Impact Of an Immune Modulator Mycobacterium-w On Adaptive Natural Killer Cells and Protection Against COVID-19

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Summary

The kinetics of NKG2C⁺ adaptive natural killer (ANK) cells and NKG2A⁺ inhibitory NK (iNK) cells with respect to the incidence of SARS-CoV-2 infection were studied in a cohort of health-care workers following administration of heat killed *Mycobacterium w* (Mw group) in comparison to a control group. COVID-19 infection correlated with a lower NKG2C⁺ ANK cells at baseline. NKG2C⁺ ANK cells at baseline did not differ, but there was a significant upregulation of NKG2C expression and cytokine release in the Mw group (p=0.009), particularly in those with lower baseline NKG2C expression (<15%), along with marked downregulation of NKG2A⁺ iNK cells (p<0.0001), and an increase in the NKG2C⁺ ANK/NKG2A⁺ iNK ratio. This translated to a significant reduction in COVID-19 and its severity in the Mw group. No impact was observed on T cell subsets. Mw was observed to have a salutary impact on the ANK cell profile which might have provided protection against COVID-19 over a prolonged period in a non-immune high-risk population.
Introduction

The rapid explosion of the novel coronavirus, SARS-CoV-2, in early 2020, across the globe overwhelmed even the most prepared health infrastructures (Blumenthal et al., 2020) and exposed the healthcare workers to an unforeseen situation, where they remained at the greatest risk of exposure to the highest viral load, in the absence of prevention or cure.

Despite a very high incidence of infections, witnessed in the Indian population as well, there was a surprising sparing of the urban slum-dwellers (Kaushal and Mahajan, 2021). We had hypothesized regarding the role of a bolstered innate immune system secondary to chronic pathogen exposure as a plausible reason for this phenomenon. The hypothesis proposed that due to an almost ubiquitous exposure to cytomegalovirus (CMV) in early childhood, there might be stronger repertoire of NKG2C expressing adaptive natural killer (ANK) cells in this cohort (Jaiswal et al., 2020c).

Early CD56bright NK cells have very high expression of NKG2A, which functions as an inhibitory check-point in the process of functional maturation of NK cells (Moretta et al., 2011). Both NKG2C and NKG2A bind to the same ligand, HLA-E, but the latter binds with several-fold greater affinity compared to NKG2C (Beziat et al., 2011). Unlike somatic mutations witnessed in T and B cells to produce precise and clonal antigen specificity, NK cells express a plethora of germline encoded activating and inhibitory receptors. The regulation of NK cell function, which is termed as ‘licensing’, occurs with stochastic expression of killer-immunoglobulin-like receptors (KIR), which bind to self- HLA-Class 1 antigens with biallelic specificity (Beziat et al., 2010). Expression of suitable KIRs for which appropriate self-HLA antigens exist enable continued inhibition of NK cells preventing autologous cytotoxicity. Thus, NKG2A+ inhibitory NK cells (iNK) are key to the prevention of autoreactivity of NK cells, prior to the KIR-driven process of licensing. In a subset of mature and licensed NK cells, exposure to CMV leads to the expression of a C-lectin type
activating receptor, NKG2C, which is encoded by the KLRC2 gene (Lopez-Verges et al., 2011). These cells are characterized by upregulation of NKG2C (ANK) and downregulation of the inhibitory counterpart, NKG2A (Della Chiesa et al., 2015). This subset of NK cells, now called NKG2C^+ ANK cells, exhibits the classic adaptive features, such as, clonal expansion, persistence and recall memory more akin to memory cytotoxic T cells, than canonical NK cells (Sun and Lanier, 2009).

While the major subset of ANK cells express NKG2C, which is the phenotypical defining feature, several myeloid (FCERG, SYK, EAT-2, PLZF16) and B lymphoid (SYK, EAT-2) genes are downregulated, and certain T lymphoid genes (CD3e, BCL11B) are upregulated in ANK cells (Schlums et al., 2015). In a small subset of ANK cells, the adaptive functions might be demonstrable with epigenetic distribution of myeloid and lymphoid associated genes as described above, even without upregulation of NKG2C expression (Muccio et al., 2018; Schlums et al., 2015). Hence, for the sake of clarity, we shall describe ANK cells in the context of this study as NKG2C^+ ANK cells.

