019, submitted manuscript*). Since MCL patients typically have few circulating malignant B cells, we had a unique opportunity to compare peripheral NK-cell repertoire and function in the same patients before and after one month of monotherapy with ibrutinib or zanubrutinib. We found that the repertoire of NK-cell ligands on B cells of MCL patients was essentially

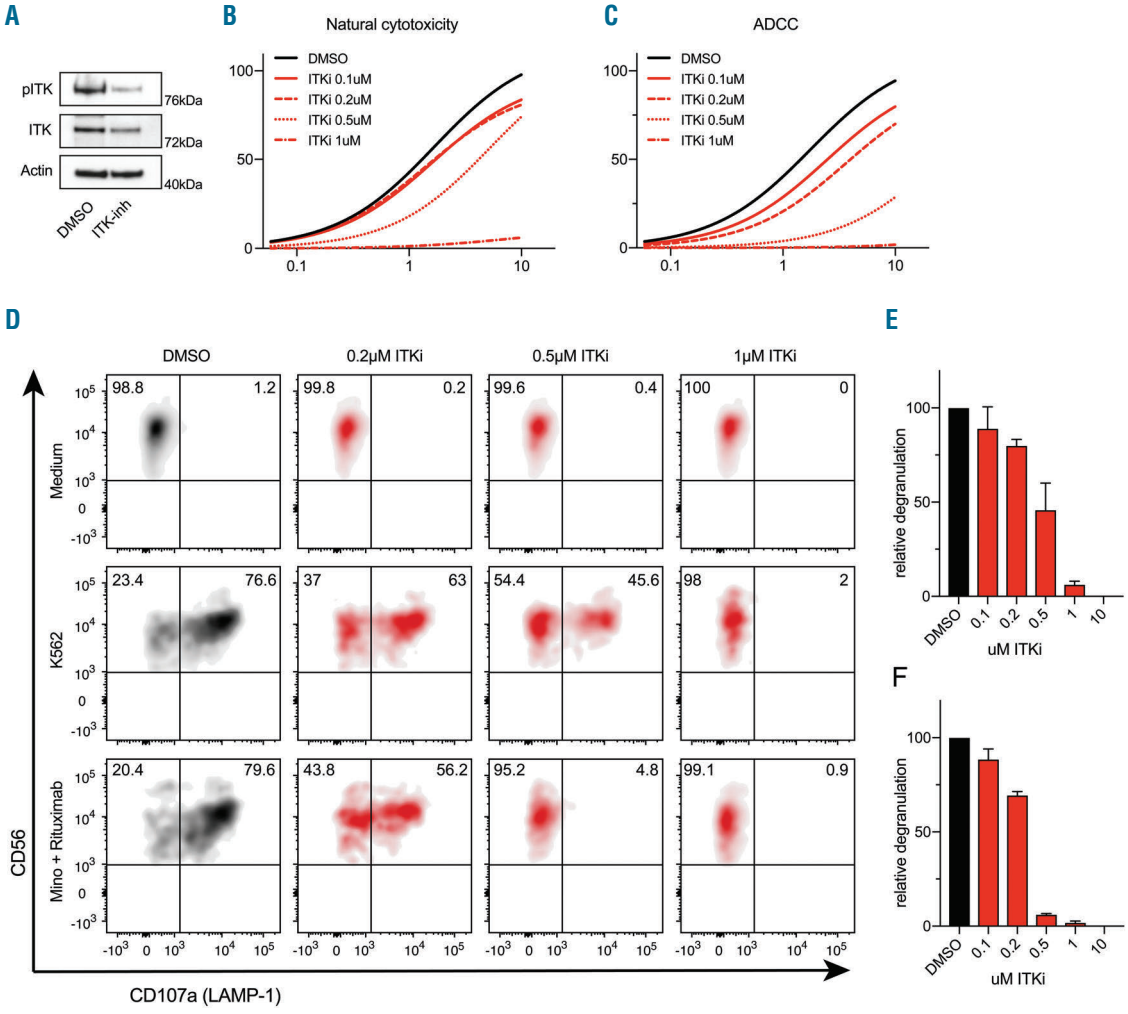

Figure 1.IL-2-inducible kinase (ITK) is essential for natural killer (NK)-cell function. (A) Isolated primary human NK-cells were stimulated for 4 hours (h) with 1000IU IL-2 in the presence of dimethyl sulfoxide (DMSO) or 10 μM of the ITK inhibitor 5-aminomethylbenzimdazole. Shown are the western immunoblots of phosphorylated ITK, total ITK, and actin. (B-F) The effect of ITK inhibition (ITKi) on NK-cell function. Primary human NK cells were mixed at various effector-to-target ratio with K562 (B) or Mino (treated with rituximab) targets (C) in the presence of vehicle or various concentrations of the ITKi. NK-cell cytotoxicity against target cell was determined using a 4-h ^{51}Cr release assay, and extrapolated using a Michaelis-Menten equation (n=3 independent donors). (D-F) Primary human NK cells were incubated with or without K562 or Mino (treated with rituximab) target cells and various concentrations of the ITKi, and NK-cell degranulation was assessed by measurement of CD107a surface labeling in the CD56^{dim} lymphocytes. (D) Shown are representative plots from one donor NK cells. Summary of degranulation relative to the DMSO control for natural cytotoxicity (E) and ADCC (F) (n=3 independent donors).

indistinguishable from that of healthy donors, and treatment of patients with BTK inhibitors had no effect on the expression of these ligands. (See *Online Supplementary Table S1* for patient information, panels used for phenotyping in *Online Supplementary Figure S1*, gating in *Online Supplementary Figure S2*, and *Online Supplementary Figures S3* and *S4* for B-cell analyses).

