Iranian Journal of Blood & Cancer

Journal Home Page: www.ijbc.ir

Review

CAR-NK Cells: A Systematic Review of Emerging Alternative on Immunotherapy Against Leukemia

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ARTICLE INFO

Article History:
Received: 30/12/2021
Accepted: 7/6/2022

Keywords:
Chimeric Antigen Receptors
Leukemia
Natural Killer Cells
Cancer
Systematic Review

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Abstract

Background: Cancer is a public health emergency. It has a high mortality rate despite numerous studies on pharmaceutical therapies. Chimeric antigen receptor-natural killer (CAR-NK) cells are promising immunotherapy that could be used to treat cancer, especially leukemia. However, the evidence is still unclear. Thus, this systematic review aims to summarize the evidence regarding the use of CAR-NK cells as a therapy for leukemia.

Method: This systematic review was conducted in accordance with the preferred reporting items for systematic reviews and meta-analysis (PRISMA) statement guidelines. The literature search was done using PubMed, ProQuest, ScienceDirect, and EBSCOHost with "chimeric antigen receptor", "natural killer cell", and "leukemia" as the primary keywords until 20 March 2020. Data collection and extraction were done by three independent reviewers. Extending a risk-of-bias approach to address in-vitro studies for assessing the risk of bias was utilized in the quality assessment of the studies.

Results: The search strategy identified 221 studies. Three relevant articles met our inclusion criteria. All the included studies had a low risk of bias. The main findings from available data were as follows: (a) cytotoxicities of CAR-NK cells were found highest in cell lines expressing antigen for CAR (CD19+ cancer cells) (b) CAR-NK cells had low cytotoxicities against cells that didn't express antigen for CAR (e.g. SR-91); (c) all studies did not result in aberrant growth of the transduced CAR-NK cells.

Conclusion: The use of CAR-NK cells showed promising results in treating leukemia based on its cytotoxicity against CD19+ cancer cell lines.

Please cite this article as: Prasetya A, Soetedjo R, Arifin ES. CAR-NK Cells: A Systematic Review of Emerging Alternative on Immunotherapy Against Leukemia. IJBC 2022; 14(2): 85-91

Abbreviations: ALL, Acute Lymphoblastic Leukemia; CAR, Chimeric Antigen Receptor; CAR-NK, Chimeric Antigen Receptor-Natural Killer; CB, Cord Blood; CLL, Chronic Lymphocytic Leukemia; CRS, Cytokine Release Syndrome; DNA, Deoxyribonucleic acid; FDA, Food and Drug Administration; GVHD, Graft Versus Host Disease; iC9, Inducible caspase-9; iC9.CAR19.CD28-zeta-2A-IL-15, Anti-CD19 CAR in Combination with the Human IL15 Gene and the Inducible Caspase-9 Suicide Gene Separated Using 2A Sequence Peptides; IL-2, Interleukin-2; mRNA, Messenger ribonucleic acid; NK, Natural Killer; NT, Non-transduced; NT-CB-NK, Non-Transduced Cord Blood-Natural Killer; PCR, Polymerase chain reaction; PRISMA-P, Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols; SCGM, Serum-free cell growth medium; T; Transduced; WHO, World Health Organization.

1. Introduction:

Leukemia was first discovered in 1847 by dr. Rudolf Virchow, the father of modern pathology [1]. It is still one of the potent killers of humanity today. According to the World Health Organization (WHO), there were approximately 13.4 cases per 100,000 people in Indonesia in 2018, with a crude mortality rate of 4.2. This high mortality rate is due to a lack of effective treatment [2]. Currently, chemotherapy is the primary treatment of choice for leukemia. The downside, however, is that chemotherapy could cause many, severe adverse effects [3]. Alternative treatments are needed in this situation. Immunotherapy is one of the most studied alternatives. It mainly works by reinforcing the immune system to fend off cancer cells [4].

One of the most extensively researched immunotherapy is chimeric antigen receptor (CAR)-engineered immune cells. It is a molecule designed for redirecting T cells to target certain types of cells, making it an excellent tool for targeting cancer-specific antigens [5]. Tisagenlecleucel, a CAR T cell therapy, has been approved by the food and drug administration (FDA) to treat young adults and children with acute lymphoblastic leukemia (ALL) who were unresponsive to standard treatment. This treatment method is customized to each patient to prevent graft versus host disease (GVHD). Nevertheless, several adverse effects could manifest such as cytokine release syndrome (CRS) and neurological complications [6].