In the context of haploidentical hematopoietic cell transplantation (HCT), NKG2C^+ ANK cells were found to afford protection, not only against leukemia, but also a range of viral infections, other than CMV. It was suggested that high NKG2C^+ ANK cells was essential in maintaining a non-inflammatory milieu without compromising anti-tumor effect post-HCT (Jaiswal et al., 2020a; Jaiswal et al., 2020b; Jaiswal and Chakrabarti, 2019; Jaiswal et al., 2021b). Based on these seminal findings along with the existing evidence that certain natural infections, such as Hantavirus, as well as Influenza and BCG vaccinations are capable of upregulating NKG2C^+ ANK cells in CMV-exposed populations (Abebe, 2021; Bjorkstrom et al., 2011; Sun and Lanier, 2018), we hypothesized that a novel heat killed *Mycobacterium w* (Mw), also known as *Mycobacterium indicus pranii*, an approved immunomodulator in India for adjuvant treatment of sepsis (Sehgal et al., 2015), might upregulate NKG2C^+ ANK
cells and offer protection against SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) infections in the process. We studied the impact of Mw on the incidence of COVID-19 (Corona Virus Disease 2019 caused by SARS-CoV-2) in a cohort-control study in front line health care workers and its impact on the kinetics and repertoire of NKG2C+ANK cells. in the context of KLRC2 genotype.

Results

Mw and COVID-19 infection

The characteristics and outcomes of the Mw and control groups are detailed in Table 1. All subjects were seropositive for CMV.

Twenty (3 in Mw group vs 17 in the control group) out of 100 subjects in the entire cohort had symptomatic COVID-19 infections during the study period. All infections on the Mw group had mild COVID-19 infection while 6 of 17 in control group had moderate disease and required hospital admission (p=0.01). No deaths were recorded in either group. In Mw group all three of the infections occurred beyond 150 days. In the control group, 17 subjects had COVID-19 infection at a median of 56 days (range, 18–135). Additional three mild symptomatic COVID-19 infections were documented within the first 7 days of Mw administration (Figure 1).

The incidence rates (IR) of COVID-19 were 3.57 and 24.05 /10,000 person-days in Mw and control groups respectively (IRR-0.15, 95% CI, 0.04-0.5, p=0.0004) with an efficacy rate for Mw being 82.35%, (95% CI, 50-96 %).

Thirty-three subjects fulfilled the criteria for randomization to receive a second dose of Mw. However, 24 consented for randomization and 12 eligible subjects were randomized in 1:1 to receive or not receive the second dose, 30 days of first dose. Impact of the second dose on protection could not be analysed as none of the 24 randomised subjects did not develop COVID-19 during the study period.
Mw and ANK cells

Thirty subjects from the Mw group and 15 from the control group were included in the final longitudinal analysis of immunological impact of Mw.

Baseline ANK levels did not differ, but ANK Kinetics at 60 days were different in the Mw group

There was no difference in overall NK cells between the two groups at baseline or at subsequent time-points. NKG2C⁺ANK and NKG2A⁺iNK cells expression were also similar at baseline in both groups (Figure 2A and 2B). The data from control group shown in further comparisons only pertains to the baseline and day 60, as the values in the control group for both NKG2C⁺ ANK cells and NKG2A⁺ iNK cells did not change significantly at any of the time-points.

However, only in the Mw group (Figure 2A-2D), NKG2C⁺ANK cells increased from baseline from 23.0 ± 22.9 to 25.23 ± 21.08 on day 30 (p=0.69, Figure 2C) and 40.9 ± 16.5 day 60 (p=0.0009, Figure 2C), and NKG2A⁺iNK cells reduced from 36.23 ± 14.4 to 26.22 ± 14.04 on day 30 and 6.17 ± 6.01 on day 60 (p<0.0001, Figure 2D). The absolute values as well as the log two-fold change (log2FC) were significantly different for both NKG2C⁺ANK cells (1.5 ± 1.9 vs -0.62 ± 0.89, p=0.0002, Figure 2E) as well as NKG2A⁺iNK cells (-2.9 ± 1.2 vs 0.17 ± 0.29, p<0.0001, Figure 2E) cells at day 60.

The impact of Mw on NKG2C upregulation was only observed in those with low ANK cells at baseline

A cut-off of 15% was established for NKG2C⁺ANK cells based on recursive partitioning for evaluating effect of base line value on NKG2C upregulation. Upregulation of NKG2C⁺ ANK cells was seen in those with NKG2C⁺ ANK levels below 15% (Low ANK group) [4.7 ± 2.2] on day 30 (8.9 ± 4.9, p=0.005, Figure 3A) as well as day 60 (43.3 ± 19.12, p<0.0001, Figure 3A).
In those with NKG2C\(^+\)\text{ANK} levels above 15\% (High NKG2C\(^+\)\text{ANK}), there was no significant change in NKG2C\(^+\)\text{ANK} cells at either day 30 (41.5 ± 18.02, \(p=0.97\), Figure 3B) or day 60 (38.54 ± 14.5, \(p=0.65\), Figure 3B). The log2FC evaluation revealed positive impact of Mw irrespective of baseline value. Even though Mw had a positive impact on log2FC in the High ANK group, compared to that in the control group (-0.1 ± 0.7 vs -0.8 ± 0.9, \(p=0.038\), Figure 3E), the effect of Mw on NKG2C\(^+\)\text{ANK} cells was predominantly seen in the Low NKG2C\(^+\)\text{ANK} group. (3.08 ± 4.4 vs -0.4 ± 0.9, \(p<0.0001\), Figure 3E).

