









# Macrophages protect mycoplasma-infected chronic myeloid leukemia cells from natural killer cell killing

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## Keywords

Chronic infection, inflammation, chronic myeloid leukemia, macrophages, maintenance of NK mCD16, natural killer cells, tumor environment

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## Abstract

Macrophages (M $\phi$ ) have been reported to downmodulate the cytotoxicity of natural killer (NK) cell against solid tumor cells. However, the collaborative role between NK cells and M $\phi$  remains underappreciated, especially in hematological cancers, such as chronic myeloid leukemia (CML). We observed a higher ratio of innate immune cells (M $\phi$  and NK) to adaptive immune cells (T and B cells) in CML bone marrow aspirates, prompting us to investigate the roles of NK and M $\phi$  in CML. Using coculture models simulating the tumor inflammatory environment, we observed that M $\phi$  protects CML from NK attack only when CML was itself mycoplasma-infected and under chronic infection–inflammation condition. We found that the M $\phi$ -protective effect on CML was associated with the maintenance of CD16 level on the NK cell membrane. Although the NK membrane CD16 (mCD16) was actively shed in M $\phi$  + NK + CML trioculture, the NK mCD16 level was maintained, and this was independent of the modulation of sheddase by tissue inhibitor of metalloproteinase 1 or inhibitory cytokine transforming growth factor beta. Instead, we found that this process of NK mCD16 maintenance was conferred by M $\phi$  in a contact-dependent manner. We propose a new perspective on anti-CML strategy through abrogating M $\phi$ -mediated retention of NK surface CD16.

## INTRODUCTION

Natural killer (NK) cell spontaneously recognizes and eliminates cancer or pathogen-infected cells without prior sensitization.<sup>1</sup> This is attributed to the expression of germline-encoded activating and inhibitory receptors through which the NK cell receives signals to make a cellular decision on launching cytotoxicity.<sup>2</sup> CD16 is a well-recognized NK activating receptor that mediates antibody-dependent cellular cytotoxicity against target cells coated (or opsonized) with specific antibodies.<sup>3</sup> In addition, CD16 is involved in nonantibody-dependent

cellular cytotoxicity-dependent NK functions through (a) direct lysis of target cells in the absence of antibody coating,<sup>4</sup> (b) boosting serial engagement of target cells through CD16 shedding,<sup>5</sup> and (c) coupling of NK CD3 $\zeta$  adaptor molecule to CD2 activation mediator upon CD16 cleavage.<sup>6</sup> As the nonantibody-dependent cellular cytotoxicity function of NK CD16 is less explored and could be potentially important, we were prompted to further investigate such a function of NK CD16.

Upon activation, NK cell secretes cytotoxic molecules, such as perforin and granzymes, to induce target cell death.<sup>7</sup> This innate capability of NK cell makes it a key

player in cancer immunosurveillance. Indeed, individuals with low NK cell counts have increased risk of developing leukemia and other cancers.<sup>8</sup> It is therefore unclear how cancer cells thrive despite the presence of such effective killer cells.<sup>9,10</sup>

The most abundant immune cell population present in the tumor environment is the macrophage (M $\phi$ ).<sup>11</sup> Depending on environmental cues, M $\phi$  can be polarized into classically activated proinflammatory M1 or the alternatively activated anti-inflammatory M2 phenotypes.<sup>12</sup> In the tumor environment, M $\phi$  more closely resembles the “healing” M2 type, which is protumorigenic and immunosuppressive.<sup>13</sup> In solid tumor environments, it was proposed that M $\phi$  renders NK cell dysfunctional<sup>14</sup> through soluble factors such as transforming growth factor beta (TGF $\beta$ ) and prostaglandin E2, and through ligand–receptor interactions via HLAG-ILT2 and CD48-2B4.<sup>15–18</sup> However, the collaborative role of NK cell and M $\phi$  in hematological cancers remains unclear. Here, we query how M $\phi$  would modulate NK activity in chronic myeloid leukemia (CML), a hematological cancer arising from the bone marrow, characterized by abnormal proliferation of granulocytes (neutrophils, basophils, eosinophils) and myeloid progenitors as a result of constitutive activation of BCR-Abl tyrosine kinase.<sup>19</sup>

Inflammation is identified as one of the key hallmarks of cancer,<sup>20</sup> characterized by the presence of inflammatory cytokines such as interleukin-6 (IL-6), IL-8 and tumor necrosis factor-alpha (TNF $\alpha$ ) in the cancer environment.<sup>21</sup> This could be a result of chronic infection condition. Notably, mycoplasma is detected in 50% of myeloid leukemia patients,<sup>22</sup> and other patients with solid cancers.<sup>23–25</sup> In cell cultures, the presence of mycoplasma creates a low-grade chronic inflammatory condition without compromising cell viability, ideal for promoting cancer transformation.<sup>26</sup> Moreover, there are several reports supporting mycoplasma-induced production of inflammatory cytokines, such as IL-6, IL-8 and TNF $\alpha$ .<sup>27,28</sup> How these infection–inflammation conditions may promote clinical cancer progression is hitherto unexplored.

In this study, we sought to understand whether M $\phi$  could alter NK activation in CML, particularly through the modulation of NK CD16 level, in the presence or absence of mycoplasma-induced inflammatory conditions. To investigate the specific interactions between CML, NK and M $\phi$ , we performed *ex vivo* coculture of primary NK cell and M $\phi$  (derived from healthy blood donors) with CML cell lines. By systematically delineating findings under mycoplasma negative (myco<sup>-</sup>) and mycoplasma positive (myco<sup>+</sup>) conditions, we further defined specific contributions from mycoplasma-induced inflammation.

## RESULTS

### CML cells showed inflammation induced by acute and chronic mycoplasma infection

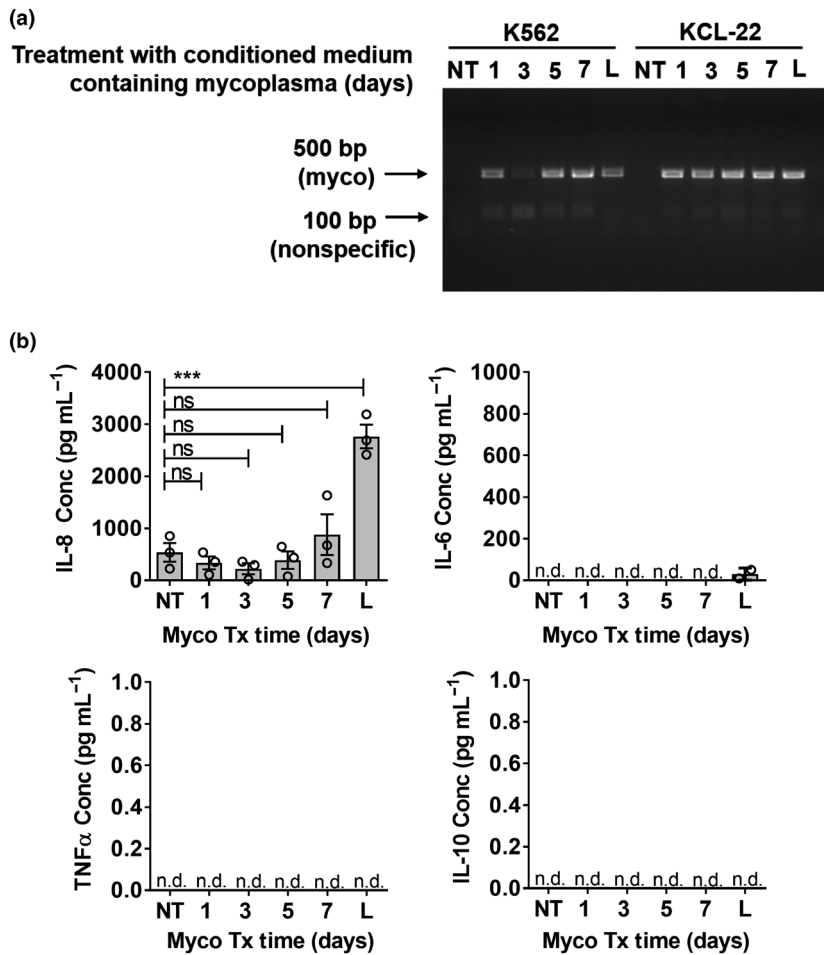
The tumor environment of CML patients is characterized by inflammation, and mycoplasma is also detected in bone marrow samples of myeloid leukemia patients.<sup>22,29</sup> Hence, to model inflammation condition in CML, we infected CML cell lines with mycoplasma, using two strategies: (1) short-term (acute) mycoplasma-infected CML cells (referred to as myco tx) that were experimentally infected with mycoplasma through addition of mycoplasma-containing culture medium for up to 7 days, and (2) long-term (chronic) mycoplasma-infected CML cells (referred to as myco<sup>+</sup> and annotated L for long-term), which were cells carrying latent infection with mycoplasma for many passages. Noninfected cultures were annotated as myco<sup>-</sup>. We determined that CML cells acutely and chronically infected with mycoplasma were mycoplasma positive (Figure 1a). In the figure, the nonspecific band detected in infected CML cell lines, but absent in noninfected controls, could be attributed to nonspecific amplification of a conserved portion of the mycoplasma genome, either from the primer sets that were used or from priming by the mycoplasma PCR products.