To confirm that ITK is important for the cytotoxic activity of primary human NK cells (as was previously shown for NK-cell lines[3]), we utilized a highly selective ITK inhibitor, 5-aminomethylbenzimdazole (IC_{50} for ITK is 0.58nM, and for BTK - 440nM) (Figure 1A).[9] NK-cell cytotoxicity (Figure 1B) and antibody-dependent cytotoxicity (ADCC) (Figure 1C) were both severely suppressed in a concentration-dependent manner, while NK-cell number and phenotype remained unaffected. Consistent with these observations, NK-cell degranulation was significantly decreased (Figure 1D-F), and is likely to be the primary reason for suppressed cytotoxicity.

Having confirmed that ITK is important for NK-cell effector function, we next investigated the effect of BTK inhibitors on the catalytic activity of both kinases. We confirmed that both ibrutinib and zanubrutinib are potent inhibitors of BTK (Figure 2A)[10,11] and, consistent with this observation, they bound to the kinase and inhibited the proliferation of the MCL cell line Rec-1, with similar potency (Figure 2B). However, zanubrutinib was almost 20-fold less potent at inhibiting ITK than ibrutinib (Figure 2A), and a 10-45-fold higher concentration of zanubrutinib was required for equivalent inhibition of PLCγ1 or IL-2 secretion (IC_{50}) (Figure 2B). Zanubrutinib is, therefore, an equally potent, but more selective inhibitor of BTK than ibrutinib *in vitro*.

Having established that off-target inhibition of ITK is greater by ibrutinib than zanubrutinib (Figure 2A and B), we assessed the effect of both drugs on NK cells *in vitro*. We co-cultured NK cells with the MCL cell-line Mino in the presence of rituximab (Figure 2C) or GA-101 (therapeutic anti-CD20 antibody by Beigene) (*Online Supplementary Figure S5*). We found that IFNγ release was severely suppressed by ibrutinib in a concentration-dependent manner, while zanubrutinib caused only minor inhibition (Figure 2C). We then assessed NK-cell mediated killing of Mino cells, and again found significant inhibition of NK-cell killing by ibrutinib, with EC_{50} of 1.17 μM, compared to 27.8 μM for zanubrutinib (Figure 2D). In summary, and in addition to previous publications, NK-cell activation and target cell killing were severely suppressed by ibrutinib,[4-6] but not zanubrutinib.