**The impact on NKG2A\(^+\)\text{iNK} downregulation was irrespective of baseline NKG2C\(^+\)\text{ANK} expression**

The downregulation of NKG2A\(^+\)\text{iNK} cells was noted in the Mw group, irrespective of baseline NKG2C expression [Figure 3C and D]. The downregulation of NKG2A\(^+\)\text{iNK} cells was significant at day 60 in the Mw group, irrespective of baseline NKG2C expression (-2.99 ± 1.21 vs 0.23 ± 0.23, \(p<0.0001\) for Low ANK group and -2.84 ± 1.28 vs 0.09 ± 0.35, \(p<0.0001\) for High ANK group) [Figure 3F]. However, this was significantly downregulated (\(p=0.005\)) at day 30 in the Low NKG2C group only. [Figure 3A].

**Mw had a sustained effect on NKG2C\(^+\)\text{ANK}/NKG2A\(^+\)\text{iNK} ratio until 100 days**

NKG2C\(^+\)\text{ANK}/NKG2A\(^+\)\text{iNK} ratio was similar between two groups at baseline. However, this increased in the Mw group at day 60 (12.3 ± 9.2, \(p<0.0001\)) and persisted until day 100 (7.44 ± 13.55, \(p=0.01\)). This was witnessed for both Low and High ANK group in the Mw cohort but not in the control cohort. [Figure 4A-4D].

**Baseline NKG2C\(^+\)\text{ANK} cells correlated with increased risk of COVID-19 infections in both Mw and Control groups**

Baseline data on immune parameters was available in 60 subjects in the overall cohort, 30 in control and 30 in Mw group. The NKG2C\(^+\)\text{ANK} cells in those who got infected (n=16) was
7.9 ± 6.1, (includes 3 who got infected within 7 days of receiving Mw) compared to 19.1 ± 13.1 in those who remained uninfected (p= 0.002).

Amongst the 30 subjects in the control group, who were tested at baseline for NKG2C+ANK cells, 10 developed COVID-19. The NKG2C+ANK cells at baseline was 9.7 ± 6.6 in those with infection compared to 17.7 ± 5.56 in those without infection (p=0.0016). Those who acquired the infection in the Mw group, also had a lower baseline NKG2C+ANK (4.76 ± 3.9 vs 20.1 ± 17.3, Figure 5, p=0.04). Interestingly, 2 of the 3 subjects, experiencing COVID-19 infections beyond 150 days in the Mw group, failed to show any increment in NKG2C+ANK levels or NKG2C/NKG2A ratios at any of the time-points. We did not find any correlation between expression of NKG2A as well as NKG2C/NKG2A ratio with development of symptomatic COVID-19 in this study. KLRC2 deletion status did not predispose to COVID-19 infections in the Mw group.

**IFN-γ release was higher in Mw group at 60 days**

IFN-γ release potential of the NKG2C+ANK cells was studied at baseline and at 60 days. This was similar between the groups at the baseline (mean-6.7 vs 6.4). However, this was significantly increased in the Mw group at day 60, compared to the control group (mean-27.96 vs 9.9, p=0.01, Figure 4E).

**KLRC2 and kinetics of ANK cells**

KLRC2 deletion was studied in the Mw group only. KLRC2 Wt+/del+ was detected in 36% of the subjects in the Mw group. This was not associated with any significant decrease in the baseline NKG2C+ANK levels (15.1 ± 17.67 vs 20.4 ± 17.96, p=0.46). There was no difference in the effect of Mw between the KLRC2 Wt+/Wt+ and Wt+/Del+ groups at day 60 either (p=0.26), although the log2FC increase tended to be higher in the Wt+/Del+ group (p=0.12) [Figure S1A and 1B]. KLRC2 deletion was detected in 2 out of 6 with COVID-19, compared to 16 out of 44 without COVID-19 (p=0.6).
Mw did not influence kinetics of naïve and memory T cell subsets

CD4 and CD8 cells remained unchanged, as were the CD45RA and CD45RO subsets at days 30 and 60 in the Mw group [Figure S2A-2B]. The same was witnessed in the control group (data not shown).

NKG2A expression on CD3+ T cells was examined at baseline and subsequent timelines. Despite a significant downregulation of NKG2A observed in NK cells, no such effect was evident in the T cells. [Figure S3]

Impact of second dose of Mw on NKG2C+ANK cells

There was no difference between in NKG2C+ANK cells at day 60(33.11 ± 19.91 vs 27.01 ± 18.34, p=0.42) and day 100(19.19 ± 12.3 vs 12.86 ± 13.87, p=0.28) in double vs single doses of Mw (Figure 6A). However, NKG2A+ iNK cell expression was significantly reduced at day 100 (7.7 ± 16.77 vs 24.85 ± 19.43, p=0.04) in double dose group (Figure 6B). The NKG2C/NKG2A ratio was also significantly higher in double dose group at day 100 (10.42 ± 7.19 vs 2.08 ± 3.26, p <0.001) [Figure 6C]. There was no difference in CD4+ & CD8+ T cells and memory or naïve subsets at day 60 and 100 between the randomized groups (data not shown).