To determine the inflammation status, we tested for inflammatory cytokines (IL-8/IL-6/TNF $\alpha$ /IL-10) produced into the culture supernatant of myco<sup>-</sup> (NT), myco tx (days 1, 3, 5, 7) and myco<sup>+</sup> CML (annotated L for long term) cells. Of the four cytokines tested, only IL-8 was produced at detectable levels, with significantly increased production by CML cells which were chronically infected with mycoplasma (Figure 1b and Supplementary figure 1). IL-6/TNF $\alpha$ /IL-10 were nondetectable (n.d.), except for trace level of IL-6 in chronically infected culture. The species of mycoplasma infecting and resulting in the increased IL-8 production were determined to be *Mycoplasma fermentans* and *Mycoplasma hyorhinis* (Supplementary figure 2).

Taken together, mycoplasma infection of K562 CML cells induced high production of IL-8. This was consistent with the reported upregulation of IL-8 in the serum of CML patients.<sup>29,30</sup> Hence, to simulate the inflammatory condition in the CML environment, we employed the strategy of using chronically infected (myco<sup>+</sup>) CML cells compared with noninfected counterparts (myco<sup>-</sup>) in subsequent coculture experiments with primary M $\phi$  and NK cells.

### M $\phi$ protected mycoplasma-infected CML from NK cytotoxicity

To determine the influence of M $\phi$  and NK in CML survival, we first queried the change in proportion of

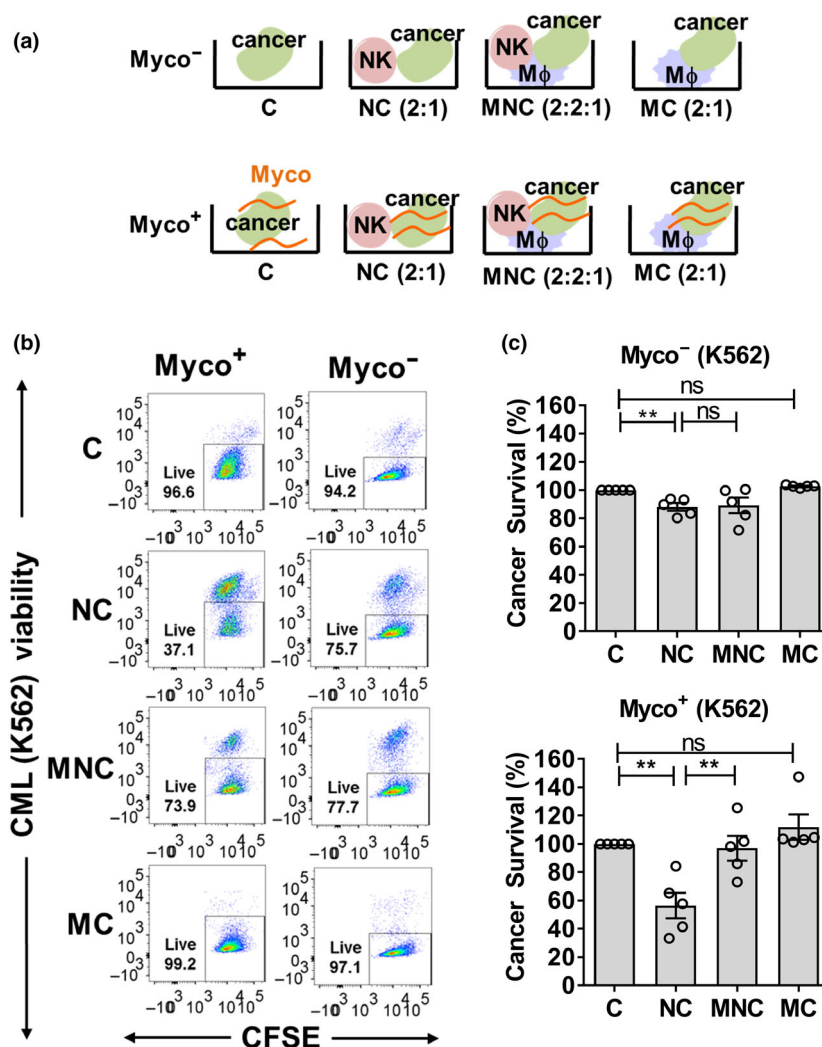


**Figure 1.** Increased production of interleukin-8 (IL-8) by chronic myeloid leukemia (CML) with chronic and acute infection of mycoplasma. Noninfected K562 cells were treated with mycoplasma-containing culture supernatant for 1, 3, 5 and 7 days (myco tx). These acutely infected cultures were compared with noninfected (NT) and chronically infected CML cultures (L). **(a)** Cell culture supernatants were tested for presence of mycoplasma via PCR. DNA bands were visualized via UV transillumination (Bio-Rad imager and Syngene Genesnap software) of SYBR safe-stained agarose gel. **(b)** Mycoplasma-infected K562 cells were seeded at 1 million cells mL<sup>-1</sup> and incubated overnight. Culture supernatants were tested for presence of IL-8, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-10 using ELISA. Results shown are mean  $\pm$  s.e.m. of three independent experiments (donors). See Supplementary figure 1 for individual replicate experiments. Statistical significance was determined using repeated measures one-way ANOVA followed by Tukey's test. \*\*\* $P < 0.001$ . L, CML cells that were long-term mycoplasma infected because of tissue culture procedures; n.d., nondetectable; ns, nonsignificant; NT, nontreated CML cells that were mycoplasma free.

innate M $\phi$  and NK cells compared with adaptive T and B cells in bone marrow aspirates of CML patients and nonleukemia orthopedic patient controls (Supplementary figure 3). We observed a low ratio of [M $\phi$ -NK]:[T-B] cells in nonleukemia controls of about 0.1, that is, the proportion of adaptive T and B cells were 10-fold more than innate M $\phi$  and NK cells. However, CML patients in the more severe "accelerated and blast crisis phase" showed significant ( $P = 0.033$ ) increase in the ratio of [M $\phi$ -NK]:[T-B] cells. This suggested a shift in the immunological profile in patients with severe CML toward antigen-independent innate immunity, prompting

us to further determine the functional roles of M $\phi$  and NK in CML survival.