NK cells are a preferable alternative because they are unable to induce GVHD and CRS, unlike T cells. NK cells also could not induce tumorigenesis, because of their short life span. However, it could not create a persistent antitumor effect, whereas CAR T cells could. Similar to T cells, CAR can also be incorporated or applied to NK cells [7]. The CAR genome could be integrated either with the help of a retrovirus or by using an electrophoran approach. Despite all of its advantages, this method of treatment has yet to be clinically proven unlike CAR T cells [6]. Thus, the objective of this study is to systematically review the literature for qualitative evidence regarding the use of CAR-NK cells for leukemia treatment.

2. Materials and Methods

2.1. Protocol and registration

The systematic search process for this review was done on 20 March 2020. We used the preferred reporting items for systematic review and meta-analyses protocols (PRISMA-P) guidelines as our study protocol [8].

2.2. Study designs

In this review, we only included study designs such as laboratory (in-vitro) and animal studies (in-vivo).

2.3. Intervention

Interventions included in this review involved animal and laboratory studies that assessed the current leukemia therapy, particularly using CAR NK cells.

2.4. Study population, timing, and setting

There is no restriction placed on the study population, timing, and settings.

2.5. Comparators

There were no comparators' constraints implanted in this review.

2.6. Outcomes

The outcome sought in this review was the efficacy and safety of CAR-NK cells as immunotherapy in leukemic patients.

2.7. Languages

We did not restrict the languages of the articles that we included in order to determine all of the possible studies that were published, but we only used English terms in the search process.

2.8. Publication status

All of the articles that we selected were published in various scientific journals.

2.9. Information sources

Electronic databases that we used were ScienceDirect, EBSCOHost, PubMed, and ProQuest.

2.10. Search strategy

We searched the articles using "chimeric antigen receptor", "natural killer cell", "neoplasm", and "leukemia" as our MeSH (Medical Subject Headings) terms along with their synonyms (**Table 1**).

2.11. Study selection

The screening was done by all three authors

Table 1: Characteristics of the included studies of MMP-2 polymorphism

Databases	Keywords	Number of Articles
PubMed	("Receptors, Chimeric Antigen"[Mesh]) OR "chimeric antigen receptor"[Title/Abstract]) OR "chimeric antigen receptors"[Title/Abstract]) OR CAR[Title/Abstract])) AND ("Killer Cells, Natural"[Mesh]) OR NK cell[Title/Abstract]) OR "NK cell"[Title/Abstract]) OR "NK cells"[Title/Abstract]) OR "natural killer cell"[Title/Abstract]) OR "natural killer cells"[Title/Abstract]) OR "killer cell"[Title/Abstract]) OR "killer cells"[Title/Abstract]) OR "K cell"[Title/Abstract]) OR "K cells"[Title/Abstract]) AND ("Neoplasms"[Mesh]) OR Neoplasms[Title/Abstract]) OR Neoplasm[Title/Abstract]) OR Tumor[Title/Abstract]) OR Tumors[Title/Abstract]) OR Cancer[Title/Abstract]) OR Cancers[Title/Abstract]) AND ("Leukemia"[Mesh]) OR leukemia[Title/Abstract]) OR leukemias[Title/Abstract]) OR leucocythaemia[Title/Abstract]) OR leucocythaemias[Title/Abstract]) OR leucocythemia[Title/Abstract]) OR leucocythemias[Title/Abstract])	85
ProQuest	AB ("NK cell" OR "NK cells" OR "natural killer cell" OR "natural killer cells" OR "K cell" OR "K cells" OR "killer cell" OR "Killer cells") AND ab("chimeric antigen receptor" OR "chimeric antigen receptors" OR CAR) AND ab(cancer OR cancers OR neoplasm OR neoplasms OR tumor OR tumors) AND ab(leukemia OR leukemias OR leucocythaemia OR leucocythaemias OR leucocythemia OR leucocythemias)	28
Science Direct	("NK cell" OR "NK cells" OR "natural killer cell" OR "natural killer cells") AND ("chimeric antigen receptor" OR "chimeric antigen receptors" OR CAR) AND (cancer OR cancers OR neoplasm OR neoplasms OR tumor OR tumors) AND (leukemia OR leukemias)	59
EBSCO	AB ("NK cell" OR "NK cells" OR "natural killer cell" OR "natural killer cells" OR "K cell" OR "K cells" OR "killer cell" OR "Killer cells") AND AB ("chimeric antigen receptor" OR "chimeric antigen receptors" OR CAR) AND AB (cancer OR cancers OR neoplasm OR neoplasms OR tumor OR tumors) AND AB (leukemia OR leukemias OR leucocythaemia OR leucocythaemias OR leucocythemia OR leucocythemias)	49

independently from a list of search results compiled in a document review platform. To avoid bias, each stage was screened by two authors. Duplicates and irrelevant articles were excluded using Endnote X9 and sysrev.com. The authors screened the titles and abstracts obtained through the search before excluding any work that did not meet the inclusion criteria. Selected studies on this stage were screened further through the full text of the records to determine their eligibility. Any disagreements in each stage of the selection were resolved by discussion.

