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TGF-β regulated leukemia cell susceptibility against NK targeting through the down-regulation of the CD48 expression

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ABSTRACT

Transforming growth factor-β (TGF-β) is known to function as a dual role regulatory cytokine for being either a suppresser or promoter during tumor initiation and progression. In solid tumors, TGF-β secreted from tumor microenvironment acts as a suppresser against host immunity, like natural killer (NK) cells, to favor tumor evasion. However, besides solid tumors, the underlying mechanism of how TGF-β regulates leukemogenesis, tumor progression, immunoediting, and NK function is still not clear in detail. In this study, we found that TGF-β induced leukemia MEG-01 and U937 cells to become less sensitive to NK-92MI targeting by down-regulating CD48, a ligand for NK activating receptor 2B4, but not down-regulating other tumor-associated carbohydrate antigens (TACAs). In CD48-knockdown cells, cells responding to NK-92MI targeting displayed a phenotype of less NK susceptibility and cell conjugation. On the other hand, when NK cells were treated with TGF-β, TGF-β suppressed NK recognition, degranulation, and killing activity in time-dependent manner by regulating ICAM-1 binding capacity instead of affecting expressions of activating and inhibitory receptors. Taken together, both leukemia cells and immune NK cells could be regulated by TGF-β through suppressing leukemia cell surface CD48 to escape from host surveillance and down-regulating NK cell surface ICAM-1 binding activity to impair NK functions, respectively. Our results suggested that TGF-β had effect in leukemia similar to that observed in solid tumors but through different regulatory mechanism.

1. Introduction

The immunosuppressive cytokine transforming growth factor-β (TGF-β) is a multifunctional polypeptide secreted by many cell types, including T cells, macrophages, and even tumor cells. The roles of TGF-β and underlying regulatory mechanisms have been found to be very complex and intricate (Massague, 2008; Roberts and Wakefield, 2003; Bierie and Moses, 2009). Studies show that TGF-β suppresses tumor initiation and early development by the inhibition of cell cycle progression and apoptosis, and by down-regulation of growth factors, cytokines, and chemokines. Additionally, in a healthy immune system, immune response can be blunted by TGF-β in order to minimize the self-reactivity (Flavell et al., 2010; Gorelik and Flavell, 2000). However, TGF-β is also considered as pro-oncogenic for being produced by many tumor types in large amounts to exert adverse effect from pro-tumor immunity, and for its association with the progression of aggressive diseases under host-tumor immune surveillance (Bruna et al., 2007; Li et al., 2006). For example, firstly, the cytotoxic T lymphocytes (CTL) are markedly and directly suppressed by the presence of TGF-β, resulting in incapability to eradicate tumors when challenged with EL-4 thymoma and B16-F10 melanoma tumor cells (Gorelik and Flavell, 2001). Secondly, tumor progression can be favored while two distinct phenotypes present in T cells, the suppression of T-cell proliferation and cytotoxic gene program (Thomas and Massague, 2005). The $CD4^+$ T

Abbreviations: CTL, cytotoxic T lymphocytes; LC-AA, ligand-complex-based adhesion assay; NCRs, natural cytotoxicity receptors; NK, natural killer; Treg, regulatory T cells; sLe^a, sialyl Lewis a; sLe^x, sialyl Lewis x; SLAM, signaling lymphocyte activation molecule; SL-AA, soluble-ligand-based adhesion assay; TGF-β, transforming growth factor-β; TACAs, tumor-associated carbohydrate antigens

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cells has been shown to be converted into functionally inhibited induced regulatory T cells (Treg) by TGF-β through up-regulation of Foxp3 expression (Trapani, 2005; Nakamura et al., 2001; Ghiringhelli et al., 2005; Shevach, 2009).

Other than the effect on T cells, TGF-β can exert its influence on other blood cells. For example, exogenous administration of TGF-β suppresses B cell proliferation and immunoglobulin secretion (Kehrl et al., 1991; Cazac and Roes, 2000). TGF-β reduces natural killer (NK) cell proliferation, and inhibits expressions of NKp30 and NKG2D to suppress NK-mediated cytotoxicity (Castriconi et al., 2003; Lee et al., 2004). In addition to the systemic effects, TGF-β regulates infiltration of both inflammatory and immune cells as well as cancer-associated fibroblasts of the tumor microenvironment, causing direct changes of tumor cells in a pro-oncogenic status (Flavell et al., 2010; Whiteside, 2008). Besides the relationship between TGF-β and the solid tumor, the malignant hematologic cells and leukemia cells have been reported to be affected by elevated TGF-β in both bone marrow and the circulating system as evident of high TGF-β levels observed in the bone marrow and serum of leukemia patients (Shehata et al., 2004; Rameshwar et al., 1998). Also recent research pointed out that the TGF-β-FOXO signaling is responsible for the stemnness property in chronic myeloid leukemia (Naka et al., 2010), suggesting the regulatory role of TGF-β in leukemia. However, currently the detail mechanism is still not clear regarding how TGF-β affects leukemia and the host immuno-response along the course of disease progression.

Human tumor/leukemia cells can be recognized and eliminated by the host immune systems, such as CTL and NK cells. The ability of NK cells to kill tumor/leukemia depends on the activation signalings initiated from cell surface activating receptors. NK cell activating receptors can be classified as (1) the natural cytotoxicity receptors (NCRs), such as NKp30, NKp44, and NKp46, (2) members of the signaling lymphocyte activation molecule (SLAM)-related receptors, including 2B4, NTB-A, and CRACC, and (3) others like NKG2D, and DNAM-1 (Lanier, 2008). 2B4-mediated activating signaling is known for the importance of NK cells against target cells derived from hematopoietic origins, as 2B4 ligand (CD48) is widely expressed by these cells (Claus et al., 2008). CD48, a glycosylphophatidylinositol (GPI) linked protein, belongs to SLAM family and constitutively expresses on most hematopoietic cells, including neutrophils, eosinophils, mast cells, lymphocytes, monocytes, and NK cells (Thorley-Lawson et al., 1982; Yokoyama et al., 1991; Katsuura et al., 1998; Assarsson et al., 2005; Boles et al., 2001). CD48 participates in several immunological processes, such as adhesion and activation of immune cells, through its interaction with cell surface 2B4 and CD2 from other cells. CD48 expression can be upregulated by interferons, granulocyte-colony stimulating factor (G-CSF), or IL-4 specifically found in neutrophils, B cells, and Eosinophils (Yokoyama et al., 1991; Katsuura et al., 1998; Munitz et al., 2007; Rocha-de-Souza et al., 2008; Munitz et al., 2006). In T cells, CD48 regulates T cell signaling to promote proliferation, and contributes to the priming and effector functions of $CD8^+$ T cells (Moran and Miceli, 1998; Musgrave et al., 2003, 2004). In NK cells, it has been shown that NK effector functions are also subjected to the regulation of CD48 signaling in a similar way like those observed on $CD8^+$ T cells (Assarsson et al., 2005; Chavin et al., 1994; Messmer et al., 2006).