Next, we compared the effects of the two BTK inhibitors on patient NK-cell phenotype and function following one month of monotherapy. We found that the number of NK cells in MCL patients was within the healthy donor range, and remained unaffected by treatment with either inhibitor (Figure 3A). NK-cell effector function depends on the balance of germline-encoded cell surface activating, inhibitory and dual-function receptors,[12] where CD57, CD62L and FcεRγ are typically associated with activation,[13] polyfunctionality[14] and memory NK-cell[15] phenotypes, respectively. t-SNE analysis of over 20 surface markers of NK cells showed no consistent difference between healthy donors and MCL patients. (Example of 2 patients in *Online Supplementary Figure S6*. All data of NK-cell panel 2 in *Online Supplementary Figure S7*. All other t-SNE data available on request). This was confirmed by further detailed analysis of the activating receptors NKp30, NKp44, NKp46, NKG2C, NKG2D, DNAM-1 and KIR2DS4 (Figure 3B, and *Online Supplementary Figure S8A* and *B*) or inhibitory receptor NKG2A, and KIRs: 2DL1, 2DL2, 2DL3 and 3DL1, which

A

	IC_{50} (nM)		
Enzyme	Ibrutinib	Zanubrutinib	Zanu/Ibru
BTK	0.18	0.3	1.7
ITK	3	56	18.7
Selectivity	17	187	

B

		IC_{50} (nM)		
Targets	Assays	Ibrutinib	Zanurutinib	Zanu/Ibru
BTK	BTK-pY223	3.5	1.8	0.5
	Rec-1 proliferation	0.34	0.36	1.1
	BTK occupation	2.4	2.2	0.9
ITK	ITK occupation	130	3290	25
	p-PLCγ1	77	3477	45
	IL-2 production	260	2536	9.8

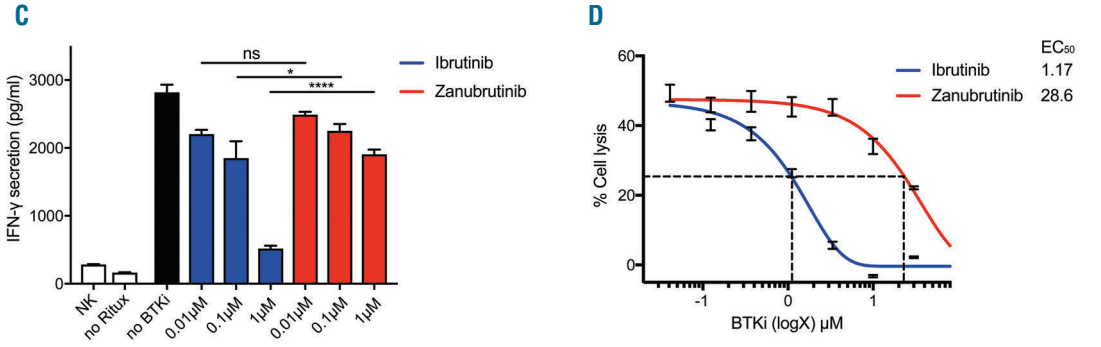

Figure 2. Zanubrutinib is an equally potent, but more selective, inhibitor of Bruton's tyrosine kinase (BTK) than ibrutinib. (A) Zanubrutinib and ibrutinib were tested for inhibition of BTK and IL-2-inducible kinase (ITK) using time-resolved fluorescence-resonance energy transfer (TR-FRET). (B) IC_{50} values of BTK occupation assays, inhibition of Rec-1 tumor cell proliferation, inhibition of cellular BTK activation, ITK occupation assays, p-PLCγ1 cellular assay and IL-2 production assays. (C and D) *In vitro* studies of the effect of ibrutinib and zanubrutinib on NK-cell function. (C) Mino cells and NK92MI cells were co-seeded and treated with vehicle or various concentrations of BTK inhibitors in the presence of rituximab; interferon (IFN)-γ levels in the conditioned medium were measured as a readout of the assay. (Left) Two bars show IFN-γ production by NK cells alone and by NK cells co-cultured with MINO cells without added rituximab. (D) Mino cells and NK92MI cells were co-seeded and treated with vehicle or various concentrations of BTK inhibitors. Cytotoxicity of the target cells was determined by lactate dehydrogenase release into the culture medium.

were all comparable between healthy donors and MCL patients (Figure 2B and *Online Supplementary Figure S8C*). CD57, CD62L and FcεRγ expression levels in MCL patients were also indistinguishable from controls (Figure 3B and *Online Supplementary Figure S8D*). Importantly, NK-cell phenotype remained unchanged following treatment with ibrutinib or zanubrutinib (Figure 3B and *Online Supplementary Figure S8*. Example of t-SNE analysis is shown in *Online Supplementary Figure S6*). We also investigated whether the inhibitors influenced NK-cell cytotoxicity in this context, by assaying NK cells from individual MCL patients prior to and following one