Safety Profile of Mw

Mw was safe with no systemic adverse effects. Only one subject had mild fever (37.5° C) for less than two hours, 24 hours after administration, which was self-limiting. However, 14% had pain at the local sites, with 12% developing pain and erythema lasting for more than 72 hours. In 8% of the subjects, ulceration was noted at the local site with long-term scars similar to that observed with BCG vaccination. Those with severe local reactions were all older (35 years and above), compared to the median age of the group (28 years).
Discussion

The identification of NK cells with ability to persist and mount recall responses has challenged the traditional compartmentalization of adaptive and immune responses (Lanier, 2013; O'Leary et al., 2006). The NKG2C+ANK cells explored in this study, which are CD56 dim and express NKG2C, CD16 and CD57 receptors, are known to be induced by primary exposure to CMV (Cichocki et al., 2016). Not only are these cells endowed with the enhanced potential for cytokine release and cytotoxicity, they are also noted for an enhanced antibody-dependent cytotoxicity response (Rolle et al., 2018). However, in CMV exposed patients, these NKG2C+ANK cells have been found to proliferate in response to a variety of viral pathogens, including HIV, HCV, Hantavirus, influenza, and pox viruses (Bjorkstrom et al., 2011; Mele et al., 2021; Nattermann et al., 2005; Thomas et al., 2012; Zhang et al., 2007) and is suggested for protection against COVID-19 (Jaiswal et al., 2020c; Vietzen et al., 2021).

More importantly, these NKG2C+ANK cells were shown to exhibit a strong anti-viral effect against the above pathogens (Nikzad et al., 2019). Thus, even though the inducing event for NKG2C expression might be CMV exposure, these cells have a broad spectrum of reactivity against a host of pathogens, including unrelated pathogens such as mycobacteria, candida, and plasmodium falciparum (Covian et al., 2019; Hart et al., 2019; Kleinnijenhuis et al., 2014). The unique nature of this non-antigen specific recall response against a wide variety of pathogens, puts NKG2C+ANK cells at the forefront in the battle against novel pathogens such as SARS-CoV-2, where antigen-specific adaptive response generated from T and B cells are absent at the outset of the pandemic (Jaiswal et al., 2020c).

BCG vaccine has been shown to induce long-term heterologous memory response of NK cells (Covian et al., 2019). This has been studied, both in the context of infections as well as cancer (Esteso et al., 2021). Influenza vaccination has been shown to induce proliferation of NKG2C+ANK cells with potential for enhanced cytokine release (Riese et al., 2020).
Response to influenza vaccine is also linked to upregulation of NKG2C expression on NK cells (Riese et al., 2020). These considerations prompted us to explore a novel immunomodulator, Mw, derived from heat-killed mycobacteria (Mycobacterium indicus pranii or Mycobacterium w, which has been extensively studied in leprosy [Kamal et al., 2017], tuberculosis [Gupta et al., 2012], sepsis [Sehgal et al., 2015], and some forms of cancers including BCG resistant bladder cancer [Subramaniam et al., 2016]), as prophylaxis against COVID-19. This study was meant to explore if Mw could favorably influence the repertoire of NKG2C+ANK cells and if this would protect the most vulnerable population i.e., healthcare workers, at the peak of the COVID-19 pandemic.

Prophylaxis with Mw was associated with a 6-fold reduction in the incidence of symptomatic COVID-19 in this high-risk cohort. This protective efficacy was accompanied by a sharp increase in NKG2C+ANK cells between 30- and 60-days following exposure to Mw. Interestingly, upregulation of NKG2C+ANK cells was more evident in those with low baseline expression of NKG2C. However, a more critical observation was the steeper decline in the expression of the inhibitory counterpart, NKG2A+iNK cells, which continued through 100 days. This resulted in an increasing NKG2C/NKG2A ratio through the study period.

Eligible subjects of the cohort were further randomized to receive a second dose of Mw, which resulted in a continued downregulation of NKG2A+ iNK cell and a further increase in NKG2C/NKG2A ratios in those receiving the second dose. The importance of the ratio between NKG2C and NKG2A expression on NK cells would be appreciated further in the context of the fact that the inhibitory impact of NKG2A overrides that of NKG2C, as it binds to the ligand HLA-E with several fold greater affinity than NKG2C (Beziat et al., 2011).