The cell-cell contact, conducted by integrins, between NK and target is the essential step to initiate activation of NK cytotoxicity. Once activated, NK cells exocytose their cytotoxic granules containing pro-apoptotic components, granzyme, and perforin to kill tumor/leukemia cells. Integrins are well-characterized cell adhesion proteins and it is known that this integrin-mediated cell adhesion process is highly regulated not only by the balance between signals from activating and inhibitory receptors but also by integrin itself in expression levels, clustering, and activation state (Hynes, 2002; Luo et al., 2007). NK cells express the integrin LFA-1 and macrophage receptor 1, both of which are composed in dimmer form, CD11a and CD18 for former, and CD11b and CD18 for latter. Both integrin LFA-1 and macrophage receptor 1 can interact with ICAMs located on target cells. And interestingly LFA-1 has been reported to be induced into the high-affinity conformation by the NK activating receptors (Bryceson et al., 2009; Osman et al., 2007). The immunoediting concept describes that the tumor/leukemia cells have the potential to escape from host surveillance and based on this, numerous mechanisms of such tumor immune evasion have been studied. For example, loss of MHC class I expression on tumor cells prevents CTL recognition but induces NK activation. However, the tumor/leukemia cells can impair NK function through a number of mechanisms, including suppressing NK surface activating receptors, and releasing soluble factors with immunosuppressive properties such as TGF-β (Whiteside, 2008; Vitale et al., 2014; Vesely et al., 2011; Zitvogel et al., 2006; Stringaris et al., 2014; Waldhauer and Steinle, 2008; Raulet and Guerra, 2009; Fauriat et al., 2007). It is known that the elevated levels of TGF-β are often observed in the serum of cancer patients with systemic inhibition of immune function, including weakened NK cell response (Lee et al., 2004; Ikushima and Miyazono, 2010; Friese et al., 2004), suppressed cytotoxic T activity (Gorelik and Flavell, 2001), induced regulatory T cell generation (Trapani, 2005; Nakamura et al., 2001; Ghiringhelli et al., 2005), and poor prognosis.

In this study, we aimed to address the role of TGF-β in disease progression, especially focusing on how immune cells and leukemia cells responded to TGF-β treatment. We found that TGF-β weakened NK functions and enabled leukemia cells to be less sensitive to NK targeting. Most importantly, we showed that CD48-2B4 signaling was the responsible signal pathway that mediates TGF-β effect to reduce the susceptibility of leukemia cells and helped these cells to escape from NK-mediated immunosurveillance. Based on these findings, we suggested that TGF-β signaling may serve as a therapeutic target for the leukemia treatment.

2. Material and methods

2.1. Cell culture and reagents

Human NK cell line and leukemia cells were purchased from Bioresource Collection and Research Center. NK-92MI cells were cultured at 37 °C with 5% CO_2 in α-MEM medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), 12.5% horse serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. All leukemia cell lines were grown in RPMI 1640 medium with 10% FBS, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. All reagents were purchased from Life Technologies. To study the effect of TGF-β on susceptibility against NK targeting or the cytotoxicity of NK-92MI in killing leukemia, 1×10^6 leukemia or NK cells were seeded and treated with 5 ng/ml TGF-β (PeproTech) for different designated time periods.

2.2. CD107a degranulation assay

Fluorescent-labeled (Claret Far Red Fluorescent Cell Linker Kit; Sigma-Aldrich) effector cells, NK-92MI, were incubated with equal number of target leukemia cells at 37° for 1 h. After co-incubation, the cell mixture was stained with phycoerythrin (PE)-conjugated CD107a antibody (eBiosceience). Samples were resuspended in FACS buffer (2% FBS in PBS) and then were subjected to FACSCalibur (BD Biosciences) or CytoFLEX (Beckman Coulter Inc) flow cytometry equipment, and results were analyzed using FlowJo or CytExpert software. To determine the surface CD107a levels of NK cells, CD107a positive rate of fluorescent-labeled NK-92MI cells was analyzed.

2.3. Flow cytometric analysis

The expression of cell surface-bound receptors and their corresponded ligands on NK or leukemia cells, respectively, were assayed by flow cytometry (FACS) with the fluorescent-conjugated antibodies against 2B4, CD11a, CD18 (BioLegend), CD48 (Abcam), NKG2D,

NKp30, NKp44, NKp46 (BioLegend). After staining, samples were re-suspended in FACS buffer and were subjected to FACSCalibur or CytoFLEX flow cytometry equipment and results were analyzed using FlowJo or CytExpert software.

2.4. Establishment of CD48-knockdown cells

Lentiviral virions were produced by transient transfection into 293 T cells (American Type Culture Collection) with lentiviral vector, encoding shRNA for human CD48 (pLKO.1-sh431647) and control plasmids as well as pCMVdeltaR8.91 and pMD.G (National RNAi Core Facility, Academia Sinica, Taiwan) by Turbofect Transfection Reagent (Thermo Scientific). Supernatants with viral particles were collected after 48 h, and then were used to transduce K-562, MEG-01, or U937 cells in the presence of protamine sulfate (Sigma-Aldrich). These infected cells were recovered from puromycine (2 μg/ml; Bioshop) selection for further experiments.