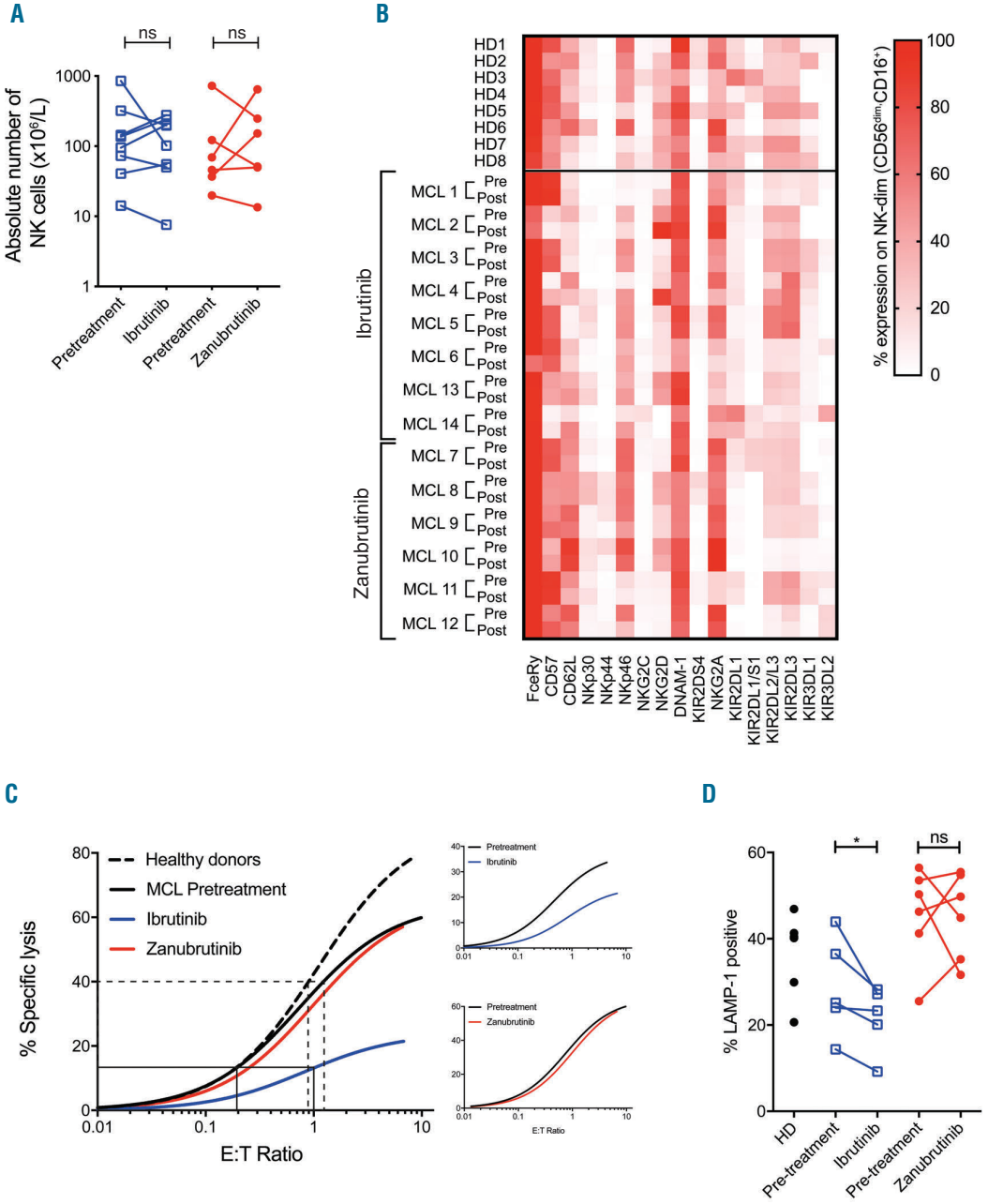

Figure 3. Ibrutinib, but not zanubrutinib inhibits natural killer (NK)-cell cytotoxic function in mantle cell lymphoma (MCL) patients. (A) NK-cell number ($CD3^-/CD16^+/CD56^{dim}$) in 14 MCL patients pre- and post-treatment with ibrutinib or zanubrutinib. (B) Peripheral blood mononuclear cells (PBMC) from mantle cells lymphoma (MCL) patients or healthy donor controls (HD) were stained for surface and intracellular markers. (See *Online Supplementary Figure S1* for staining panels and *Online Supplementary Figure S2* for representative gating). Heatmap of surface receptor expression profiles of NK cells ($CD3^-CD16^+CD56^{dim}$) of eight healthy donors and 14 MCL patients before and after treatment with ibrutinib or zanubrutinib. (C) PBMC taken prior to (black line) or after therapy with ibrutinib (blue line) or zanubrutinib (red line) were incubated with ^{51}Cr-labeled K562 target cells for 4 hours (h) at the indicated effector to target cell ratios (normalized for the percentage of NK cells). NK-cell cytotoxicity against K562 target cell was determined using ^{51}Cr release assay and extrapolated using the Michaelis-Menten equation. (Left) Average cytotoxicity of healthy donors (n=8), pooled pre-treated patients (n=12), and patients treated with ibrutinib (n=6) or zanubrutinib (n=6). (Right) Average cytotoxic activity of NK cells from MCL patients before and after treatment with ibrutinib (n=6) or zanubrutinib (n=6). See *Online Supplementary Figure S8* for all individual graphs. (D) PBMC were incubated for 3 h in the absence or presence of K562 target cells, and NK-cell degranulation was assessed by measurement of CD107a surface labeling in the $CD3^-/CD16^+/CD56^{dim}$ lymphocytes. Data points represent degranulation (-background levels) prior to and after treatment with ibrutinib (blue squares) or zanubrutinib (red dots). *P<0.05 (paired t-test). ITKi: IL-2-inducible kinase inhibitor.