2.12. Data extraction and data items

Data extraction of the included studies was done on a web-based word processor and any discrepancies were resolved through discussion. The extracted data includes authors, the type of studies, means of transduction, intervention, and result.

2.13. Risk of bias in individual studies

To reduce the risk of bias, all authors independently assessed included studies based on the extending a risk-of-bias approach to address in-vitro studies for assessing the risk of bias. The studies were classified as definitely low risk, probably low risk, probably high risk, or definitely high risk [9].

3. Result

3.1. Literature Search

The search strategy yielded a total of 221 articles (Figure 1). Duplicates were removed, resulting in 106 articles being reviewed. The titles and abstracts of these articles were retrieved and reviewed for relevance, sparing 12 articles upon elimination. From these articles, we identified one non-randomized control trial, six reviews, one editorial, and one news. The authors then decided to exclude these nine articles, leaving three articles to be analyzed qualitatively (**Table 2**) [10-12].

3.2. Risk of Bias for In-Vitro and In-Vivo Studies

Table 3 summarized the risk of bias based on the authors' assessments. In all three studies, there was a probably high risk of bias regarding blinding of personnel and a probably low risk of bias regarding potential threats to internal validity.

3.3. Transduction of Chimeric Antigen Receptor

Liu et al. obtained NK cells from cord blood and peripheral blood of patients with chronic lymphocytic leukemia (CLL). Purified NK cells were then cultured

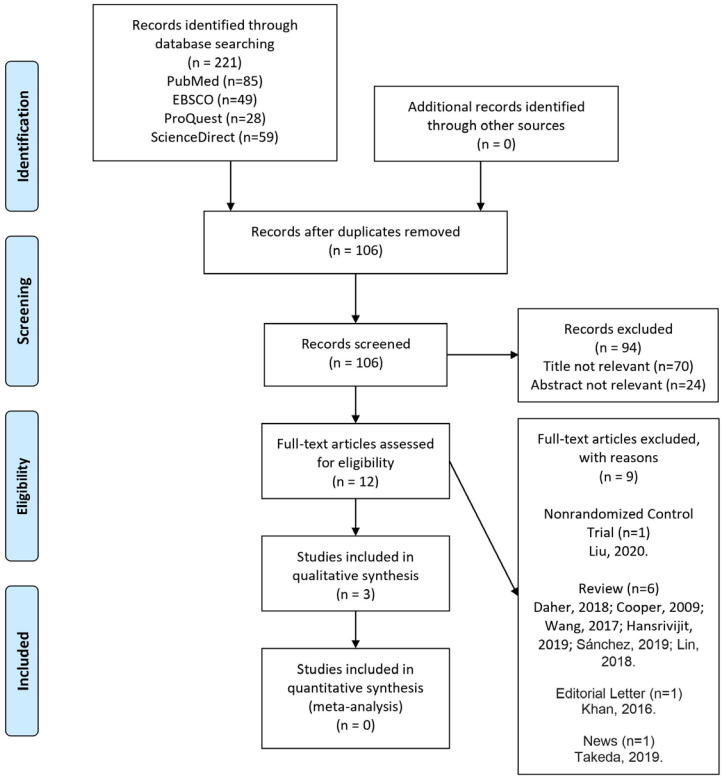

Figure 1: Flow diagram of the identification and selection of studies included in the analysis.