We performed *ex vivo* mono, duo and triocultures using primary M $\phi$  and NK cells from the peripheral blood of healthy donors, and myco<sup>-</sup>/myco<sup>+</sup> K562 CML cells (Figure 2a). All treatments were normalized to cancer alone (C) because of the background viability of C sample. In the duoculture of NK + CML (NC), we observed a decrease in CML survival under both myco<sup>-</sup> and myco<sup>+</sup> conditions, demonstrating NK-mediated killing of CML (Figure 2b, c and Supplementary figure 4). In the absence of mycoplasma infection, a comparison



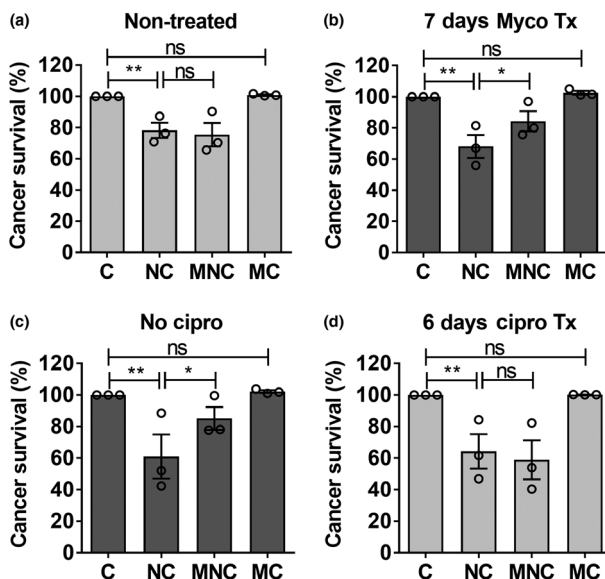
**Figure 2.** Macrophage (Mφ) attenuated natural killer (NK) cell cytotoxicity against mycoplasma-infected chronic myeloid leukemia (CML). **(a)** Experimental setup of mono, duo and triocultures with Mφ, NK and CML cells (cell density ratios in parentheses). Cells were cocultured for 24 h prior to measurement of CML survival based on flow cytometry. Chronically infected cultures were annotated myco<sup>+</sup>, whereas noninfected cultures were annotated myco<sup>-</sup>. **(b)** Representative flow cytometry plots showing carboxyfluorescein succinimidyl ester-stained cancer cells and % live cells based on negative staining for fixable viability dye. **(c)** CML survival measured under myco<sup>+</sup> and myco<sup>-</sup> conditions, upon coculture with Mφ, NK cells and normalized to cancer-alone control. Results shown are mean ± s.e.m. of five independent experiments (donors). See Supplementary figure 4 for individual replicate experiments. Statistical significance was determined using repeated measures one-way ANOVA followed by Fisher's LSD test. \*\**P* < 0.01. C, CML alone; MC, Mφ + CML; MNC, Mφ + NK + CML; NC, NK + CML; ns, nonsignificant.

between Mφ + NK + CML (MNC) and Mφ + NK (NC) showed no further modulation of myco<sup>-</sup> CML survival mediated by Mφ. Interestingly, with mycoplasma-infected CML, the NK-mediated killing of myco<sup>+</sup> CML was attenuated in the presence of Mφ (Figure 2b, c and Supplementary figure 4), suggesting that Mφ protected myco<sup>+</sup> CML from NK killing. We also observed that duoculture of Mφ + CML (MC) did not reduce CML survival, indicating that the killing activity against CML was mediated by NK, and not Mφ. We further

cocultured NK and Mφ with another CML cell line, KCL-22 (Supplementary figure 5). When KCL-22 was myco<sup>-</sup>, there was further decrease in KCL-22 survival in MNC trio cultures. However, when KCL-22 was myco<sup>+</sup>, the Mφ appeared to protect KCL-22 from NK killing, consistent with that of K562. Taken together, our findings suggest that there is no observable Mφ protection of myco<sup>-</sup> CML cells from NK cytotoxicity. Mycoplasma infection of the CML cells prompted Mφ-mediated protection from NK cytotoxicity.

### Only myco<sup>+</sup> CML cells were protected by M $\phi$ from NK cytotoxicity

Henceforth, we performed further studies using one of the two CML cell lines, K562, simply referred to as CML. To confirm that the M $\phi$ -protective effect was specific to mycoplasma infection, we tested cocultures of (1) 7-day myco tx CML cells with M $\phi$  and NK cells and (2) CML cells cleared of mycoplasma infection by ciprofloxacin treatment, with M $\phi$  and NK cells. Supplementary figure 6 shows the efficacy of ciprofloxacin treatment which eliminated mycoplasma infection in CML cells. Consistent with the observation for chronically infected CML (Figure 2c), we also observed M $\phi$  protection of 7-day myco tx CML from NK killing (Figure 3a, b and Supplementary figure 7a, b). On the contrary, when chronically infected CML was cleared of mycoplasma by ciprofloxacin treatment (6-day cipro tx), the M $\phi$  protective effect was abrogated (Figure 3c, d and Supplementary figure 7c, d), supporting the



**Figure 3.** Macrophage (M $\phi$ ) protection of chronic myeloid leukemia (CML) was specific to infection–inflammation condition induced by mycoplasma. Natural killer (NK) cells and M $\phi$  were cocultured with (a, b) noninfected CML treated with mycoplasma-containing medium, or (c, d) chronically infected CML treated with 10  $\mu\text{g mL}^{-1}$  ciprofloxacin. CML survival was subsequently measured based on negative staining for fixable viability dye and normalized to cancer alone control. Results shown are mean  $\pm$  s.e.m. of three independent experiments (donors). See Supplementary figure 7 for individual replicate experiments. Statistical significance was determined using repeated measures one-way ANOVA followed by Fisher's least significant difference test. \* $P < 0.05$ ; \*\* $P < 0.01$ . C, CML alone; MNC, M $\phi$  + NK + CML; MC, M $\phi$  + CML; NC, NK + CML; ns, nonsignificant.

importance of mycoplasma infection condition in mediating M $\phi$  protection of CML from NK killing.

We further queried whether inflammation induced by lipopolysaccharide (LPS) or poly(I:C) would create an environment conducive to M $\phi$  protection of CML from NK cytotoxicity (refer to Supplementary methods). Stimulation of inflammation in the CML by LPS or poly(I:C) was confirmed by induced IL-8 production by CML cells (Supplementary figures 8a, b and 9a, b). We found that M $\phi$  protection was only conferred on mycoplasma-infected CML but not on LPS- or poly(I:C)-treated CML (Supplementary figures 8c and 9c).

Altogether, we have demonstrated that M $\phi$  protection of CML from NK cytotoxicity was specific to mycoplasma-induced inflammation condition because (1) infection of “clean” CML cells with mycoplasma-containing medium resulted in M $\phi$  protection of the myco tx CML from NK cytotoxicity, (2) there was abrogation of M $\phi$ -protective effect of CML when the mycoplasma was eradicated by ciprofloxacin treatment of CML, and (3) inflammation induced by treatment of CML with LPS or poly(I:C) alone did not confer M $\phi$  protection of CML from NK cytotoxicity.

### IL-8 was not involved in M $\phi$ protection of myco<sup>+</sup> CML from NK cytotoxicity

The high production of IL-8 by myco<sup>+</sup> CML and the high level of IL-8 in the serum of CML patients<sup>29,30</sup> led us to query whether IL-8 was involved in the observed M $\phi$ -mediated protection of myco<sup>+</sup> CML from NK cytotoxicity (Figure 2c). We thus attempted to neutralize IL-8 in trioculture of M $\phi$ , NK and myco<sup>+</sup> CML (MNC). However, we did not observe modulation of M $\phi$ -protective effect toward myco<sup>+</sup> CML with increasing doses of IL-8 neutralizing antibody (Supplementary figure 10), indicating that IL-8 does not contribute directly to the M $\phi$ -mediated protection of CML.

### NK degranulation was suppressed by M $\phi$ in MNC trioculture

The changes in CML survival observed in mono, duo and triocultures of M $\phi$ , NK and CML were interpreted as modulation of NK cytotoxicity against CML (Figure 2c). To further substantiate that NK activation (and hence cytotoxicity) was modulated, we measured NK degranulation marker, CD107a, under mono, duo and triocultures over two time points (4 and 24 h). The 4-h incubation is the commonly reported time point for degranulation assays.<sup>31</sup> Because we performed cocultures of M $\phi$ , NK and CML cells for 24 h, we also performed the degranulation assay during the last 4 h of the 24-h

coculture, as outlined in Figure 4a and Supplementary figure 11a. At the 24-h time point, comparing NC duoculture with N alone culture, there was an increase in the proportion of CD107a<sup>+</sup> NK, suggesting an increase in NK activation and degranulation in NC coculture. By contrast, there was no significant increase in CD107a<sup>+</sup>-activated NK cells in cocultures with myco<sup>-</sup> CML (Figure 4a), consistent with the higher NK cytotoxicity observed for myco<sup>+</sup> than myco<sup>-</sup> CML cells (Figure 2c). At the 4-h time point, the increase in the proportion of CD107a<sup>+</sup> NK in NC duo culture was also observed (Figure 4b and Supplementary figure 11b).

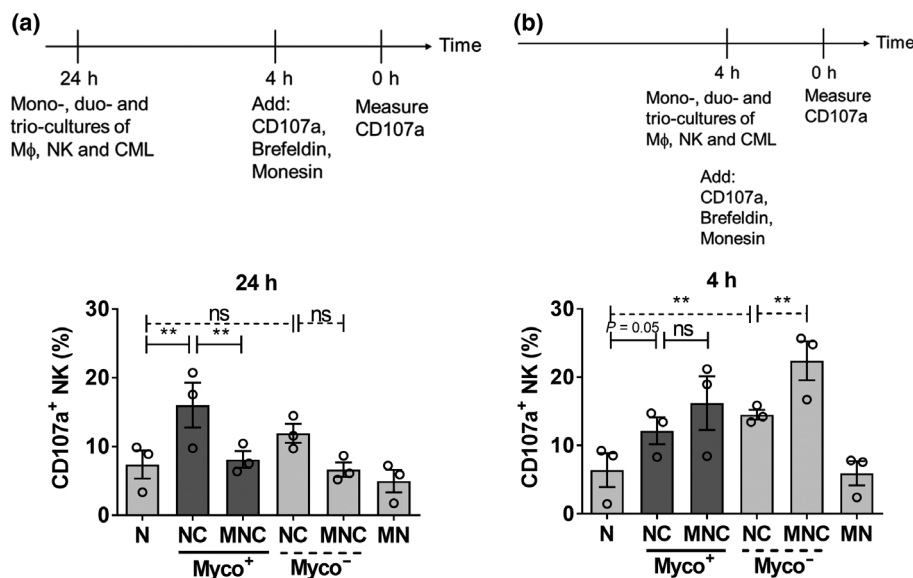
The increase in CD107a<sup>+</sup> NK in the NC duo culture was attenuated under MNC triocultures at the 24-h time point (Figure 4a), suggesting a suppression of NK activation in the presence of Mφ. This corroborates the observation that Mφ protected myco<sup>+</sup> CML from NK cytotoxicity (Figure 2c). Interestingly, for the 4-h time point in MNC triocultures, we observed a trend toward further increase in CD107a<sup>+</sup> NK instead of attenuation, suggesting that there was a gradual Mφ-mediated suppression of NK activity over time.

Activated NK are also known to secrete cytokines and chemokines such as interferon-γ (IFNγ), TNFα and macrophage inflammatory protein-1α (MIP-1α). Hence, we also tested for these secreted NK factors in the mono, duo and triocultures. However, we found no significant

modulation of NK IFNγ, TNFα and MIP-1α production (Supplementary figure 12a–c). Taken together, the data suggested that (1) the decrease in CML survival in NC duo cultures (Figure 2c) was due to increase in NK degranulation and cytotoxicity (Figure 4a); (2) the increase in CML survival in MNC triocultures compared with NC duo cultures (Figure 2c) was due to suppression of NK degranulation and cytotoxicity mediated by Mφ (Figure 4) and (3) NK IFNγ, TNFα and MIP-1α cytokines production did not influence the killing of CML in the coculture system. The observed Mφ protection of CML from NK cytotoxicity was mediated by the suppression of NK degranulation capability, prompting us to further query the mechanism of NK suppression.

### Maintenance of NK membrane CD16 in MNC trioculture

To clarify the mechanism of Mφ-mediated suppression of NK killing ability, we first characterized the changes in the expression of NK-activating membrane receptors, membrane Nkp46 and membrane CD16 (mCD16), in triocultures of NK, Mφ and CML cells (MNC). There were marginal changes in Nkp46<sup>+</sup> NK in mono, duo and triocultures regardless of myco<sup>+</sup> or myco<sup>-</sup> conditions (Supplementary figure 13). Comparing NC with N alone,

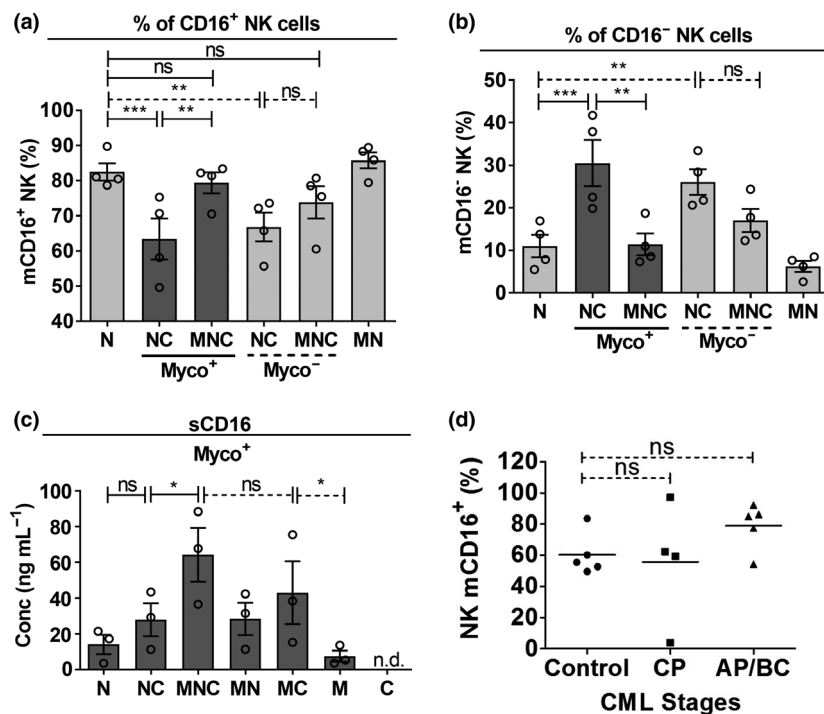


**Figure 4.** Macrophage (Mφ) suppressed natural killer (NK) degranulation over time in MNC trioculture. NK, Mφ, and myco<sup>-</sup> and myco<sup>+</sup> chronic myeloid leukemia (CML) were incubated in mono, duo and triocultures according to the experimental strategies outlined in (a) (24 h) and (b) (4 h). CFSE<sup>-</sup>CD14<sup>-</sup>CD56<sup>+</sup> NK cells were then gated and CD107a<sup>+</sup> NK cells were determined. Results shown are mean ± s.e.m. of three independent experiments (donors). See Supplementary figure 11 for individual replicate experiments. Statistical significance was determined using repeated measures one-way ANOVA followed by Tukey's test. \*\**P* < 0.01. CFSE, carboxyfluorescein succinimidyl ester; MN, Mφ + NK; MNC, Mφ + NK + CML; N, NK alone; NC, NK + CML; ns, nonsignificant.

the proportion of mCD16<sup>+</sup> NK was significantly reduced in NC, and the reduction was higher for myco<sup>+</sup> than for myco<sup>-</sup> CML (Figure 5a and Supplementary figure 14a). As shedding of surface CD16 on NK cells has previously been associated with elevated NK cell effector responses,<sup>32,33</sup> we interpreted the reduction in mCD16<sup>+</sup> NK to be an increase NK activation. We found that the reduction in NK mCD16 level in NC was rescued in MNC, and again to a higher extent for myco<sup>+</sup> CML than for myco<sup>-</sup> CML cultures, suggesting a suppression of NK activation in MNC compared with NC. We also observed a reciprocal pattern for CD16<sup>-</sup> NK (Figure 5b and Supplementary figure 14b). In summary, Mφ mediated a rescue of NK mCD16 level in MNC trioculture.

The increase in shedding of CD16 from NK indicates NK activation, whereas a decrease in shedding indicates

suppression of NK activity. NK mCD16 is regulated post-translationally by metalloproteinases (MMPs, also functioning as sheddase), which mediates cleavage upon activation of NK.<sup>33–36</sup> Hence, we queried whether the Mφ-mediated perturbations in NK mCD16 level were due to modulation of NK CD16 shedding.<sup>32</sup> As a proxy for MMP–sheddase activity, we measured changes in the expression level of membrane CD62L (mCD62L) on NK cells, a sheddase substrate that is often comodulated with CD16.<sup>32</sup> We found a significant decrease in NK mCD62L in NC duo culture (Supplementary figure 15), corroborating the drop in NK mCD16 (Figure 5a), and a recovery of NK mCD62L when in MNC trioculture compared with NC duo culture, and this was consistent with the rescue of NK mCD16 level in MNC. The data for NK mCD62L appeared to suggest the potential



**Figure 5.** Natural killer (NK) cell membrane CD16 (mCD16) level was maintained *in vitro* in MNC trioculture under infection–inflammation condition of chronic myeloid leukemia (CML) cells and *in vivo* in CML patients. **(a, b)** NK, macrophage (Mφ) and myco<sup>-</sup>/myco<sup>+</sup> CML were incubated in mono, duo and triocultures. CFSE<sup>-</sup>CD14<sup>-</sup>CD56<sup>+</sup> NK cells were gated. Percentage of **(a)** CD16<sup>+</sup> and **(b)** CD16<sup>-</sup> NK were determined on NK upon coculture with Mφ and CML. NK mCD16 level was determined specifically on CD56<sup>dim</sup> NK, which is the cytotoxic counterpart of the NK population. **(c)** NK, Mφ and myco<sup>-</sup> and myco<sup>+</sup> CML cells were incubated in mono, duo and triocultures. Culture supernatants were collected and measured for concentration (conc) of soluble CD16 (sCD16) using ELISA. For **(a–c)**, results shown are mean ± s.e.m. of three or four independent experiments (donors). See Supplementary figure 14 for individual replicate experiments. **(d)** % mCD16<sup>+</sup> of NK cells in bone marrow exudates of CML patients at various stages (CP, AP and BC) compared with that in control, which represents non-CML patients' bone marrow. Each data point represents information extracted from one patient. Closed circles represent non-CML orthopedic controls; closed squares represent CP patients; closed triangles represent AP/BC patients. Control patients  $n = 5$ ; CP chronic phase patients  $n = 4$ ; AP/BC accelerated phase/blast crisis patients  $n = 5$ . All statistical significance was determined using repeated measures one-way ANOVA followed by Tukey's test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . AP, accelerated phase; BC, blast crisis phase; CFSE, carboxyfluorescein succinimidyl ester; CP, chronic phase; MN, Mφ + NK; MNC, Mφ + NK + CML; N, NK alone; NC, NK + CML; ns, nonsignificant.

involvement of MMP–shedase in modulating the NK mCD16 level. Therefore, we measured soluble CD16 (sCD16) in the culture supernatants of the cocultures of NK, M $\phi$  and myco<sup>+</sup> CML. Consistent with reduced mCD16 level in NC compared with N alone (Figure 5a), a trend toward increased CD16 shedding was observed (Figure 5c and Supplementary figure 14c). Owing to the rescue of NK mCD16 in MNC compared with NC (Figure 5a), we further envisaged a decrease in the level of NK sCD16 shed into MNC culture supernatant compared with that in NC. Instead, we observed a further increase in sCD16 in MNC compared with NC (Figure 5c). It is plausible that despite continuous shedding of CD16 from NK in MNC triocultures, the level of mCD16 on NK was actively maintained.

Both the M $\phi$  and NK could have contributed to the pool of sCD16 in the culture supernatant, as evidenced by the increased shedding of sCD16 in (1) NC compared with N and (2) MC compared with M (Figure 5c). Thus, we queried whether the surge in sCD16 detected in MNC (compared with MC or NC) was solely resulting from M $\phi$ . We observed that the shedding of M $\phi$  sCD16 in the MC culture supernatant could also be attributed to the increase in mCD16 expression on M $\phi$  in MC compared with that in M (Supplementary table 3). Interestingly, there was no further increase in the mCD16 level on M $\phi$  in MNC compared MC (Supplementary table 3). Hence, we reasoned that the increased level of sCD16 in the MNC trioculture supernatant is attributed to the combined shedding of CD16 from both M $\phi$  and NK.

In MNC, the NK mCD16 level was not downregulated and was maintained compared with baseline (N alone sample; Figure 5a). Such maintenance in the NK mCD16 level was also observed on the NK cells from CML patients' bone marrow compared with nonleukemia individual's bone marrow, regardless of the stage of CML (Figure 5d). Taken together, we propose that mycoplasma-infected CML cells likely escape NK cytotoxicity through M $\phi$ -mediated suppression of NK activity, which is associated with the maintenance of the NK mCD16 level.

#### **M $\phi$ protection of CML from NK killing was independent of tissue inhibitor of metalloproteinase 1**

To understand how M $\phi$  mediates the maintenance of NK mCD16 in MNC trioculture, we investigated the potential of tissue inhibitor of metalloproteinase (TIMP) in modulating the NK mCD16 level. TIMPs are endogenous inhibitors of MMP–shedase, which are involved in post-translational regulation of the NK mCD16 level.<sup>33,34</sup> TIMPs are also detected in mononuclear cells isolated from myeloid leukemia

patients<sup>37</sup> and in M $\phi$ .<sup>38</sup> We therefore sought to verify whether modulation of shedase activity by TIMPs may have an influence on NK activation. We first determined the level of M $\phi$  TIMP in cocultures with NK and myco<sup>+</sup>/myco<sup>-</sup> CML (Supplementary figure 16a). Interestingly, M $\phi$  TIMP-1 expression was increased in MNC only under the myco<sup>+</sup> condition. Thus, we asked whether manipulation of the level of TIMP-1 secreted by M $\phi$  would influence the observed M $\phi$  protection of myco<sup>+</sup> CML from NK cytotoxicity. We neutralized the secreted TIMP-1 with TIMP-1 neutralizing antibody (refer to Supplemental methods) in MNC and determined changes in M $\phi$  protection of myco<sup>+</sup> CML (Supplementary figure 16b, white bars). Comparing between MNC trioculture treated with TIMP-1 neutralizing antibody and trioculture treated with immunoglobulin G (IgG) isotype control, no difference in CML survival was observed. Because it has been suggested that M $\phi$  suppresses NK activity via TGF $\beta$ ,<sup>15,17</sup> we also treated MNC trioculture with TGF $\beta$  neutralizing antibody, which showed an observable but insignificant decrease in CML survival compared with IgG isotype control-treated trioculture (Supplementary figure 16b, gray bars). Taken together, the data suggest that the maintenance of NK mCD16 level is independent of TGF $\beta$  and M $\phi$  TIMP-1 modulation of MMP–shedase activity.

#### **M $\phi$ protection of CML and maintenance of NK CD16 level was contact dependent**

We further clarified whether the mechanism of M $\phi$  protection of CML from NK cytotoxicity was contact dependent. We disrupted contact between M $\phi$  and NK + CML by using transwell assays. Comparing Figure 6a, b (also Supplementary figure 17a, b), we found that the M $\phi$  protection of CML from NK cytotoxicity was abrogated in the presence of transwell, as there was no increase in CML survival in MNC compared with NC. However, with myco<sup>-</sup> CML, we observed no difference in M $\phi$  protection, regardless of contact (no transwell; Figure 6c and Supplementary figure 17c) or without contact (with transwell; Figure 6d and Supplementary figure 17d).

Because we have shown the association between the M $\phi$ -protective effect of CML cells and the maintenance of NK CD16 level (Figure 5), we further queried whether the M $\phi$ -mediated maintenance of NK CD16 level was contact dependent. We performed the same transwell assay and measured the percentage of CD16<sup>+</sup> NK cells in cocultures of NK, M $\phi$  and myco<sup>+</sup>/myco<sup>-</sup> CML. We found that the maintenance of NK CD16 level in MNC trioculture (Figure 6e and Supplementary figure 17e) was abolished when the contact interaction between M $\phi$  and NK-CML was disrupted (Figure 6f and Supplementary figure 17f), suggesting that M $\phi$ -mediated maintenance of

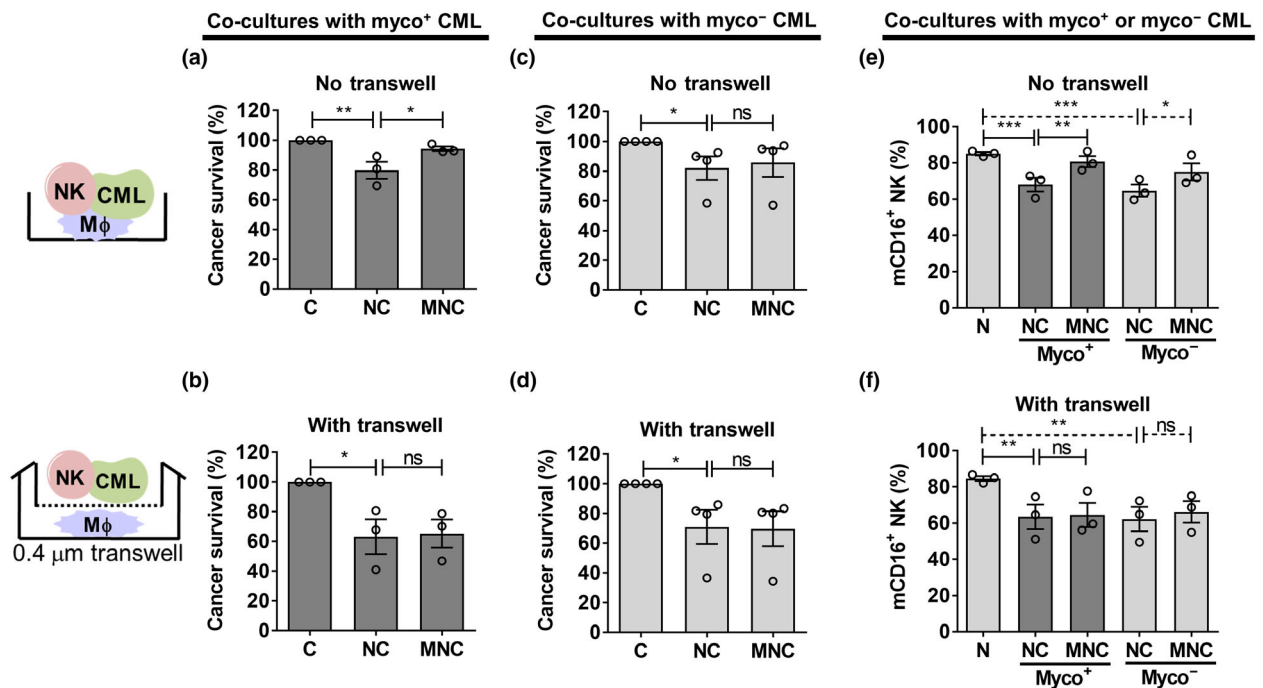


NK CD16 level was contact dependent. We therefore propose that under the inflammatory condition induced by mycoplasma infection, the CML cells escape NK cytotoxicity through M $\phi$ -mediated maintenance of NK mCD16 level in a contact-dependent manner.

## DISCUSSION

We aimed to understand the influence of M $\phi$  and NK cells on CML survival, particularly on how M $\phi$  modulates NK cytotoxicity. We focused on CML because of the lack of studies on M $\phi$ -NK interactions in hematological tumors, and that solid tumors are well known to be resistant to immunotherapeutic efforts, whereas hematological malignancies are responsive owing to the accessibility of immune cells to the leukemic cells.<sup>39,40</sup> Moreover, we found an increase in the ratio of innate to adaptive immune cells in CML bone marrow compared with nonleukemia controls, further prompting us to focus our investigation of M $\phi$  and NK cells on CML.

The key findings that led us to propose the mechanism for M $\phi$  protection of CML from NK cytotoxicity are shown in Figure 7. M $\phi$  protection was demonstrated when the reduction in cancer survival between NC and C (i.e. NK killing) was attenuated in the presence of M $\phi$  (in MNC trioculture). The attenuation was only observed in MNC for myco<sup>+</sup> K562 CML cells. When myco<sup>+</sup> K562 CML cells were treated with ciprofloxacin to eliminate mycoplasma infection, the attenuation of NK killing was not observed in MNC. These two observations led us to reason that mycoplasma infection prompted M $\phi$ -mediated protection of CML from NK killing. We found that M $\phi$  protected mycoplasma-infected CML from NK cytotoxicity through attenuating NK degranulation. We further observed that the protection of CML from NK killing in MNC trioculture was associated with the maintenance of NK mCD16 level in a contact-dependent manner. This suggested that M $\phi$  in the MNC culture might have signaled the restoration of NK mCD16 level, leading us to inquire how modulation of mCD16 might influence the NK activation level.



**Figure 6.** Macrophage (M $\phi$ ) protection of chronic myeloid leukemia (CML) from natural killer (NK) cell cytotoxicity was abrogated when cell–cell contact interactions were disrupted. Transwell assay was performed as illustrated in this figure. **(a, b)** M $\phi$ , NK cells and myco<sup>+</sup> CML cells were cocultured in the presence and absence of transwell for 24 h. CML survival was then assessed based on negative staining for viability dye and normalizing to cancer-alone control. **(c, d)** M $\phi$ , NK cells and myco<sup>-</sup> CML were assessed as described in **a** and **b**. For **a–d**, see Supplementary figure 17 for individual replicate experiments. **(e, f)** M $\phi$ , NK cells and myco<sup>+</sup> or myco<sup>-</sup> CML were cocultured in the presence and absence of transwell for 24 h and then assessed for percentage mCD16<sup>+</sup> NK cells by flow cytometry. Results shown are mean  $\pm$  s.e.m. of three or four independent experiments (donors). Statistical significance was determined using repeated measures one-way ANOVA followed by Fisher's least significant difference test (**a–d**) or Tukey's test (**e, f**). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . C, CML alone; mCD16, membrane CD16; MC, M $\phi$  + CML; MNC, M $\phi$  + NK + CML; N, NK alone; NC, NK + CML; ns, nonsignificant.