month of ibrutinib or zanubrutinib monotherapy. Prior to therapy, NK cells from MCL patients had slightly lower cytotoxicity than healthy donors (Figure 3C). However, following treatment with ibrutinib, their cytotoxic activity was reduced by more than 75% in 5 of the 6 patients studied (Figure 3C; individual assays are shown in *Online Supplementary Figure S9A*). In contrast, zanubrutinib had no significant effect on NK-cell cytotoxicity (Figure 3C; individual assays shown in *Online Supplementary Figure S9B*).

Natural killer cells kill their targets predominantly via the cytotoxic secretory granule exocytosis pathway. Consequently, inefficient granule exocytosis may result in reduced NK-cell cytotoxicity. We assessed granule exocytosis by measuring externalization of the granule marker LAMP-1 (CD107a) on NK cells incubated with K562 target cells (representative plot is shown in *Online Supplementary Figure S10*). Whereas NK cells from untreated MCL patients had normal degranulation, ibrutinib therapy significant inhibited granule exocytosis as shown by reduced CD107a externalization. In contrast, the NK cells of MCL patients treated with zanubrutinib showed no such defect (Figure 3D). This observation is consistent with inhibition of ITK, that also affects PLCγ and IL2 secretion (Figure 2B).

In summary, we demonstrate that advanced MCL has no intrinsic impact on the repertoire or cytotoxic activity of patient NK cells. By testing a unique cohort of MCL patients prior to and following BTK inhibitor monotherapy, we found that whereas neither BTK inhibitor affected NK-cell repertoire, ibrutinib, but not zanubrutinib, significantly suppressed NK-cell cytotoxicity, most likely due to off-target inhibition of ITK that impaired degranulation. Our findings demonstrate the differential effects of first- and second-generation BTK inhibitors ibrutinib and zanubrutinib on NK-cell function, highlighting the need for careful consideration of the most appropriate combination therapies when using this class of drugs to treat blood cancers.

Thijs W.H. Flinsenberg,[1] Charnelle C. Tromedjo,[1] Nan Hu,[2] Ye Liu,[2] Yin Guo,[2] Kevin Y.T. Thia,[1] Tahereh Noori,[1] Xiaomin Song,[2] Han X. Aw Yeang,[1,3] Daniela G. Tantalo,[1] Sasanka Handunnetti,[4,5] John F. Seymour,[4,5] Andrew W. Roberts,[4,5] David Ritchie,[4,5,6] Rachel Koldej,[4,6] Paul J. Neeson,[1,3] Lai Wang,[2] Joseph A. Trapani,[1,3] Constantine S. Tam[4,5] and Ilia Voskoboinik[1,3*]*

**CST and IV contributed equally to this work as co-senior authors.*

[1]Cancer Immunology Program and Rosie Lew Immunotherapy Program, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; [2]BeiGene LTD, Beijing, China; [3]Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, Victoria, Australia; [4]Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, Victoria, Australia; [5]Department of Clinical Haematology, Peter MacCallum Cancer Centre & Royal Melbourne Hospital, Melbourne, Victoria, Australia and [6]The ACRF Translational Research Laboratory; Royal Melbourne Hospital, Parkville, Victoria, Australia

Acknowledgments: we would like to thank Associate Professor Kaylene J. Simpson (Head of the Victorian Centre for Functional Genomics) and Dr. C. Kearney for help with the ITK experiments.

Funding: we acknowledge the support of EMBO (ALTF 219-2017 for TF), CLL Global Research Foundation for CT and IV, National Health and Medical Research Council for IV and JT.

Correspondence: THIJS FLINSENBERG/ILIA VOSKOBOINIK/ CONSTANTINE TAM
thijsflinsenberg@gmail.com/ilia.voskoboinik@petermac.org/ constantine.tam@petermac.org
doi:10.3324/haematol.2019.220590

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Pal Singh S, Dammeijer F, Hendriks RW. Role of Bruton's tyrosine kinase in B cells and malignancies. Mol Cancer. 2018;17(1):57.
2. Dubovsky JA, Beckwith KA, Natarajan G et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. Blood. 2013;122(15):2539-2549.
3. Khurana D, Arneson LN, Schoon RA, Dick CJ, Leibson PJ. Differential Regulation of Human NK Cell-Mediated Cytotoxicity by the Tyrosine Kinase Itk. J Immunol. 2007;178(6):3575-3582.
4. Da Roit F, Engelberts PJ, Taylor RP, et al. Ibrutinib interferes with the cell-mediated anti-tumor activities of therapeutic CD20 antibodies: Implications for combination therapy. Haematologica. 2015; 100(1):77-86.
5. Kohrt HE, Sagiv-Barfi I, Rafiq S, et al. Ibrutinib antagonizes rituximab-dependent NK cell-mediated cytotoxicity. Blood. 2014; 123(12):1957-1960.
6. Rajasekaran N, Sadaram M, Hebb J, et al. Three BTK-Specific Inhibitors, in Contrast to Ibrutinib, Do Not Antagonize Rituximab-Dependent NK-Cell Mediated Cytotoxicity. Blood. 2014; 124(21):3118-3118.
7. Kuo H, Hsieh S, Whang J, Huang Y, Sirisawad M, Chang BY. Ibrutinib Potentiated NK Cell-Mediated Cytotoxicity in Mouse Models of B-Cell Lymphomas. Blood. 2016;126(22):4140.
8. Tam CS, Anderson MA, Pott C, et al. Ibrutinib plus Venetoclax for the Treatment of Mantle-Cell Lymphoma. N Engl J Med. 2018; 378(13):1211-1223.
9. Riether D, Zindell R, Kowalski JA, et al. 5 aminomethylbenzimidazoles as potent ITK antagonists. Bioorganic Med Chem Lett. 2009; 19(6):1588-1591.
10. Rule S, Chen RW. Expert Review of Hematology New and emerging Bruton tyrosine kinase inhibitors for treating mantle cell lymphoma – where do they fit in ? lymphoma – where do they fit in ? Expert Rev Hematol. 2018;11(9):749-756.
11. Tam C, Grigg AP, Opat S, et al. The BTK Inhibitor, Bgb-3111, is safe, tolerable, and highly active in patients with relapsed/refractory B-cell malignancies: initial report of a Phase 1 first-in-human Trial. Blood. 2015;126(23):832.
12. Freud AG, Mundy-bosse BL, Yu J, Caligiuri MA. Review the broad spectrum of human natural killer cell diversity. Immunity. 2017; 47(5):820-833.
13. Nielsen CM, White MJ, Goodier MR, Riley EM. Functional significance of CD57 expression on human NK cells and relevance to disease. Front Immunol. 2013;4:422.
14. Juelke K, Killig M, Luetke-Eversloh M et al. CD62L expression identifies a unique subset of polyfunctional CD56 dim NK cells. Blood. 2010;116(8):1299-1307.
15. Schlums H, Cichocki F, Tesi B, et al. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. Immunity. 2015;42(3):443-456.