Thus, for NKG2C+ANK cells or even canonical NK cells to exert its cytotoxic effect, downregulation of NKG2A+ iNK cells is of paramount importance.
In the context of SARS-CoV-2 infection, in-vitro studies have demonstrated that the viral spike protein-1 (SP-1) upregulates NKG2A on NK cells and HLA-E on the infected lung epithelial cells, causing a strong inhibition of cytotoxicity of NK cells (Bortolotti et al., 2020). The expression of NKG2A in presence of SP-1 increased from 16% to 80%, resulting in significant compromise of IFN-γ release and activation. Downregulation of NKG2A has also been shown to be a marker for immunological and clinical recovery in COVID-19 (Zheng et al., 2020). Our group has demonstrated correlation between high expression of NKG2A and marked suppression of NKG2C, with adverse outcome following severe COVID-19 lung disease, despite viral clearance (Jaiswal et al., 2021a). Hence, it might be inferred that if any intervention can achieve the reverse, i.e., downregulation of NKG2A and upregulation of NKG2C expression, this might offer protection against COVID-19.

In our study, a lower baseline NKG2C+ANK cells predisposed to COVID-19 in the control group. The same in the Mw group was associated with COVID-19 in the first 2 weeks, but not thereafter until 150 days. It is also worth noting that COVID-19 occurring in the Mw group beyond 150 days was associated with failure in modulation of the adverse NKG2C+ANK cell profile in 2 out of 3 subjects, which is probably further indicative of the protective impact of a favorable NKG2C+ANK cell profile following Mw exposure. Apart from phenotypic alteration, Mw seemed to influence the cytokine release potential as well, as evidenced by an increase in IFN-γ release at day 60 in the Mw group, compared to both baseline as well as the control group. In addition, a second dose of Mw at 30 days did prolong the favorable NKG2C+ANK profile to 100 days, suggesting a booster effect on the same.

Despite favorably impacting the NKG2C+ANK cell profile, we did not observe any effect of Mw on T cell subsets in the study period. While our study did explore the effect of Mw on NKG2C+ANK and NKG2A+iNK subsets, the mechanistic pathway needs further exploration.
Prior studies on Mw have explored its effect on TLRs via the monocyte/macrophage pathway (Das et al., 2016; Kumar et al., 2015; Pandey et al., 2012). Both BCG and Mw have been shown to activate macrophages via Myd88 dependent pathways (Kumar et al., 2019). However, Mw was shown to be a stronger agonist for TLR2 than BCG and also inducing pure Th1 response. Despite limited data on TLR-based pathways operational in NK cell maturation and activation, TLR2 pathway has been shown to activate NK cells (Martinez et al., 2010; Sivori et al., 2014). In fact, it has been demonstrated that NK cell recognition of CMV might be operational via the TLR2 pathway (Szomolanyi-Tsuda et al., 2006) and this might suggest as to why Mw, being a strong TLR2 agonist might skew NK cells in favor of an adaptive pathway.

Another salient observation in our study was that the upregulation of NKG2C was dominantly noted in those with a lower baseline NKG2C+ ANK cell and this effect was only marginal in those with higher baseline NKG2C+ ANK cells. However, the downregulation of NKG2A+iNK cells was observed independent of NKG2C expression. This could be explained by the fact that upregulation of NKG2C expression might be more tightly controlled, unlike downregulation of NKG2A, in mature and licensed NK cells, where an inhibitory regulation mediated by NKG2A is physiologically redundant due to KIR-mediated inhibitory control. More importantly, this also demonstrates that downregulation of checkpoint receptor NKG2A is an important determinant of a favorable ANK profile. Further studies on transcriptional regulation of NKG2C and NKG2A might help understand this better. Thus, within the limitations of a small cohort, the findings are suggestive of a salutary effect of Mw on a favorable NKG2C+ANK profile and at the same time indicative of the fact that a favorable NKG2C+ANK profile might be protective against COVID-19.

Another study had suggested that KLRC2 deletion might predispose to severe COVID-19 (Vietzen et al., 2021). A third of the Mw cohort had KLRC2 deletion genotype and tended
to have a slightly lower baseline NKG2C+ANK cells and tended to have a greater impact of Mw on the log2FC at day 60. It is possible that the adverse effect of KLRC2 genotype was mitigated by Mw. Unfortunately, KLRC2 genotype evaluation of the control group was not part of the study. Its evaluation could have shed some light on the predisposition of KLRC2 deletion genotype if any, independent of NKG2C expression, on COVID-19. The absence of any observation on the monocyte/macrophage pathway might be deemed as another limitation of the study, particularly when a NK-monocyte crosstalk might have been at play (Michel et al., 2012). Comparison of gene expression by RNAseq on NK and monocyte subsets pre- and post- Mw might help in better understanding of this phenomenon, which is a part of our ongoing project. The suggested mechanistic pathway as to how Mw might be favorably influencing ANK mediated protection against COVID-19 has been depicted in Figure 7.

In conclusion, Mw did seem to offer protection against symptomatic COVID-19 in a high-risk population at the peak of the pandemic in the absence of antigen-specific immunity. This might have been possible through upregulation of NKG2C+ANK cell and simultaneous downregulation of NKG2A+iNK pathways, which need further exploration at a mechanistic level. If borne out in a randomized setting, these findings might usher a novel approach to bolster heterologous immunity in the current and future pandemics and identify immunological vulnerability for developing COVID-19.