2.5. RNA extraction and quantitative real-time PCR

The relative *CD48* levels were assessed by quantitative real-time PCR. In brief, total RNA was obtained using Novel Total RNA Mini Kit (NovelGene), and complementary DNA was synthesized from the total RNA template using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) as previously described (Huang et al., 2018). Target transcripts in the cDNA samples were quantified using TaqMan gene expression assay kits (Applied Biosystems) with *CD48* (Hs00171172_m1) and *GAPDH* (Hs99999905_m1) as primers and probes. For each target, PCR was performed and the PCR product was detected by the StepOnePlus Real-Time PCR system (Applied Biosystems).

2.6. Conjugate formation assay

Heterotypic cell conjugates were quantitatively determined by a double fluorescence assay as described previously (Y-HL et al., 2017). Briefly, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich)-labeled target cells were co-incubated with equal numbers of far red fluorescent-labeled NK-92MI cells. The double-colored conjugates were counted by FACSCalibur and the conjugates were calculated as a percentage of the total target cells.

2.7. CFSE-based cytotoxic assay

The sensitivity of selected leukemia cells against NK-92MI was performed by using CFSE-based cytotoxicity assay. Briefly, the effector cells were co-cultured with equal number of CFSE-labeled target cells for 4 h. The cell mixture was stained with 7-aminoactinomycin D (Biotium) then the percentage of dead cells within CFSE-positive target cells was detected by CytoFLEX and results were analyzed using CytExpert software.

2.8. Soluble-ligand-based adhesion assay (SL-AA)

The SL-AA was performed according to previous research (Konstandin et al., 2006; Urlaub et al., 2017). Basic buffer for all reaction is 0.5% BSA in PBS supplemented with or without 1 mM $CaCl_2$ and 2 mM $MgCl_2$. NK-92MI cells with TGF-β treatment for different designated time periods were washed twice and were then incubated with ICAM-Fc (R&D System) for 15 min at 37 °C. Reaction was terminated by adding pre-heated (37 °C) paraformaldehyde to a final concentration of 2%. Cells were resuspended with ice-cold buffer, the bound ICAM-Fc was stained with PE-conjugated goat anti-human Fcγ fragment IgG (Jason Immuno-Research) for 20 min at 37 °C incubator. After the reaction, cells were pelleted, washed then analyzed by CytoFLEX and FlowJo software.

2.9. Ligand complex-based adhesion assay (LC-AA)

The LFA-1 binding capacity was detected by LC-AA as previous described (Huang et al., 2018). In brief, the ICAM-1-Fc complexes were prepared by mixing recombinant human ICAM-1-Fc (50 μg/ml) and PE-labeled $F(ab)_2$ fragment of goat anti-human Fcγ fragment specific (40 μg/ml) in buffer without cations for at 4 °C 16 h. CD99-Fc (R&D System) was used as a negative control. NK-92MI with TGF-β treatment for different time period were incubated with 2B4 (1 μg/ml; Invitrogen; clone C1.7) or isotype control for 10 min. After washing, cells were resuspended in buffer, followed by addition of ICAM-1-Fc complexes before being reaction at 37 °C for 15 min. The cells were fixed by adding pre-heated (37 °C) paraformaldehyde. After 5 min, fixation was stopped by adding ice-cold buffer, and then the cells were pelleted then analyzed by CytoFLEX and FlowJo software.

3. Results

3.1. Susceptibility of leukemia cells against NK-92MI targeting was regulated by TGF-β

It has been demonstrated that the tumor-secreted TGF-β promotes the escaping of cancer cell from NK-mediated targeting in patients with solid tumors, like breast cancer, colon cancer, and lung cancer (Hasegawa et al., 2001; Kong et al., 1996; Narai et al., 2002). To address whether the TGF-β has similar effect on non-solid type tumor, like leukemia, we first performed NK degranulation assay to examine the responses from a selection of TGF-β treated leukemia cell lines against killing from a human NK cell line, NK-92MI, due to its application in clinical trail for years (Tonn et al., 2001, 2013; Arai et al., 2008). Selected leukemia cell lines, K-562, MEG-01, and U937, were treated with TGF-β for 5 days and their susceptibilities to NK-92MI killing were compared to those of untreated control. Among the three selected leukemia cells, except K-562 cells, showed significant decreases in susceptibility after the TGF-β treatment against NK-92MI killing, indicating the cell susceptibility against NK killing was affected by the TGF-β treatment (Fig. 1). These results indicated that, like the effect on solid tumors, TGF-β could exert a similar effect on leukemia in enhancing escape from NK-mediated targeting.

3.2. The TGF-β down-regulated leukemia cell surface corresponding ligands for NK activating receptors

The TGF-β-regulated expressions of glycan-related genes in solid tumors have been shown to be highly correlated with disease

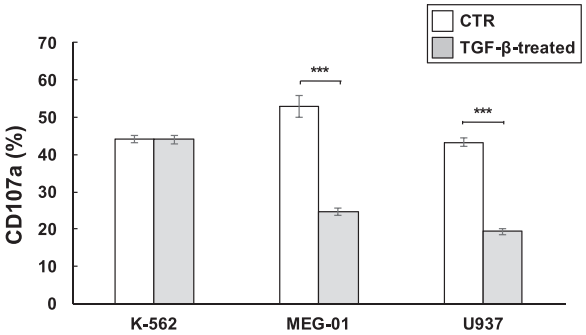

Fig. 1. TGF-β promotes the leukemia cell escape from NK-mediated targeting. Three different leukemia cells were first cultured for 5 days with the presence of 5 ng/ml TGF-β or not as the control (CTR) before the incubation with fluorescence-labeled NK-92MI at 1:1 ratio. Response from TGF-β treated leukemia cells against NK targeting was determined by the NK degranulation assay for the expression of cell surface CD107 on $CD107a^+$ NK-92MI cells. Results are presented as mean ± SD of triplicates (***$P < 0.001$). The experiments were repeated at least three times with similar results.

progression, metastasis, and poor prognosis. For example, TGF-β-mediated signal affects the expression level *GCNT2*, encoding I-branching β-1,6-N-acetylglucosaminyltransferase 2, to promote lung metastasis in patients with breast cancer (Zhang et al., 2011). We next examined whether cell surface glycans on leukemia cells were regulated by TGF-β. We measured the expression of tumor-associated carbohydrate antigens (TACAs), sialyl Lewis a (sLe^a), sialyl Lewis x (sLe^x), and onco-developmental antigen (branched I) on leukemia cells after TGF-β treatment by FACS analysis. The result showed that there were no correlation with changes on the cell surface glycan expression to the reduced sensitivity observed in TGF-β treated K-562, MEG-01 and U937 cells (data not shown), suggesting TGF-β didn't regulate leukemia cell sensitivity through the expression of TACAs like that described in the breast cancer above.

