Cholangiocarcinoma-exposed cytokine-induced killer cells developed senescence and exhaustion

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ABSTRACT

The existence of cellular exhaustion and senescence have been well-documented in T cells, but not yet in CIK cells. The exposure of CIK cells to HubCCA1 cholangiocarcinoma cells resulted in impeding the proliferation of CD3⁺CD56⁺ subset and T cell (CD3⁺CD56⁻) subset but not NK cell (CD3⁻CD56⁺) subset. The tumor exposure could not raise the proportion of Treg subset until after the co-culture for 3 d. The blocking on the otherwise the most rapidly proliferating CD3⁺CD56⁺ subset lowered the proportion of this subset in the total CIK cells, while relatively sparing the proportion of T cell subset. These 3 subsets of the survived CIK cells were inflicted with increasing senescence markers (KLRG1 and CD57) upon the tumor exposure. However, CD28, a T cell activation marker, was upregulated in CD3⁺CD56⁺ subset and NK cell subset upon tumor exposure, but was suppressed in T cell subset. For exhaustion, the co-culturing tumor cells raised the exhaustion markers (CTLA-4, PD-1 and Tim-3) on CIK cells. The pre-inoculation with DCs could slightly lower the level of PD-1 in untreated CIK cells, but was unable to withstand the upregulation of PD-1 induced by the tumor exposure. The co-culture of CIK cells with dendritic cells (DCs) could alleviate only CD57, but not other studied markers. In summary, the exposure of CIK cells to tumor cells rendered the development of senescence and exhaustion to the CIK cells. The co-culture of CIK cells with DCs upregulated CD28, but had limited role in preventing senescence and exhaustion.
METHODS

**Generation of CIK cells and DCs from peripheral blood mononuclear cells**

PBMCs were isolated from whole blood by Ficoll gradient centrifugation. The cells were allowed to adhere over the tissue culture container at a density of $5 \times 10^6$ cells/mL for 1 h at 37°C in RPMI 1640, 10% FBS. The adherent cells were processed into DCs. DCs were maintained in RPMI 1640, 10% FBS, 400 U/mL GM-CSF, 500 U/mL IL-4 and matured by adding 1,000 U/mL TNF-α. The non-adherent PBMCs were prepared as CIK cells by adding IFN-γ (1,000 U/mL) and incubated at 37°C, 5% CO₂ for 24 h. Then 50 ng/mL OKT3 and 300 IU/mL IL-2 were added. After 21 d, an aliquot of CIK cells were inoculated with DCs at a S:R ratio of 1:10 for 5 d. CIK cells were maintained up to day 26 prior to the co-culture with HubCCA1 cholangiocarcinoma cells for 3 d. The cellular ratio of CIK cells to HuCCA1 cells was $3 \times 10^5 : 3 \times 10^4$ (cells : cells) on each well of the 24-well plate.

**Fluorescence-activated cell sorting (FACS) analysis**

CIK cells were washed once in PBS containing 5% FBS and resuspended in 30 µL PBS/BSA. The cells were incubated with 0.25-0.5 µL of the corresponding primary monoclonal antibodies (1 mg/mL) for 30 min at 25°C, washed twice with PBS, and resuspended in 30 µL of PBS/FBS. Flow cytometry analysis on 10,000 cells was performed using a CytoFLEX (Beckman Coulter). Data were analyzed using FlowJo version 10.0.5.
Figure 1 The proliferation of all subsets of CIK cells were suppressed by the co-culturing HubCCA1 cells. The co-culture of CIK cells with the HubCCA1 cells resulted in the stagnant of cell numbers in all CIK subsets (A) which otherwise would be increasing. Both CD3^+CD56^+ subset and T cell (CD3^+CD56^-) subset were lowered significantly. Since CD3^+CD56^+ subset is generally the most rapidly proliferating subset, its proportion as reflected by the percentage of subset (B) was therefore the most affected subset among others. The already low proportion of NK cell subset was not clearly affected by the co-culture.
Figure 2 The co-culture with HubCCA1 affected the proportion of Treg subset of CIK cells. The proportion of Treg subset in CIK cells could not be heighten by the co-culture with HubCCA1 until for least 3 days. Instead, the CIK cells that had been pre-inoculated with mDCs could readily raise the Treg proportion without the tumor cells.
Figure 3 All CIK cell subsets were evaluated for their induction to senescence and exhaustion after the co-cultured with HubCCA1 cells based on KLRG1 (A), CD57 (B), CTLA-4 (C), PD-1 (D) and Tim-3 (E). The co-culture of CIK cells with HubCCA1 upregulated all markers. The pre-inoculation with mDCs could not prevent CIK cells from senescence / exhaustion based on the studied markers except CD57 (B), where the pre-inoculation was beneficial to CIK cells. The senescence / exhaustion was maintained in CIK cells even after the separation of CIK cells from the tumors based on KLRG1 and CTLA-4 (F).
Figure 4 The pre-incubation of CIK cells with mDCs and the co-culture with tumor cells raised CD28 in CD3^+CD56^+ subset (A) and T cell (CD3^+CD56^+) subset (B), but NK cells (CD3^+CD56^+) subset (C) only responded to the co-culturing tumor.
CONCLUSION

CIK cells were venerable to senescence / exhaustion induction upon tumor exposure. The pre-inoculation of CIK cells with DCs prior to the tumor exposure upregulated CD28 on the former that might explain the enhancement of the anti-tumor activity of CIK cells. However, the pre-inoculation with DCs had limited role in preventing senescence and exhaustion. Attempt to reverse these immunosuppressive conditions might unlock the anti-tumor action of CIK cells.
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