**Methods**

In a single-center non-randomised cohort control study, 50 HCWs from a single department in the hospital were administered 0.1 ml Mw (Sepsivac, Cadila Pharmaceuticals, India) intradermally in each arm on day 1 of the study (Mw group) and 50 randomly selected HCWs from the rest of the institution were enrolled in a Control group. In addition, in the Mw group, those without any local site reaction, who consented for second dose, were
administered an additional dose of 0.1 ml Mw, 30 days after the first dose. The observation period of the study was 15 days after administration of the first dose of Mw to 180 days (September 2020 to February 2021).

All HCW included in the study had nasopharyngeal swab evaluated for SARS-CoV-2 by reverse transcriptase-polymerase chain reaction (RT-PCR), on development of symptoms suggestive of COVID-19 or following contact with a patient, HCW or a family member in contact with COVID-19 patient. Body temperature, pulse rate, oxygen saturation and self-reporting of symptoms was evaluated before and after each working day (Wu and McGoogan, 2020). COVID-19 was diagnosed and its severity was graded as per established criteria. The duration of observation was 6 months from September 2020 to February 2021. In addition, blood was collected, at baseline, days 30, 60, and 100 from all the subjects, for evaluating the kinetics of NKG2C^+ANK cells and T cells. All subjects of this study also underwent evaluation for CMV status (seropositive or seronegative).

All subjects provided written informed consent for participating in the study. The study was approved by institutional ethics committee and registered with Clinical Trials Registry of India (CTRI/2020/10/028326).

**Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) for Detection of SARS-CoV-2**

All Covid tests were done by Truenat Real Time Reverse Transcriptase Polymerase chain reaction (RT-PCR) test. Nasopharyngeal swabs were collected using standard nylon flocked swab and inserted into the viral transport medium (VTM) provided from the same company (Molbio diagnostics Pvt. Ltd. Goa, India). Samples were transported immediately to the laboratory maintaining proper temperature and processed as per manufacturer’s guidelines.
Pvt. Ltd. Goa, India]. The target sequence for this assay is E gene of Sarbeco virus and human RNaseP (internal positive control). Confirmatory genes were RdRP gene and ORF1A gene.

**Immunological monitoring**

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples, by density gradient centrifugations using HiSep™ LSM 1077 media. For surface staining, 0.5 × 10^6 cells were washed with phosphate-buffered saline (PBS) and stained with the following antibodies which were used for phenotypic analysis: CD3(APC-H7, SK-7) CD16 (PE-Cy7, B73.1), CD56 (APC R700, NCAM16.2), CD57 (BV605, NK-1), NKG2A (PE-Cy7, Z199), CD4 (APC-H7), CD8 (Per-CP Cy), CD45RA (FITC), CD45RO (BV605), from BD Biosciences, (San Jose, CA) and NKG2C (PE, REA205) from Miltenyi Biotec, Germany. Cells were then incubated for 30 minutes. Viability was assessed with 7-AAD viability dye (Beckman Coulter). For intracellular staining, cells were stained for IFN- gamma using monoclonal antibodies for interferon-gamma (IFN-γ) (4S.B3) and perforin (Alexa647, DG9) (BD Biosciences) after fixation and permeabilization with appropriate buffer (BD Biosciences and e-biosciences, San Diego, CA, USA). Flow Cytometry was performed using 10 colour flow cytometry (BD FACS Lyrics) and the flow cytometry data was analyzed using FlowJo software (v10.6.2, FlowJo). Unstained, single stained (one antibody/sample) as well as fluorescence-minus-one (FMO) samples were used as controls for the acquisition as well as the subsequent analysis. Statistical divergences were determined by the GraphPad Prism software.

The gating strategy has been described earlier(Jaiswal et al., 2021b). NKG2C^ANK cells were defined as CD56^{dim}NKG2C^NKG2A^CD57^{+} subset of NK cells. NKG2C/NKG2A ratio was calculated as relative % of NKG2C^NKG2A^- ANK cells / NKG2C^NKG2A^+ iNK cells (Figure S5).
KLRC2 (NKG2C) genotyping