Target recognition is a key step for the activation of NK cell cytotoxicity, achieved by the interaction between NK cell activating receptor(s) and their corresponding ligand(s) on target cells. Whether TGF-β impact is through this interaction on both sides of NK cells and leukemia is still not clear. Hence we examined the TGF-β effect on key leukemia cell surface ligand expressions for interacting with activating receptors presented on NK-92MI cells. We found after TGF-β treatment, the presence of CD48, a ligand for highly expressed cell surface 2B4 on NK-92MI, were significantly affected in the MEG-01 and U937, but not in K-562 (Fig. 2A). Additionally, treatments with higher doses (10 and 20 ng/ml) of TGF-β didn't further reduce the expression levels of ligand CD48 on tested leukemia cells (data not shown). And the quantitative RT-PCR also showed that the TGF-β induced decrease in the *CD48* transcripts in all selected leukemia cells, including MEG-01 and U937 cells, but not K-562 cells (Fig. 2B), correlating with the observation in the FACS analysis above. Taken together, this data suggested that CD48 served as a downstream effector of TGF-β in leukemia MEG-01 and U937 and was responsible for the lower sensitivity against NK-mediated targeting to promote escape from host immunity due to the reduction of the surface CD48 expression (Figs. 1 and 2).

3.3. CD48 Knockdown in the leukemia cells contributed low susceptibility in the leukemia against NK targeting

Given the positive correlation observed above between reduction in CD48 presence and lower leukemia cell sensitivity to NK-92MI killing, we further examined how CD48 contributed to the susceptibility of leukemia cells in response to NK-92MI killing. We first conducted *CD48* knockdown by infection with lentivirus carrying shRNA (sh-431647) targeting at *CD48* into two TGF-β responsive leukemia cells, MEG-01 and U937, and irresponsive K-562. *CD48* knockdown was confirmed by the quantification of *CD48* transcript and the presence of surface CD48 ligand by quantitative RT-PCR and FACS analysis, respectively (Fig. 3A and B). Resulted showed that both *CD48* transcript and ligand were significantly reduced as opposed to the mock control in all examined cells and that they are ready for the further study.

To test the effect of *CD48*-knockdown, we compared the changes of cell sensitivity between mock and *CD48*-knockdown cells against NK-92MI targeting by performing both FACS-based cytotoxic assay and CD107a degranulation assay. The results showed that the susceptibility of the *CD48*-knockdown cells in MEG-01 and U937 against NK-92MI was significantly lower than that in mock controls, indicating that TGF-β regulated cell susceptibility to NK killing through the manipulation of CD48 expression (Fig. 3C and D). Very surprisingly, TGF-β irresponsive K-562 cells with *CD48* knockdown exhibited the same low susceptibility phenotype as two other cells did, which further strengthened the important role of CD48 in the regulation of cell susceptibility.

Next, we examined whether the effector-target interaction was affected by *CD48* knockdown as this step is important for NK cell to initiate cytotoxicity. We conducted conjugation formation assay to compare this interaction between *CD48* knockdown cells and controls. The result showed that this effector-target interaction was greatly weakened in all tested cells, suggesting weaker interaction as result of reduced presence of cell surface CD48 that leading to lower cell susceptibility against NK targeting (Fig. 3E). Based on these results, TGF-β signaling on leukemia cells induced the down-regulation of cell surface CD48, resulting in a weaker effector-target interaction to escape cytotoxicity from NK-92MI cells (Figs. 13).

3.4. TGF-β treatment impaired the cytotoxic activity of the NK-92MI through reducing ICAM-1 binding capability

Other than the TGF-β effect in promoting tumor/leukemia cell escape from host immunity, it has also been demonstrated that the TGF-β released from tumor microenvironment can compromise the function of immune cells, including CTL and NK cells (Bruna et al., 2007; Castriconi et al., 2003). Next, we examined how TGF-β impacted NK functions, including target recognition, interaction, and killing activities toward leukemia cells by conjugate formation, CD107a, and cytotoxic assays, respectively. We examined how exposure of NK-92MI to TGF-β at different periods of time can affect their cell functions. NK-92MI cells were first treated with TGF-β for 24, 48, and 72 h and were analyzed for the changes of NK functions and the expressions of both cell surface activating and inhibitory receptors. Agreeing to the results from previous study, treatment of TGF-β impaired NK killing and degranulation activities in a time-dependent manner (Fig. 4A and B), likely as the result of weakened conjugation for target-effector interaction (Fig. 4C). Furthermore, the TGF-β only slightly reduced surface activating receptors NKG2D along the time course (Fig. 4D), but had no significant effect on the expression of other activating receptors 2B4, NKp30, NKp44, and NKp46 (Fig. 4D) along with inhibitory receptors, ILT2 and PD-1 (data not shown). Based on these results, TGF-β exerted its effect on NK function at the early stage of target recognition and interaction.

For NK targeting, the critical effector-target interaction is first initiated by recognitions between activating receptors and corresponding ligands on NK and target cells, respectively. Importantly, integrins on NK cells work as co-receptors to stabilize the conjugation and to relay its own signal pathway to downstream effectors in response to the outside stimulation. We next investigated whether cell surface integrins were also influenced by TGF-β treatment by examining the expression levels of CD11a and CD18, two subunits of surface integrin LFA-1. Results showed that the expressions of both CD11a and CD18 were not affected by the TGF-β treatment during a time course from 24 up to 72 h (Fig. 5A). Next, we examined the effect of TGF-β on LFA-1 binding affinity and capacity by performing soluble-ligand-based adhesion assay (SL—AA) and ligand-complex-based adhesion assay (LC—AA), respectively. We found that the ICAM-1 binding affinity and capacity were much lower in the TGF-β-treated group (Fig. 5B and C). These results demonstrated that LFA-1 mediated effector-target interaction was impaired by TGF-β treatment, supporting TGF-β influence for lower NK cytotoxicity occurred at the very upstream, i.e., the effector-target interaction, leading to the suppressed degranulation and cytotoxicity activities toward leukemia cells (Figs. 4 and 5).