Table 2: 6-MP tolerated dose intensity based on c.415C>T genotype in pediatric ALL

Authors	Study Design	Cell Lines	Means of Transduction	Interventions	Result
Liu et al., 2018	In-vivo and in-vitro	K562, CLL patients' cells, Raji	Retrovirus	Suicide gene iC9 was also transduced into the CB-NK cells. The gene could be activated to control the T-NK cells' growth with the help of AP1903.	Superior capacity was observed in T-CB-NK cells to eliminate CD19+ CLL cells compared to the NT-NK cells.
Boissel et al., 2009	In-vivo	K652, REH, SUP-B15, SR-91, CLL patients' cells	Electroporation	NK cells were gamma irradiated 4h before or 20h after electroporation to prevent aberrant growth.	Higher cytotoxicity was observed in CD19-CAR T-NK cells compared to NT NK cells against CD19+ cancer cell lines (REH and SUP-B15).
Shimasaki et al., 2012	In-vivo and in-vitro	380, RS4, 11, 697, OP-1, Jurkat, CEM-37, U937, K562, Raji	Electroporation	NK cells were expanded from healthy donors by co-culturing peripheral blood mononuclear cells and irradiated K562-mb15-41BBL cells for 7 days	Ten times higher antileukemic activity with NK cells that electroporated with anti-CD19-BB-ζ mRNA against the 380 cell line in a murine model. Higher cytotoxicity was observed in expanded and electroporated NK cells with anti-CD19-BB-ζ mRNA against CD19+ cancer cell lines.

Table 3: Risk of bias summary: review authors' assessments about each risk of bias item for each included study.

No	Risk of Bias Question[10]	Liu et al.	Boissel et al.	Shimasaki et al.
1	Was administered dose or exposure level adequately randomized?	++	++	++
2	Was allocation to study groups adequately concealed?	++	++	++
3	Was experimental conditions identical across study groups?	++	++	++
4	Were research personnel blinded to the study group during the study?	-	-	-
5	Were outcome data complete without attrition or exclusion from analysis?	+	++	+
6	Can we be confident in the exposure characterization?	++	++	++
7	Can we be confident in the outcome assessment (including blinding of assessors)?	+	+	-
8	Were all measured outcomes reported?	+	--	++
9	Were there no other potential threats to internal validity?	+	+	+

++	Definitely low risk of bias
+	Probably low risk of bias
-	Probably high risk of bias
--	Definitely high risk of bias

in a serum-free cell growth medium (SCGM) with the addition of irradiated feeder cells (Clone 9) and interleukin-2 (IL-2). Stimulated NK cells were mixed with anti-CD19 CAR in combination with the human IL-15 gene and the inducible caspase-9 suicide gene, which were separated using 2A sequence peptides (iC9. CAR19.CD28-zeta-2A-IL-15) encoding retroviral supernatants. CAR19 functions as a receptor to target the cluster of differentiation 19 (CD19) on tumor cells. Inducible caspase-9 was a suicide gene inserted as a tool to pharmacologically eliminate NK cells. The IL-15 gene, on the other hand, was inserted for NK cell proliferation and survival. The median efficiency of the transduction process was about 66.6% [10].

Boissel et al. used eletrophorans instead of a retroviral agent for transduction. An electrical pulse was used to create pores in the targeted cells. Two methods involved using deoxyribonucleic acid (DNA) sequence or messenger ribonucleic acid (mRNA). Polymerase chain reaction (PCR) was used to prepare the DNA sequence, while plasmid was used for the mRNA sequence. The resulting cells were then cultured in myelocult medium in a 5% CO2 incubator at 37oC. Flow cytometry was used to determine the expression of CD19-CAR proteins [11]. Shimasaki et al. implemented similar methods by electroporation using primary and expanded NK cells (activated NK cells) from peripheral blood mononuclear cells in which anti-CD19-BB-ζ mRNA was added. These cells were then cultured in leukemic cell line mediums such as 380, RS4;11, 697 OP-1 Ramos, Raji, Jurkat, CEM-C7, K562, and U937 [12].

3.4. Cytotoxicity Assays

Liu et al. tested iC9/CAR.19/IL-15 transduced cord blood-natural killer (T-CB-NK) cells and non-transduced cord blood-natural killer (NT-CB-NK) cells that were co-incubated with primary CD19+ CLL cells obtained from patients (n=6) at varying effector:tumor (E:T) ratios. The T-CB-NK cells were superior in eliminating CD19+ CLL cells compared to the NT-CB-NK cells. On the other hand, the T-CB-NK and NT-CB-NK cells showed similar capacity at killing K562 feeder cells as a positive control. This revealed that the efficacy of T-CB-NK cells on the CD19+ CLL cells was enhanced because of the expression of the CAR.19 receptor [10].

Boissel et al. used cell lines in addition to K562 such as SR-91, REH, and SUP-B15. Assessment of cytotoxicities was done using different types of NK cells that were as follows: non-electroporated NK cells and electroporated NK cells without nucleic acid, NK cells electroporated with green fluorescent protein (GFP) mRNA, or NK cells electroporated with αCD19-CAR

mRNA. The assessment was also done at varying E:T ratios similar to that of Liu et al.. All types of NK cells showed similar results against K562 cell lines. A higher cytotoxicity percentage was found when using αCD19-CAR-expressing NK cells against CD19+ cell lines such as REH and SUP-B15. The killing percentage was universally low in SR-91 cell lines since they were both CD19– and NK resistant. Primary CLL cells were also tested and showed similar results with REH and SUP-B15 [11].

Shimasaki et al. examined the cytotoxicity of NK cells targeting CD19+ cells in different cell lines. This cytotoxicity was measured at varying E:T ratios against B-lineage acute lymphoblastic leukemia (ALL) cells (380, OP-1, and RS4;11). A higher result of cytotoxicity was found in NK cells that were electroporated with anti-CD19-BB-ζ mRNA compared to non-electroporated NK cells. Expanded NK cells were constantly superior in cytotoxicity to primary NK cells. The author also assessed the anti-leukemic activity of electroporated NK cells in murine models. Similar results were found against B-lineage ALL as in the in-vitro study [12].

3.5. Potential of Aberrant Growth

In-vitro culture without growth factors did not show any evidence of aberrant growth. No evidence of anomaly was found in mice that were treated. Inducible caspase-9 was successfully activated both in-vitro and in-vivo by a molecule dimerizer called rimiducid (AP1903) [12]. Precautions were also taken by Boissel et al. in which NK-92 cells were γ-irradiated prior to infusion into patients to prevent in-vivo cell proliferation [10].

4. Discussion

All studies concluded that CAR-NK cells were superior compared to primary NK cells against CD19+ cells. The NK cells that were incorporated with the CD19-CAR gene showed higher cytotoxicity against CD19+ cancer cells compared to CD19– cell lines. Some examples of CD19+ cancer cells were REH, SUP-B15, B-lineage ALL cells, and CLL cells. CAR-NK cells also had higher toxicity against lymphoma cell lines when compared with primary NK cells [10,11,12]. CAR T cells targeting CD19+ leukemia were also found to be effective with a remission rate of 80% [5]. For this reason, the FDA has approved CAR T cells therapy for the treatment of relapsed adverse effects such as CRS and neurological syndrome [5]. In a study by Fitzgerald et al., 46% of participants with refractory ALL developed CRS after CAR T cells therapy [13]. T cells could also induce GVHD, therefore they must be taken from the patient themselves [6]. Unlike T cells, NK cells do not have the potential to induce GVHD and CRS, making them a safer alternative [7,14].

Various studies found mixed results regarding the means of transduction and its effect on cytotoxicity. Some studies stated that electroporation was superior compared to retroviral transduction as means of expressing the transduced CAR [12,15]. One study showed that these two methods had similar effects on cytotoxicity [12]. Another study stated that retroviral transduction has a higher impact on cytotoxicity compared to electroporation [15]. Transduction of mRNA was preferred over DNA since mRNA will not be integrated into the host DNA and hence does not carry the risk of genomic mutation [11]. This risk of genomic mutation can be negated by using electroporation [16]. However, electroporation leads to short-term expression of the transduced gene compared to retroviral transduction [17].

The authors acknowledge that there are some limitations in the studies. A major limitation is that all studies were conducted in in-vitro and in-vivo settings. As a result, the findings could not accurately represent CAR-NK cells usage in real patients, thus more research is needed. Different means of transduction could have affected the result of each study. Different cell lines used in the studies could have influenced the result. However, all studies reported the methods transparently and holistically.

Future human clinical trials should be performed to assess CAR-NK cells' effects on the human body. Currently, there is an ongoing phase 1 and 2 clinical trials of 11 patients regarding the use of CAR-transduced NK cells [18]. Chimeric antigen receptor-natural killer cells could be an alternative to CAR-T cells for treating malignancies. Furthermore, because CAR-NK cells have fewer side effects and complications, they may eventually replace CAR T cells as the immunotherapy of choice in treating hematologic malignancies.

5. Conclusion

All studies included showed that CAR-NK cells had higher cytotoxicity against CD19 + cell lines. Therefore, CAR-NK cells could be potentially used as a treatment for leukemia. However, there are still

unresolved questions regarding effects on the human body and other types of tumors, requiring further research. The authors believe that CAR-NK cells could play an important role in the field of cancer immunotherapy.

Conflicts of Interest

All authors declare no competing interests.

Acknowledgment

We would like to thank all of the authors and contributors to the included studies.

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