KLRC2 gene encodes for NKG2C and is located in chromosome 12p13. For categorizing subjects KLRC2 wildtype homozygous (\(Wt^+/Wt^+\)), KLRC2 deletion homogyzous (\(Del^+/Del^+\)) and KLRC2 heterozygous (\(Wt^+/Del^+\)), PCR-based KLRC2 genotyping was carried out. DNA was isolated from the peripheral blood using QIAGEN QIAamp® DNA blood mini kit method (Hilden, Germany). PCR amplification was carried out with forward and reverse primer sequences as previously described (Miyashita et al., 2004). PCR amplification was carried out in 20μl volume, containing 1x PCR master mix which has premixed taq polymerase, dNTPs, PCR buffer (Thermo fisher scientific, Waltham, MA 02451, United States), 1.65 pmol forward primers and reverse primers for KLRC2 deletion and wild type KLRC2 genes (see Table S1), 100pg-1μg genomic DNA. Amplification was performed using a T100 thermal cycler (Bio-Rad, Hercules, CA). Cycling temperature profiles were adopted from a previous study (Miyashita et al., 2004) with minor modifications. Briefly, the reaction mixture was subjected to one cycle of denaturation at 95°C for 10 min followed immediately by 29 cycles of 95°C for 20 s, 50°C for 30 s, 72 °C for 40 s; and a final extension at 72°C for 10 min before cooling to 4°C. A non-template control was included in each batch of PCR reactions. PCR products were identified by running the entire PCR product on a 2% agarose gel for 60 minutes at 70 Volts. The size of the amplicons was determined by comparison against the migration of a 100-bp DNA ladder (GeneDireX, Inc, Taoyuan County, Taiwan). Agarose gels were visualized and documented using the Gel doc XR+ gel documentation system (Bio-Rad, Hercules, CA). An illustration of the PCR assay is shown in supplementary figure (Figure S6).

Statistics

Lymphocyte subsets have been represented as % of the parent population. Binary variables were compared between the groups using chi square test. The continuous variables were
analysed using independent sample t-test considering Levene’s test for equality of variances and non-parametric tests (Mann-Whitney U test). P value < 0.05 was considered to be significant. GraphPad Prism (version 8.0 for Windows, GraphPad Software, La Jolla, CA, USA) was used for the statistical assessment (unpaired low-parametric Mann–Whitney or Kruskal–Wallis test and Spearman correlation). Recursive partitioning analysis was carried out using rpart package (https://cran.r-project.org/web/packages/rpart/index.html) in R (https://cran.r-project.org/) to generate optimal cut off for ANK cells at baseline.

The efficacy of Mw in reducing the incidence of COVID-19 infections was calculated in terms of attack rates incidence risk ratio (IRR), absolute risk reduction (ARR) and intervention efficacy (supplement Table S2). This was calculated in terms of infections occurring at 2 weeks after administration of Mw is customary in prophylactic studies as well as those occurring any time between during the study period.

**Authorship Contribution:** SRJ, BK and SC designed the study. AM, RL, GB and HM performed the study. SRJ, AS, AM, GB and HM collected the data. SRJ, AS, JA and SC analyzed the data; SRJ and SC wrote the manuscript. All the co-authors reviewed and approved the manuscript.

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Legends of Figures

Figure 1: Impact of Mw vaccination on COVID-19 compared to a control group: Points and connecting line plot show the infection trend in Mw group (n=50) and the Control group (n=50). The x-axis shows the time in weeks and y-axis shows the Cumulative Incidence (CI) in %. Red and black shaded circles represent Mw group and Control group respectively.

Figure 2: Impact of Mw on both Adaptive NK (ANK) cells and inhibitory NK (iNK) cells:
A) Scatter dot with bar plot showing the expression of NKG2C+ ANK cells (Mw group -n=30 and control group- n=15) and B) NKG2A+ iNK cells (Mw group-n=30 and control group-n=15) with and without of Mw vaccine at baseline and day 60. C) Scatter dot with bar plot showing kinetics of NKG2C+ ANK cells, and D) NKG2A+ iNK cells expression in Mw group (n=30) at Baseline, day 30, day 60 and day 100. E) log2FC expression of NKG2C+ and NKG2A+ in both Mw group (n=30) and Control group (n=15) at day 60 after normalization with baseline. Red upside and downside shaded triangles represent NKG2C+ ANK and NKG2A+ iNK respectively for Mw group. Black upside and downside shaded triangles represent NKG2C+ ANK and NKG2A+ iNK respectively for Control group. ****- p < 0.0001, **- p <0.01, ***- p <0.001 and ns= p value not significant.

Figure 3: Impact of Mw on upregulation of NKG2C+ ANK cells with respect to NKG2C expression at baseline: Scatter dot with bar plot showing the kinetics of A) NKG2C+ ANK cells (n=15) and C) NKG2A+ iNK (n=15) cells expression in Mw group at baseline, day 30 and 60 in respect to <15% NKG2C at baseline. B) NKG2C+ ANK (n=15) cells and D) NKG2A+ iNK (n=15) cells expression in Mw group at baseline, day 30 and 60 in respect to >15% NKG2C at baseline. Log2FC expression of E) NKG2C+ ANK and F) NKG2A+ iNK cells at day 60 after normalization with baseline value in Mw group (>/<15 %, n=15) and control group. Red upside and downside shaded triangles represent NKG2C+ ANK and NKG2A+ iNK respectively for Mw group. Black upside and downside shaded triangles represent NKG2C+
ANK and NKG2A+ iNK respectively for Control group. ****- p < 0.0001, **- p < 0.01, *- p < 0.05 and ns= p value not significant.