4. Discussion

Lots of studies demonstrate that the TGF-β secretion from the cells in solid tumor microenvironment could greatly affect the disease progression (Yang et al., 2010). However, little is known about the TGF-β effect in the hematological disorder, especially regarding disease progression and the impact on host immunity. In this study, we showed that the TGF-β could affect both of immune cells (NK) and malignant cells (leukemia) by suppressing NK functions and by enhancing the escape of leukemia from NK-92MI-mediated surveillance, respectively. First from the leukemia aspect, treatment of the TGF-β on those selected leukemia cell lines showed that reduced susceptibilities were observed from two selected cell lines, suggesting NK cell targeting can be compromised in the environment with the presence of TGF-β (Fig. 1). While

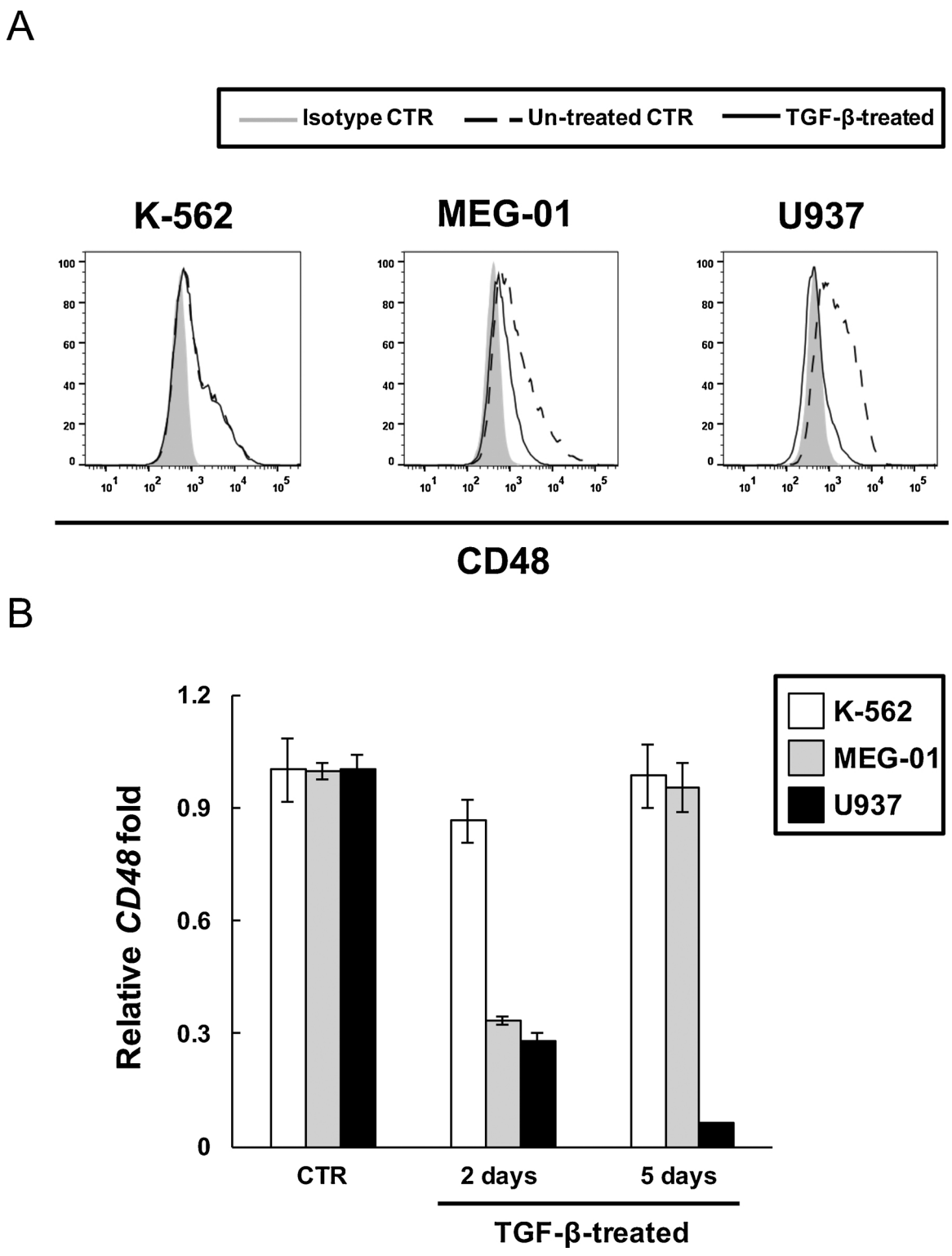

Fig. 2. The TGF-β effect on the *CD48* expression in leukemia cells. (A) Cell surface expression of CD48 on leukemia cells after TGF-β stimulation. Cell surface CD48 was measured from three leukemia cell lines after TGF-β treatment for 5 days. The shaded represents the expression pattern from cells incubated with the isotype control. The solid and dashed lines represent the cells incubated with anti-CD48 with or without TGF-β stimulation, respectively. The experiments were repeated more than three times. (B) Expression of the *CD48* transcript in leukemia cells after TGF-β stimulation. Leukemia cells K-562, MEG-01, and U937, were treated with TGF-β for 2 and 5 days before total RNA was extracted from these cells and reverse transcribed into cDNA. Real-time PCR was performed to quantify the *CD48* expressions and the obtained results were normalized to *GAPDH* levels in the respective samples. The data is presented as the mean ± SD of three independent experiments.

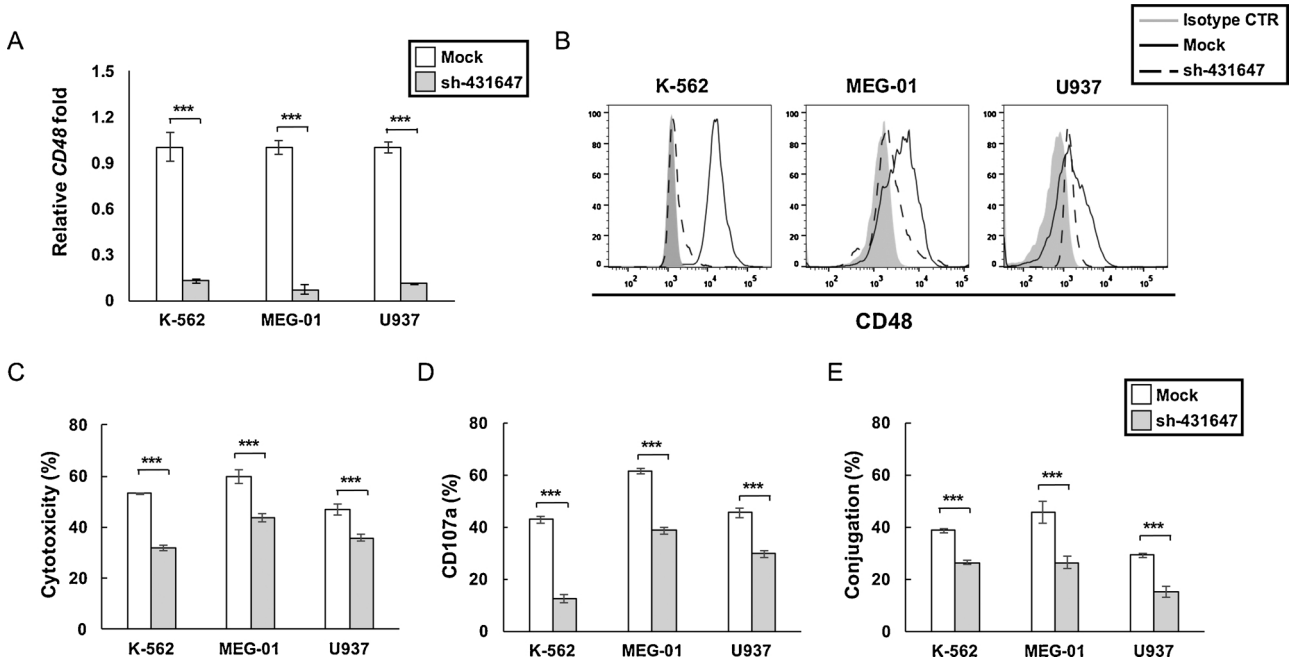

Fig. 3. *CD48* knockdown in leukemia cells decreases the cell susceptibility against NK-92MI targeting. Three different leukemia cells were first infected with lentivirus for *CD48* knockdown, either carrying shCD48 (sh-431647) gene or the shlacZ as the mock control. After puromycin-based selection for one week, the cells were harvested and *CD48* knockdown expression was confirmed by real-time PCR (A) and flow cytometry (B). (A) For each examined leukemia cell, its *CD48* expression was first normalized to *GAPDH* and compared to that of the mock control. (B) For the surface CD48 expression of each leukemia cell, the mock control and sh-431647 leukemia cells, K-562, MEG-01 and U937, were analyzed. The shaded area presented in each panel is the result obtained from cells incubated with antibody isotype control. And the dashed and solid lines represent expressions of CD48 from the CD48-knockdown and mock control with CD48 antibody, respectively. (C) Cytotoxicity assay for CD48-knockdown leukemia cells against NK-92MI. Results are presented as mean ± SD of triplicate (***$P < 0.001$). (D) *CD48* knockdown leukemia cells were analyzed for the NK susceptibility. Surface CD107a staining was performed by incubation of *CD48* knockdown target cells with fluorescence-labeled NK-92MI as described in Fig. 1. Results are presented as mean ± SD of triplicate (***$P < 0.001$). (E) Conjugation assay were conducted by incubation of CFSE-labeled mock or *CD48* knockdown target cells with fluorescence-labeled NK-92MI. Results are shown as means ± SD of triplicate (***$P < 0.001$). The experiments were repeated at least three times with similar results.

examining whether the expression of leukemia cell surface ligands could be influenced by TGF-β treatment, we found that CD48, identified as a ligand for NK activating receptor 2B4, was greatly down-regulated in the MEG-01 and U937 cells but not in K-562 cells. CD48 is a member of the SLAM family, and is involved in the adhesion and activation of immune cells (Milstein et al., 2008; Gonzalez-Cabrero et al., 1999). Due to its high binding affinity to NK activating receptor 2B4, the expression levels could very likely impact the NK-mediated cytotoxicity. Our result also strongly suggested that CD48-mediated signaling(s) was a main regulatory pathway for TGF-β-induced cell susceptibility against NK-targeting, as evident of that irresponsive K-562 cells with unaltered expression of CD48 transcript and protein didn't have reduced susceptibility as compared to those of two responsive cell lines (Figs. 1 and 2). This suggestion could be further supported by the *CD48*-knockdown in K-562 cells that leaded to reduced cell surface CD48 expression and, subsequently, low susceptibility (Fig. 3). The irresponsibility of K-562 cells in response to TGF-β stimulation might result from the signaling defect upstream of *CD48* transcription as *CD48*-knockdown showed that once CD48 expression can be manipulated, the susceptibility was greatly affected (Fig. 3). Taken together, we identified that the CD48 expression served as the key regulatory factor for TGF-β-regulated cell susceptibility against NK targeting.

It has been demonstrated that, as an important phenotype, the expression of surface glycans in solid malignant tumors is highly influenced by TGF-β (Zhang et al., 2011), suggesting the cell surface expression of TACAs and the aberrant hypersialyated formation of hematological malignant cells may be regulated by TGF-β. Our result showed that TGF-β treatment didn't result in the same effect on surface glycans from the solid tumors as we did not observe *GCNT2* activation in all examined leukemia cells (data not shown) as opposed to the enhanced expression in the breast cancer cells (Zhang et al., 2011). Taken together, our results suggested that hematological diseases and solid tumor responded differently toward TGF-β stimulation.

This is the first time that we showed a connection of CD48 expression to TGF-β in leukemia cells as both the *CD48* transcript and protein expression were suppressed by TGF-β (Fig. 2). It's still not well addressed regarding to the role of CD48 along the leukemia formation and its detail interaction with TGF-β in the leukemia patients. Recently, it has been reported that both oncogenic fusion proteins, PML-RARA and AML-ETO, specifically down-regulated CD48 to render the leukemia cells with immune evasion properties (Elias et al., 2014). Interestingly, of our examined leukemia cells, MEG-01 cells were derived from chronic myelogenous leukemia (CML) patient with BCR-ABL fusion protein. On the other hand, U937 cells were derived from histiocytic lymphoma without expressing any oncogenic fusion gene. As both cells shared same response to TGF-β stimulation, resulting in suppressed CD48 expression and lower susceptibility, this suggested very likely there are multiple signaling pathways involved in relaying TGF-β signal to *CD48* expression. Taken together, CD48 down-regulation is known as a common phenotype in several types of leukemia, including acute and chronic myelogenous leukemia, and lymphoma, to promote leukemia escape from NK-mediated killing. Hence, understanding how *CD48* is regulated by TGF-β stimulation, including identifying signaling pathways, epigenetics, and regulatory transcriptional factor(s) will greatly benefit treatments in these subclasses of leukemia as the CD48-mediated signaling can serve as a treatment target. Interestingly, in MEG-01 cells, the expression level of *CD48* transcript was observed to restore back to the untreated levels after 5-day treatment, but the cell surface CD48 expression still remained in the lower levels (Fig. 2). This activity might result from the desensitization from MEG-01 cell toward

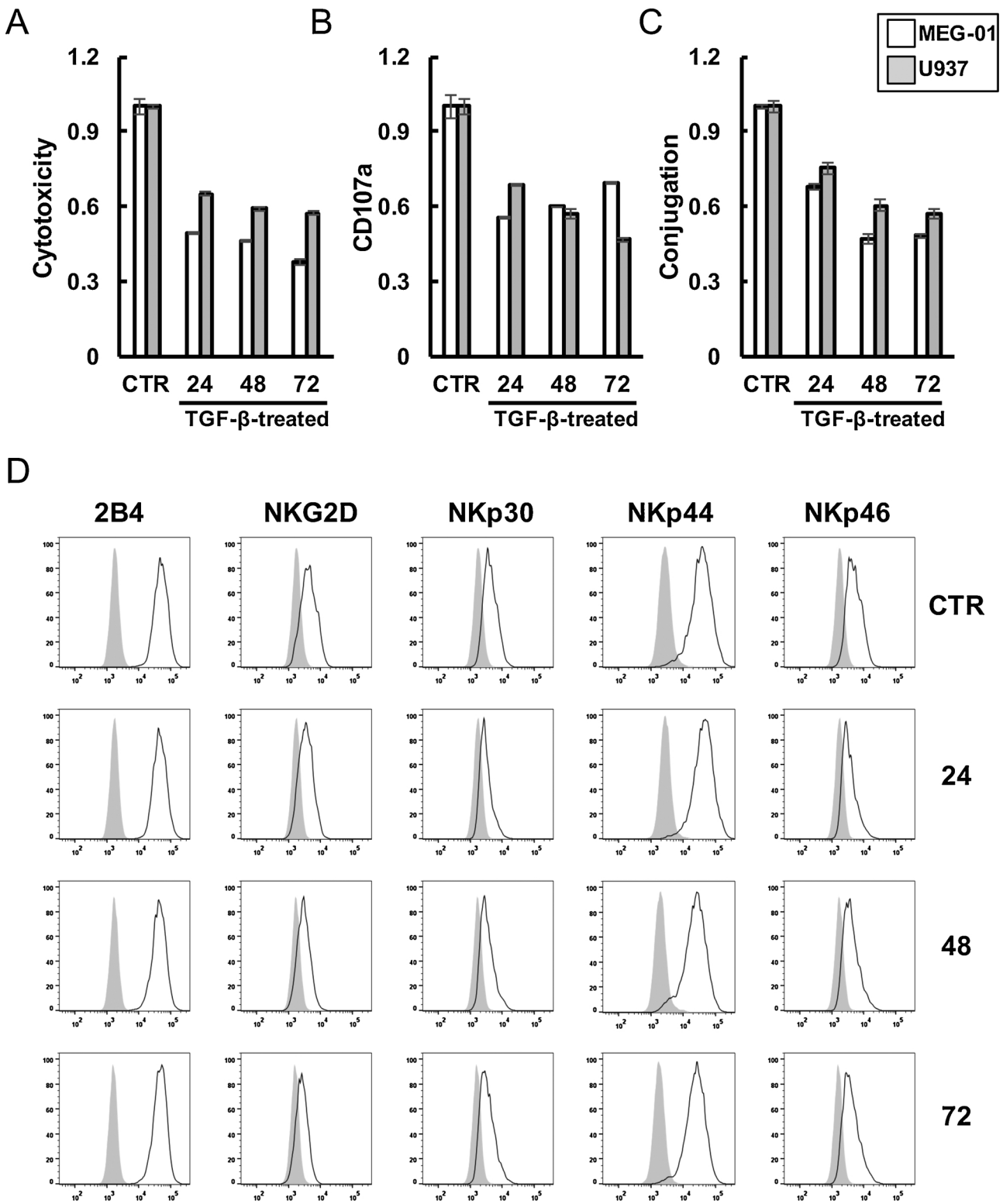

Fig. 4. NK-92MI cell functions are suppressed by the TGF-β. The TGF-β effect on NK-92MI cell functions was examined in a time course manner toward two selected leukemia cells. The NK-92MI cells treated with TGF-β were collected from time point, 24, 48, and 72 h. Collected cells, along with control cells (no TGF-β treatment) were subjected to FACS-based cytotoxicity (A), CD107a assay (B), and conjugate formation assay (C) as described previously. Results obtained from each time point were compared to that of the control (arbitrarily set as 1.0). The representative result is shown as means ± SD from three independent experiments. (D) Flow cytometric analyses of the expressions of NK-92MI cell surface activating receptors in response to TGF-β treatment at different time points. Results were displayed with the open and shaded areas representing incubations with examined antibodies and isotype control, respectively. The experiments were repeated at least three times with similar results.

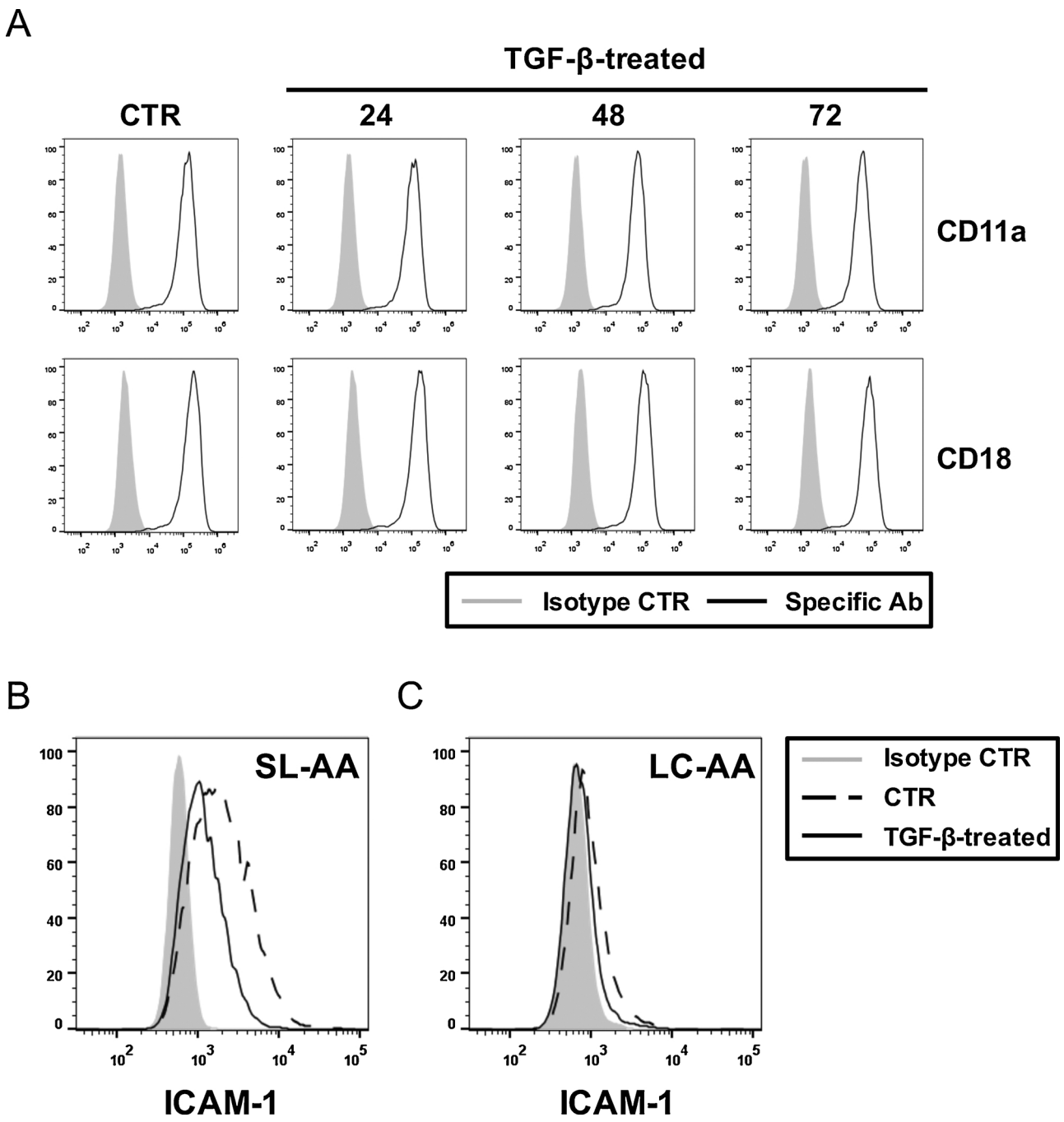

Fig. 5. TGF-β impairs the ICAM-1 binding affinity of NK-92MI. (A) Representative flow cytometric staining pattern for CD11a and CD18 on NK-92MI responding to TGF-β treatment along a time course with three time points. The open and shaded areas represent the results obtained from cells incubated with indicated antibodies and isotype control, respectively. The experiments were repeated at least three times with similar results. TGF-β effect on LFA-1 binding affinity (B) and capacity (C) in NK-92MI cells was examined. LFA-1 binding and capacity were determined by measuring the presence of ICAM-Fc complex. The shaded areas represented the incubations with the antibody control (CD99-Fc). The solid and dashed lines represented the results from NK-92MI cells with or without TGF-β treatment, respectively. This result is a representative of repeated experiments more than three times.

the prolonged TGF-β stimulation.

When examining how TGF-β influenced the NK function, we found that multiple aspects in the NK function were impaired, including the target recognition, interaction, and degranulation for target killing (Fig. 4A–C). And, surprisingly, of those important cell surface receptors examined, we didn't find altered expressions among them (Fig. 4D). These results suggested that TGF-β influence was not through these receptors or at least not through their cell surface expressions. It's been known that the co-receptor LFA-1 is not only important for NK adhesion but also contributes to granule polarization necessary for degranulation (Bryceson et al., 2005). By our results, we showed that LFA-1 was under the regulation of TGF-β. We discovered that TGF-β effect was not through the expressional change on both LFA-1 subunits, CD11a and CD18, but instead was via significant decrease in ICAM-1 binding affinity and capacity (Fig. 5). This, to our knowledge, is the first evidence to link TGF-β influence onto NK cell surface LFA-1 function. In this study, we found that TGF-β promoted leukemia cell escaping from host immunity through down-regulation of target-NK signaling by suppressing CD48 expression. And it also impaired NK cytotoxicity by decreasing conjugate formation, degranulation, and ICAM-1 binding.

5. Conclusions

In this study, we demonstrated that the TGF-β enhanced the escape of leukemia cell from NK-mediated surveillance exerting its different effects on both immune cell and malignant cell. In leukemia cells, for the first time, we showed the TGF-β down-regulated CD48 in transcription and protein levels to regulate the CD48-mediated signalings. For the NK cells, our results suggested that the TGF-β lowered the integrin-mediated binding affinity to decrease the effector-target interaction, degranulation, and killing activity of NK-92MI cells.

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Authors' contributions

Yuh-Ching Twu contributed to the experimental design, data analysis and interpretation, and manuscript preparation. Yi-Jen Liao and Tzeon-Jye Chiou contributed to data interpretation. Chin-Han Huang, Hsin-Ting Huang, and Yen-Hsi Lin performed the research and analyzed the data.

Declaration of Competing Interest

No financial conflict or commercial of interest has been identified for any of the authors.

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