To determine whether M $\phi$  protection of mycoplasma-infected CML from NK cytotoxicity is specific to inflammation induced by mycoplasma, we have further used LPS and poly(I:C) to induce a “general”/sterile inflammation. We found that M $\phi$  did not protect LPS- or poly(I:C)-treated CML from NK cytotoxicity, further suggesting that M $\phi$ -mediated protection might be specific to inflammation induced by mycoplasma infection. Future studies may involve the use of other chronic infectious pathogens such as lymphocytic choriomeningitis virus,<sup>41</sup> and bacteria associated with persistent infections<sup>42</sup> to determine whether the phenomenon extends beyond mycobacterium-induced inflammation.

The loss of CD16 (cleavage) in activated NK cells was reported.<sup>33</sup> Hence, by extension, the maintenance of mCD16 could be viewed as a “by-product” of a diminished activation status,<sup>33</sup> rather than a mechanism of active repression. However, if the modulation of CD16 is a by-product of NK activation, we would expect that when NK is activated to kill CML cells (in NC duo culture), the level of sCD16 would increase, and when NK was suppressed by M $\phi$  from killing CML (in MNC trio-culture), the sCD16 level would decrease compared with NC. However, the latter was not observed in our study. With reference to Figures 5a, c, the anticipated decrease in sCD16 in MNC compared with NC did not occur. Instead, a further increase in sCD16 level (threefold in MNC compared with NC) was detected in the culture supernatant. As such, the NK activation level did not concur with the level of NK CD16 shedding. This led us to propose that the maintenance of the level of NK CD16 (despite active shedding) could be a mechanism through which NK activation is suppressed.

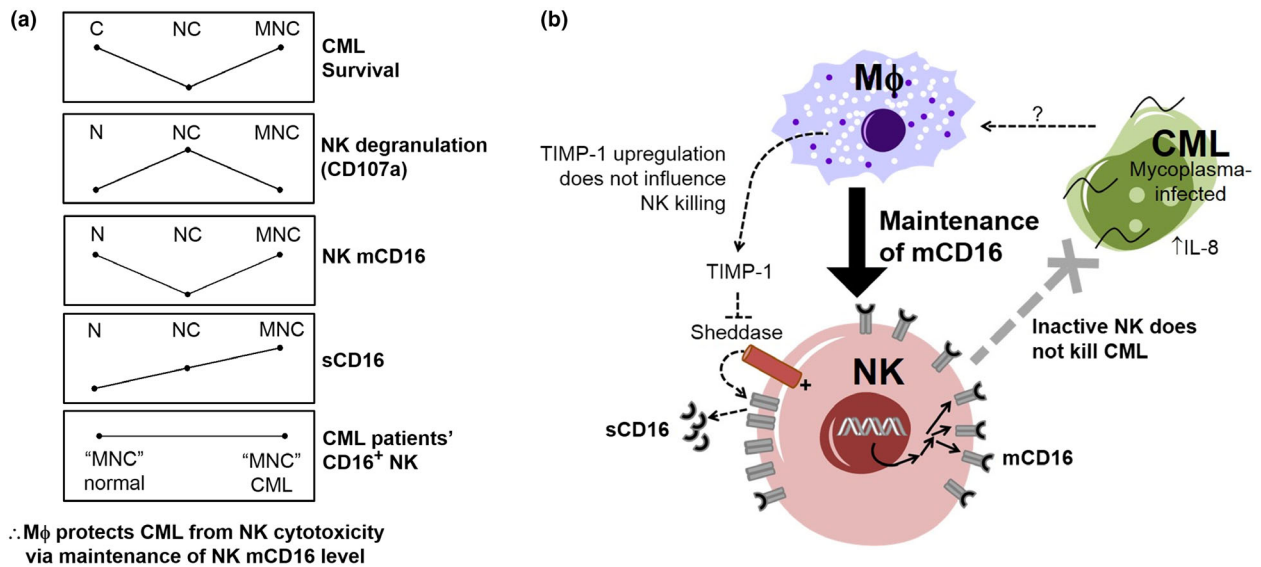
There are several proposals on correlations of NK mCD16 with NK activation level. First, it was recently reported that the presence of CD16 on NK membrane at the immunological synapse results in a tight binding between the effector (NK) and the target (cancer).<sup>5</sup> The cleavage of CD16 is mandatory for NK to be freed to attack its next target. Therefore, the maintenance of NK mCD16 level would lower NK cytotoxicity. Second, it was proposed that CD16 couples CD3 $\zeta$ , an adaptor molecule associated with the CD16 cytoplasmic domain, to CD2, an adhesion molecule capable of stimulating NK activation.<sup>6</sup> The cleavage of NK mCD16 was proposed to facilitate the coupling of CD3 $\zeta$  to CD2 molecule, which consequently enhances NK activation via CD2.<sup>43</sup> Thus, the maintenance of NK mCD16 level would presumably attenuate NK activation. However, we did not observe changes in the phosphorylation level of CD3 $\zeta$  in NK cells under mono, duo and triocultures. Future investigations

into the potential of these CD16 post-translational regulations in modulating NK activation would be beneficial.

The mechanism of M $\phi$ -mediated maintenance of NK mCD16 level is hitherto underappreciated. A plausible mechanism is through the upregulation of NK mCD16 expression in NK cells, although further exploration is warranted because the transcriptional regulation of NK CD16 expression through epigenetic modification or cytokine treatment (e.g. TGF $\beta$ -mediated downregulation)<sup>44,45</sup> was only recently investigated. We observed that M $\phi$  protected CML via a TGF $\beta$ -/TIMP-1-independent pathway. However, we found that the M $\phi$ -protective effect was contact dependent, suggesting the plausible involvement of membrane-bound factors on M $\phi$  in the modulation of NK cytotoxicity and M $\phi$ -protective effect on CML. It was reported that M $\phi$  suppressed NK activity through ligand–receptor interactions via HLAG-ILT2 and CD48-2B4,<sup>17,18</sup> but whether these interactions influence the level of NK mCD16 remain to be determined.

M $\phi$  only conferred protection on myco<sup>+</sup> CML but not on myco<sup>-</sup> CML, suggesting that the signal from mycoplasma or the mycoplasma-modulated CML “educates” M $\phi$  to confer protection on CML from NK cytotoxicity. One such potential signal was purported to be IL-8, which was the key cytokine induced in the myco<sup>+</sup> CML cells (Figure 1b). However, neutralizing IL-8 in MNC trioculture did not modulate the M $\phi$ -protective effect on mycoplasma-infected CML cells. Thus, other signals contributing to M $\phi$  protection of CML may need to be tested in future, for example, IL-4 and IL-13, which were reported to polarize M $\phi$  into immunosuppressive protumor M2 subtype and separately, reported to be induced upon mycoplasma infection.<sup>12,46</sup> Nevertheless, we observed that disruption of contact between M $\phi$  from CML and NK cells in transwell assays abrogated mCD16 maintenance and M $\phi$ -mediated protection. Therefore, it appears that the effect of M $\phi$ -mediated protection is contact dependent, perhaps through membrane ligand–receptor interactions, rather than being primarily reliant on soluble factors.

Based on our findings, we propose a mechanism of M $\phi$  suppression of NK cytotoxicity on CML under infection–inflammation condition, through maintenance of the NK mCD16 level. While the current study has focused on mycoplasma infections in CML, it aims to suggest the broader concept that infection-induced inflammation provokes cancer progression through M $\phi$ -mediated NK repression. The findings from this study hope to prompt further similar studies involving other bacterial infections and cancers.



**Figure 7.** Proposed mechanism of macrophage (Mφ) inhibition of natural killer (NK) cell cytotoxicity through modulation of NK membrane CD16 (mCD16) level. **(a)** Comparison of trends of chronic myeloid leukemia (CML) survival relative to NK activation (based on degranulation marker, CD107a), NK mCD16 level, soluble CD16 (sCD16) and NK CD16<sup>+</sup> cells in CML patients' bone marrow. The protection of CML survival in MNC tri-culture is associated with reduced NK degranulation and maintenance of NK mCD16 level. **(b)** A hypothetical model of NK mCD16 maintenance as a mechanism of Mφ-mediated suppression of NK activation, and hence, protection of myco<sup>+</sup> CML. We have also found that maintenance of NK mCD16 level is independent of Mφ tissue inhibitor of metalloproteinase 1 (TIMP-1) modulation of NK sheddase activity. It was proposed that modulation of NK mCD16 influences NK activation level via either regulation of NK attachment and detachment from target cells or activating mediators in NK activation pathway. IL, interleukin.

## METHODS

### Isolation of primary cells and Mφ differentiation

Apheresis cone from healthy donors were acquired upon donor's consent from Health Sciences Authority (HSA, Singapore) under approved HSA and NUS-Institutional Review Board protocols (201706-06 and H-17-028, respectively). Peripheral blood mononuclear cells were isolated using Ficoll-Paque (GE Healthcare, Chicago, IL, USA) gradient centrifugation. Peripheral blood mononuclear cells were apportioned for monocytes and NK enrichment. NK and monocytes were enriched from peripheral blood mononuclear cells using magnetic negative selection following manufacturer's protocol (Stem Cell Technologies, Vancouver, Canada). Enriched NK was kept frozen until autologous monocytes were differentiated into Mφ. Enriched monocytes were differentiated into Mφ by 7-day treatment with 75 ng mL<sup>-1</sup> recombinant human macrophage colony-stimulating factor (Thermo Fisher Scientific, Waltham, MA, USA) in Roswell Park Memorial Institute medium-1640 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (GE Healthcare), 1% v/v penicillin–streptomycin (Thermo Fisher Scientific), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids (Sigma-Aldrich, St Louis, MO, USA).

### Patients' samples

Acquisition of patients' samples was approved by Domain Specific Review Board (ref no: 2016/00698). All patients/

representatives gave written informed consent according to the Domain Specific Review Board guidelines. Deidentified CML patients' samples were acquired from the Cancer Science Institute of Singapore Tissue Bank. Deidentified nonleukemia bone marrow samples from total knee arthroplasty procedures were acquired from the Department of Orthopaedics, National University Hospital, Singapore. Peripheral blood mononuclear cells were extracted using Ficoll-Paque gradient centrifugation and were frozen prior to being used for polychromatic flow cytometry staining.

### Cell culture

CML cell lines (K562 and KCL-22) were cultured in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum and penicillin–streptomycin at 37°C in 5% CO<sub>2</sub> under humidified conditions.

### PCR test for mycoplasma

The presence of mycoplasma was generally detected by PCR,<sup>47</sup> using forward and reverse primers listed in Supplementary table 1. A sample was determined to be mycoplasma positive (myco<sup>+</sup>) on detection of a 500-bp band. The specific species of mycoplasma were determined using species-specific primers, also listed in Supplementary table 1. Culture supernatants were heated prior to PCR. PCR products were electrophoresed on 1.5% agarose gel and visualized using gel imager (Bio-Rad Laboratories, Hercules, CA, USA).

### Mycoplasma infection and ciprofloxacin treatment of CML cells

To infect clean CML cultures with mycoplasma, 30% noninfected CML culture medium was replaced with mycoplasma-containing medium and treated for 1, 3, 5 and 7 days. To clear infected cultures of mycoplasma, CML cultures were replaced with culture medium containing  $10 \mu\text{g mL}^{-1}$  ciprofloxacin and treated for 2, 4 and 6 days. The cell cultures were replaced with clean medium post-treatment.

### ELISA

Culture supernatants from the CML cells were tested for IL-6, IL-8, TNF $\alpha$  and IL-10 according to manufacturer's protocol (Becton Dickinson, Franklin Lakes, NJ, USA). The detection limits of IL-6, IL-8, TNF $\alpha$  and IL-10 were 4.7, 3.1, 7.8 and  $7.8 \text{ pg mL}^{-1}$ , respectively.

### Soluble CD16 measurement

sCD16 was measured in cell coculture supernatants following a reported protocol.<sup>33</sup> In brief, 96-well microplate (NUNC MaxiSorp, NUNC, Rochester, NY, USA) was coated overnight at  $4^{\circ}\text{C}$  with  $100 \mu\text{L}$  of  $10 \mu\text{g mL}^{-1}$  anti-CD16 (Becton Dickinson, clone 3G8) in phosphate-buffered saline. The plates were washed with phosphate-buffered saline containing 0.05% Tween-20 and blocked with  $200 \mu\text{L}$  phosphate-buffered saline containing 2% bovine serum albumin at  $37^{\circ}\text{C}$  for 1 h. Subsequently,  $100 \mu\text{L}$  samples/standards (Thermo Fisher Scientific) were incubated for 2 h at room temperature. The plates were then incubated with  $100 \mu\text{L}$  of  $0.5 \mu\text{g mL}^{-1}$  of biotinylated anti-CD16 (Bio-Rad Laboratories, clone DJ130c) for 1 h at room temperature. About  $100 \mu\text{L}$  of streptavidin-horseradish peroxidase (BioLegend, San Diego, CA, USA) was added for 1 h at room temperature in the dark. Finally, tetramethyl benzidine/ $\text{H}_2\text{O}_2$  substrate (Sigma-Aldrich) was added and incubated for 30 min at  $37^{\circ}\text{C}$ . The reaction was terminated with  $50 \mu\text{L}$  of  $1 \text{ M H}_2\text{SO}_4$  and absorbance at 450 nm was read with correction at 570 nm using a microplate spectrophotometer (BioTek, Winooski, VT, USA).

### NK cytotoxicity assay

CML cells were stained with  $10 \mu\text{M}$  carboxyfluorescein succinimidyl ester (Sigma-Aldrich) for 30 min at  $37^{\circ}\text{C}$ . Following coculture (of CML, M $\phi$  and NK), cells were stained with viability dye [7-aminoactinomycin D or fixable viability dye; Thermo Fisher Scientific] before analysis using flow cytometry. All carboxyfluorescein succinimidyl ester<sup>+</sup> events represented CML. To determine the specific % cancer cell survival, the following formula was used: % cancer cell survival = [(number of carboxyfluorescein succinimidyl ester<sup>+</sup>fixable viability dye<sup>-</sup> events)/(total number of carboxyfluorescein succinimidyl ester<sup>+</sup> events)]  $\times$  100%.

### Polychromatic flow cytometry

Following coculture (of CML, M $\phi$  and NK), cells were stained with fixable viability dye. Then, the cells were blocked with Fc

receptor inhibitor (Thermo Fisher Scientific), stained with primary conjugated antibodies (Supplementary table 2) and fixed with 4% paraformaldehyde. For staining intracellular proteins, IFN $\gamma$ , TNF $\alpha$ , MIP-1 $\alpha$  and TIMP-1, the coculture samples were incubated with GolgiPlug (Becton Dickinson) 4 h prior to immunostaining. Cells were fixed and permeabilized using a Fixation/Permeabilisation Solution Kit (Becton Dickinson) and stained with primary conjugated antibodies (Supplementary table 2).

### NK degranulation

Coculture (of CML, M $\phi$  and NK) was performed for 24 h. At the last 4 h of the coculture, anti-CD107a (Supplementary table 2) and protein transport inhibitor, monensin (Thermo Fisher Scientific), were added. The standard 4-h degranulation assay was also performed, where anti-CD107a and monensin were added to the cocultures and incubated for 4 h.<sup>31,48</sup> At the end of the assay, cells were harvested and processed for flow cytometry.

### Statistical analysis

At least 5000 events of the flow cytometry-gated populations were collected. Flow cytometry data were acquired on BD LSRFortessa and analyzed using FlowJo V10 software. Statistical tests were performed using GraphPad Prism 8.0.2 and described in respective figure legends. *P*-value < 0.05 was determined to be significant. Overall results were assessed based on reproducible statistical trends across at least three independent experiments.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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