**Figure 4: Mw showed sustained effect on NKG2C/NKG2A ratios until 100 days and Impact of Mw intracellular Cytokine (IFN-γ) release**: Scatter dot with bar plot showing A) NKG2C/NKG2A ratio of Control group (n=15) and Mw group (n=30) at baseline and day 60. B) Kinetics of NKG2C/NKG2A ratio at baseline, day 30, 60 and 100 in Mw group. C) & D) Kinetics of NKG2C/NKG2A ratio at baseline and day 60 on the basis of >/< 15 % NKG2C at baseline in Mw group and control group respectively. E) intracellular IFN-γ release in both control (n=5) and Mw (n=5) group at baseline and day 60. Red and black shaded circles represent Mw group and Control group respectively. ****- p < 0.0001, ***- p < 0.001, *- p < 0.05 and ns= p value not significant.

**Figure 5: Relationship between NKG2C+ANK cells and COVID-19**: Scatter dot with bar plot showing expression of NKG2C+ ANK cells at baseline in the overall cohort (no infection, n=44 & infection, n=16), Control group (no infection, n=20 & infection, n=10) and Mw group (no infection, n=24 & infection, n=6) with respect to SARS-CoV-2 infection. Black and red upside shaded triangles represent NKG2C+ ANK for Control group and Mw group respectively and unshaded upside triangles represent NKG2C+ ANK of both groups combined. * - P < 0.05 and ns= P value not significant.

**Figure 6: Impact of the Second dose of Mw on Adaptive and Inhibitory NK cells**: Scatter dot with bar plot showing expression of A) NKG2C+ ANK (day 60, single dose; n=17, double dose; n=12 and day 100, single dose; n=17, double dose; n=12), B) NKG2A+ iNK cells (day 60, single dose; n=12, double dose; n=12 and day 100, single dose; n=17, double dose; n=12) and C) NKG2C/NKG2A (day 60, single dose; n=17, double dose; n=12 and day 100, single dose; n=17, double dose; n=12) ratio with respect to single and double dose of Mw vaccine at
day 60 and 100. Red upside and downside shaded triangles represent NKG2C+ ANK and NKG2A+ iNK respectively for Mw group. Red shaded circle represents Mw group.

****- p < 0.0001, **- p < 0.01, *- p < 0.05 and ns= p value not significant.

Figure 7: The suggested mechanistic pathway of the impact of Mw on ANK cells and protection against COVID-19.
### Table 1: Characteristics and Outcomes

|                              | Control group (N=50) | Mw group (N=50) | p-value |
|------------------------------|----------------------|-----------------|---------|
| Age at vaccination, Median (Range), Years | 28 (21-55) | 28 (22-56) | 0.11 |
| <45                          | 45                   | 42              |         |
| >45                          | 5                    | 8               |         |
| Gender                       |                      |                 | 0.3     |
| Male                         | 34                   | 28              |         |
| Female                       | 16                   | 22              |         |
| SARS-CoV-2 Infection         |                      |                 |         |
| Mild                         | 17                   | 3               | 0.0012  |
| Moderate                     | 11                   | 3               | 0.02    |
| Severe                       | 6                    | 0               | 0.01    |
| 0                            | 0                    | 0               | 1.0     |
| Incidence rate/10000 person days | 24.05             | 3.57            |         |
| Time to Infection, Median(Range), Mean, Days | 56 (18-135) | 156 (151-159) | 0.00 |
|                              | 66.4 ± 39.1          | 155.3 ± 4.0     |         |

### Table 2: Incidence Rate Ratio of SARS-CoV-2 infections and Mw efficacy.

| Parameter                         | Estimate | p-value | 95% CI          |
|-----------------------------------|----------|---------|-----------------|
| Incidence Rate Ratio (IRR)        | 0.15     | 0.0004  | 0.04 to 0.5     |
| Attack rate in Mw control (ARU)   | 0.34     |         |                 |
| Attack rate in Mw treated (ARV)   | 0.06     |         |                 |
| Mw efficacy (%)                   | 82.35%   |         | 50 to 96 %      |
| Absolute Risk Reduction (ARR)     | 0.28     |         | 0.13 to 0.43    |
| Number Needed to Treat (NNT)      | 3.6      |         | 2.3 to 7.5      |
Figure 1

- Control group
- Mw group

CI in %

-10 0 10 20 30 40

2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Weeks

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Figure 2:
Figure 4
Figure 5

[Bar chart showing the percentage of NKG2C+ ANK cells across different conditions.]

perpetuity.
Figure 6

A

B

C

% NKG2C⁺ ANK cells
%

% NKG2A⁺ ANK cells
%

NKG2C/NKG2A ratio

ns

ns

ns

* ns

***

Single Dose 0D0
Single Dose 1D0
Double Dose 0D0
Double Dose 1D0

Single Dose 0D0
Single Dose 1D0
Double Dose 0D0
Double Dose 1D0

Single Dose 0D0
Single Dose 1D0
Double Dose 0D0
Double Dose 1D0

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Figure 7: