Prevention of Cytokine Release Syndrome in Chimeric Antigen Receptor T-cell Therapy

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Abstract. One of the most powerful forms of cancer immunotherapies is chimeric antigen receptor (CAR) T-cell therapy. Despite being fairly recent in terms of its development and approval, CAR T-cells show great potential as a treatment, since they can efficiently recognize and destroy tumor cells. However, due to the various toxicities associated with this treatment option, CAR T-cell therapy is usually advised as a last resort. The most prominent toxicity of CAR T-cell therapy is the onset of immunotoxicity, which includes Cytokine Release Syndrome (CRS). This review will focus on the recent definition of CRS in general, the mechanisms of CRS progression, how tumor cell pyroptosis via. CAR T-cell therapy can trigger CRS, and the strategies to prevent CRS that have shown efficiency in the application of CAR T-cell patients. By the end, this article will highlight the combination of inhibitors of CRS to improve tumor therapy in the future.

Keywords: CAR T-cell therapy, cytokine release syndrome, proinflammatory cytokines.

1. Introduction

CAR T-cell therapy is reported as a prominent application of adoptive T-cell therapy designed against cancer. Through genetic engineering, CAR T-cells express specific antibodies that recognize corresponding antigens on malignant tumor cells. Currently, all the Food and Drug Administration (FDA) approved types of CAR T-cell therapy are targeted at hematological malignancies. While it is complicated to manufacture CAR T-cells, their anti-tumor capacity has been proven with clinical trials–some of which had patients displaying complete cancer remission with the administration of CAR T-cells. Recent reports have noted that long-term results of this therapy are rather limited due to difficulties against solid tumors or a decreased expression of the target antigens on tumor cells. However, these problems are being addressed at a rapid pace. Overall, CAR T-cell therapy is not only a powerful form of cancer treatment, but also displays even greater potential in the foreseeable future.

However, CAR T-cell therapy is also plagued by numerous toxic side effects, with the most notable among them being the onset of CRS correlated with the elevated expression of multiple proinflammatory cytokines, CRS is an excess inflammatory response to tissue damage or pathogens within the body. From the publicly available trials, the proportion of patients who experience any form of CRS while also undergoing CAR T-cell therapy can go up to 100 percent and severe cases of CRS can make up to 46 percent [1]. Although the progression of CRS has minimal effects on the modified T-cells, symptoms of organ dysfunction and even life-threatening seizures characterize severe cases of CRS. Therefore, future research is needed to find more effective treatments. In this review, the focus will be on current improvements–as well as existing research gaps–in defining and preventing CRS in CAR T-cell therapy.

2. Definition of CRS

In general, proinflammatory cytokines are small proteins that participate in the immune system. Although cytokines are primarily released by T-cells and macrophages, they can also be produced by neutrophils, B-cells, and mast cells. The activation of receptors on T-cells and macrophages is induced under the presence of pathogens or signals of cell damage. As a result, kinases that regulate proinflammatory cytokine production or secretion are activated in favor of cytokine release. After

contact with their target cells, these proinflammatory cytokines can combat infections by triggering immune cell proliferation or differentiation, further proinflammatory cytokine release [2].

However, an excess release of proinflammatory cytokines can be achieved through an upregulation of their transcription, translation, or secretion. Although proinflammatory cytokines primarily target white blood cells such as monocytes and B-cells, these cells can target tissues in the kidney, liver, or other organs once activated. Therefore, if left unchecked, many proinflammatory cytokines will cause damage to even healthy cells via. higher counts of activated neutrophils or other immune cells. Symptoms of a normal fever can eventually progress to organ failure and even cases of fatality. These are the root causes to the onset of CRS and its subsequent progression.

CRS is not to be confused with another frequently observed side effect of CAR T-cell therapy named immune effector cell-associated neurotoxicity syndrome (ICANS). The reason for the confusion is that, like CRS, ICANS is also characterized by increased expression of proinflammatory cytokines–specifically in CNS. CRS, in contrast, is defined by increased cytokine activation in endothelial cells along blood vessels. Perhaps the most important distinction between CRS and ICANS is that CRS progression directly precedes the onset of ICANS. As a result, research on the prevention of CRS takes priority despite symptom progression of ICANS being more severe.

Institutions such as the American Society for Transplantation and Cellular Therapy (ASTCT) and the University of Pennsylvania have made attempts to define CRS. Despite continued controversy on recommended treatments, symptom progression of CRS is well studied. Generally, grade 1 of CRS can be identified by fevers of 38 degrees or above. However, grade 2 of CRS is characterized by additional signs of hypotension and/or signs of hypoxia. By grade 3 CRS, the patient would experience not only a continued decrease of consciousness, but also marked by occurrences of reversible seizures. Finally–by grade 4 CRS–these seizures are potentially fatal.

CRS upon the infusion of CAR T-cells is also epitomized by elevated expression of proinflammatory cytokines. Particularly, levels of proinflammatory cytokines significantly increase in CAR T-cell patients about 3-12 days after infusion of CAR T-cells. Peak expression of cytokines in the circulatory system can be observed about 7-9 days after initial infusion. Corresponding to cytokine expression, the usual symptoms of CRS such as organ dysfunction also reach peak levels about 7-12 days after infusion. However, onset of CRS can be identified among CAR T-cell patients as early as 1 day and as late as 14 days after infusion [3].

3. Mechanisms of CRS

To find treatments for patients with CRS, mechanisms behind CRS progression must be understood. When tissue damage occurs, damage-associated molecular patterns (DAMPs) will be released. These DAMPs typically include double-stranded DNA (dsDNA), HMGB1, and ATP. However, toll-like receptors (TLRs) that can recognize DAMPs can also detect pathogens such as viruses through recognition of pathogen-associated molecular patterns (PAMPs). This phenomenon is demonstrated in CRS induced by SARS COV-2.

Upon activation, TLRs can trigger either the NF-κB or the MAPK signal pathways. From these two pathways, NF-κB or activator-protein-1 (AP-1) will be brought into the nucleus, then leading to the alternated expression of target genes. Despite the NF-κB and MAPK pathways affecting different proteins, both AP-1 and NF-κB act as transcription factors via. nuclear translocation. As a result, proinflammatory cytokine synthesis is directly affected. Cytokines that are regulated by the NF-κB or MAPK pathways include IL-6 and IL-8 [1].

Signal cascades associated with DAMP recognition are more complicated. Similar to PAMPs, HMGB1 is also recognized by TLRs. However, dsDNA and ATP from tissue damage are mostly responsible for the secretion of proinflammatory cytokines rather than the regulation of their transcription or translation. Specifically, cascades induced by either ATP or dsDNA bring pro-caspase-1 proteins to inflammasome complexes. In turn, mature caspase-1 would also take part in the maturation of proinflammatory cytokines such as IL-1β and IL-18 [4]. Finally, caspase-1 can also

cause pyroptosis within affected immune cells. As a result, DAMP release is reinforced in a vicious cycle of signal cascades that contribute to the onset of CRS.

Together, the MAPK, NF-κB, and Caspase-1 signaling pathways contribute to elevated levels of IL-1β, IL-6, and INF-γ that are observed frequently in patients who experience CRS. Although IL-1β can trigger additional proinflammatory cytokine production, its main function is to ensure proliferation and differentiation of its target T-cells or B-cells. Meanwhile, INF-γ is responsible for increasing the function of neutrophils and monocytes [2]. Normally, T-cell and B-cell differentiation leads to effector T-cells and neutrophils destroying target pathogens or infected tissues. However–in the context of CRS–an overexpression of both IL-1β and INF-γ will also lead to off-target destruction of even healthy cells.

If brought to the correct sites of infection, the severity of these effects can be minimized. However, CRS is also characterized by elevated levels of MCP-1 and IL-8. Normally, IL-8 is more responsible for directing neutrophils and T-cells to specific areas of infection. Similarly, MCP-1 recruits leukocytes to sites of infection [2]. In the context of CRS, it's the healthy endothelial cells that are activated to release MCP-1 and IL-8. In turn, the effects of off-target endothelial cell destruction by neutrophils and effector T-cells will be maximized. Therefore, IL-8 and MCP-1 are just as important due to their role in the trafficking of cells affected by IL-1β and INF-γ.

From this perspective, IL-6 can be argued to be the proinflammatory cytokine most crucial to the mechanisms of CRS. Upon interaction with its receptor IL-6R, IL-6 can trigger a Jak/STAT kinase. The most important consequence of the IL-6/IL-6R interaction is endothelial activation that results from an increase in endothelial stress. This not only leads to downstream activation of IL-6 itself, but also leads to the production of MCP-1 and IL-8. Overall, IL-6 is frequently regarded as the most important factor in CRS and the subject of intensive research [5].

4. Mechanisms of CRS in CAR T-cell Therapy

As recent studies showed, it is difficult to preserve the phenotypes associated with CRS progression. However, up to 9.1 percent of CAR T-cell patients who experience CRS progresses to cases of fatality [1]. This figure 1 is up for debate, as some have reported the mortality rate for CRS to be as little as 1 percent [1]. Regardless, the frequent incidence of severe CRS (sCRS) among patients who receive CAR T-cell therapy makes it a priority to prevent sCRS progression. To achieve this objective, it is crucial to comprehend where CRS induced by CAR T-cells falls under the general mechanisms of CRS.

Unfortunately, it is still controversial as to how CAR T-cells induce CRS. Originally, due to CAR T-cells releasing proinflammatory cytokines to boost their potency against tumors, it was hypothesized that CAR T-cells were largely responsible for the onset of CRS. This was contradicted by research showing that the number of cytokines produced by the modified T-cells is insignificant compared to the amount released by antigen-presenting cells (APCs) [6]. However, considering how IL-1 and IL-6 are known to trigger amplified production of other cytokines or even themselves, it has been reported that the cytokines produced by CAR T-cells are still responsible for macrophage activation.

Yet, the cause of macrophage activation in CAR T-cell patients becomes ambiguous once the DAMPs as a result of tumor pyroptosis are taken into account. Specifically, inhibition of enzymes crucial to enhancing tumor cell pyroptosis–such as Gasdermin-E (GSDME) and caspase-3–also proved to be crucial to the prevention of CRS progression [7]. Due to conflicting research and conclusions, it is difficult to determine whether cytokines released by CAR T-cells or DAMPs resulting from tumor pyroptosis are responsible for CRS. Furthermore, if the significance of both depends on environmental factors, it is ambiguous what these factors exactly are.

Ultimately, there are a wide range of treatments for CRS if the main cause is proinflammatory cytokines produced by CAR T-cells. However, prevention of the onset of CRS due to DAMPs is more complicated. In theory, a reduction of tumor pyroptosis would prevent triggering the kinases crucial

to cytokine production or release. Yet, tumor regression should still be the main priority. Regardless of the notable risk of CRS progression, these patients have limited options due to CAR T-cell therapy already being a last resort.

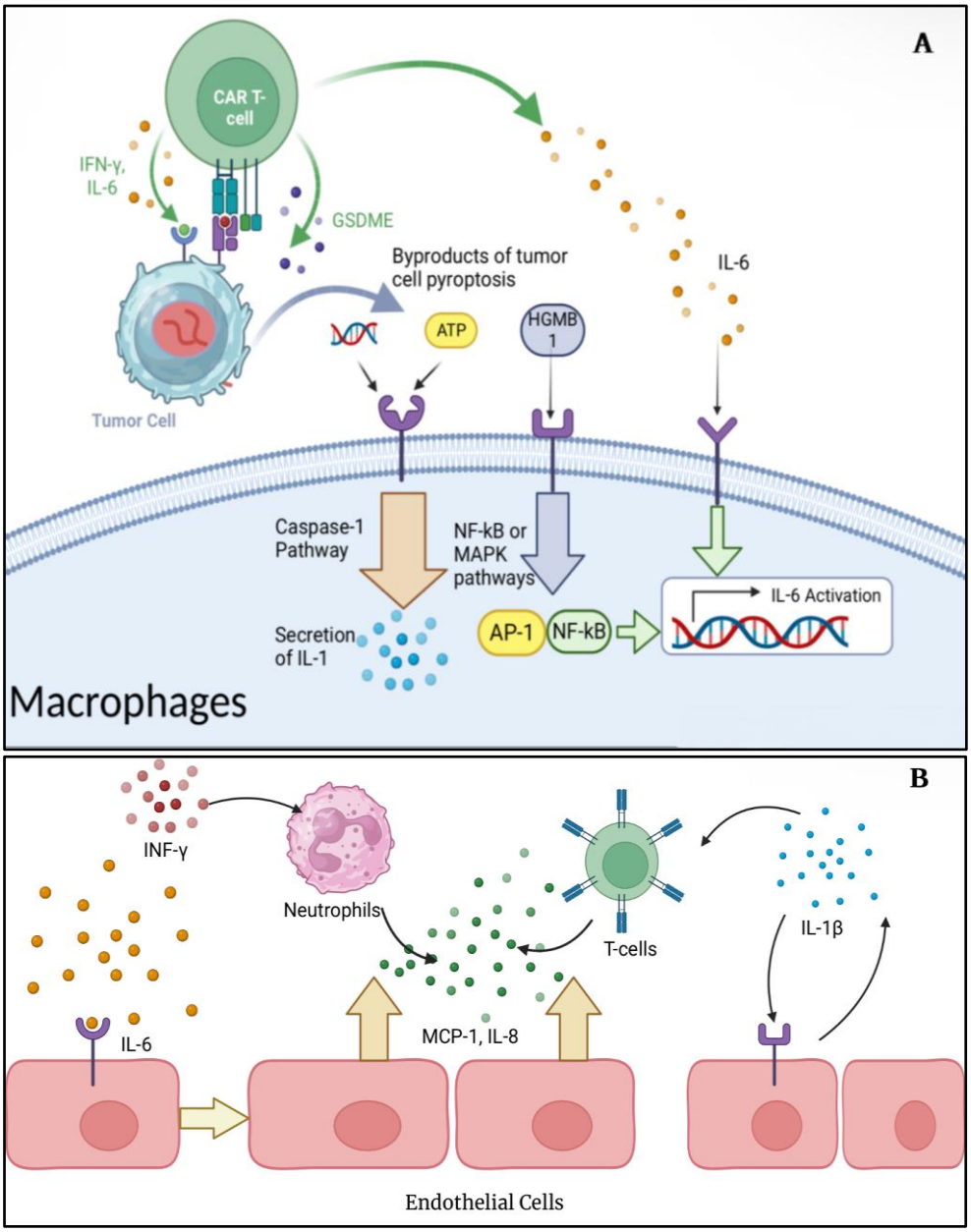

Fig. 1 Overview of the mechanisms behind CRS progression in CAR T-cell therapy. A detail the causes of proinflammatory cytokine production/secretion through either byproduct of tumor cell destruction or cytokines released by the engineered T-cells. B details the effects of cytokines on healthy endothelial cells, with IL-6 affecting endothelial cells and enhancing MCP-1 and IL-8, leading to the recruitment of neutrophils and T-cells to endothelial cells–their functions enhanced by IL-1β and INF-γ. Created using BioRender Inc.

5. Prevention of CRS in CAR T-cell Therapy

Nowadays, most of the available treatments for CRS have their limitations, despite the remarkable progress. While the inhibition of proinflammatory cytokines seems like a viable option, the amount of research on its efficacy is rather rare. Siltuximab–a drug designed to inhibit IL-6–is one of the more prominent of its kind and has displayed effectiveness in individual patients. Still, the amount of research on its applicability is limited.

However, in 2017, the FDA approved a drug named tocilizumab to treat CRS during CAR T-cell therapy. As a human-engineered monoclonal antibody, tocilizumab is designed to target either membrane-bound IL-6R or to prevent the homodimerization of gp130 via targeting soluble IL-6-R. As a result, Jak/STAT kinases triggered by IL-6R activation are averted [8].

In terms of efficacy, tocilizumab not only significantly inhibited IL-6 activity, but also proved that it did not affect the effectiveness of the modified T-cells in any significant way. In contrast to patients who took corticosteroids, tocilizumab patients continually displayed proliferation of CAR T-cells. In practice, tocilizumab is currently recommended to be administered at a dosage of 6 mg/kg per 4 weeks and used for patients experiencing grade ≥2 CRS [8].

Yet, tocilizumab is not only limited by its inability to inhibit crucial proinflammatory cytokines independent of IL-6, but also limited in terms of its long-term efficacy at inhibiting IL-6. Specifically, tocilizumab has minimal effects on the production and secretion of IL-6. Therefore, serum concentration of IL-6 will remain unaffected by tocilizumab. Furthermore, tocilizumab has no effect in the CNS, since it still shows a limited penetration of the blood-brain barrier (BBB). Despite being primarily produced by macrophages, IL-6 can also be produced in the CNS. As a result–even in the best-case scenario–tocilizumab cannot effectively treat symptoms of ICANS.

To reiterate–although driven largely by elevated levels of IL-1 and IL-6 in the CNS–ICANS is a separate phenomenon from CRS. Symptoms of decreased alertness or consciousness can progress to life threatening seizures. Similar to CRS, a substantial portion of patients who have ICANS experience symptoms that characterize grades ≥3 ICANS. Therefore, it is of high priority to not only treat symptoms of ICANS, but to prevent its onset. By definition, ICANS is preceded by CRS. Therefore–even if it is ineffective at treating ICANS–tocilizumab could prevent the emergence of ICANS by halting CRS progression.

Unfortunately, trials and studies have proven that tocilizumab can actually exacerbate the progression of ICANS [8, 9]. To explain this, elevated levels of IL-8 and MCP-1 in the CNS were also observed to be major factors in ICANS progression. Furthermore, recent data has shown an increased concentration of IL-6 in the CNS correlating with tocilizumab. Finally, in contrast to tocilizumab, IL-6 has displayed the ability to cross the blood-brain barrier. Therefore, it is possible that—by blocking sites of possible IL-6/IL-6R interactions on endothelial cells—tocilizumab forces excess IL-6 into the CNS. However, more research is needed to uncover the specific mechanisms that could explain tocilizumab's role in the onset of ICANS.

Regardless, the limitations of tocilizumab create a need for better treatments of CRS in patients. This need was answered in the form of IL-6 knockdown in the engineered T-cells. To achieve knockdown, multiple short hairpin RNA (shRNA) can be designed to target IL-6 mRNA transcripts [10]. Similar to tocilizumab, this knockdown of IL-6 did not change the anti-tumor efficacy of CAR T-cells. However, what makes this treatment option stand above tocilizumab and the rest was a striking downregulation of the concentration of IL-6 directly. Currently, the knockdown of IL-6 in CAR T-cells is under review via. clinical trials.

In individual case studies focused on the efficacy of ssCART19 cell therapy, only grade 1 CRS developed in the most severe cases. Therefore, knockdown of other proinflammatory cytokines–such as INF-γ–in adoptive T-cell therapy were also developed. Furthermore, the knockdown of IL-6 in CAR T-cells also resulted in observations of higher counts of CD19 CAR T-cells [11]. Theoretically, this would mean ssCART19 cell therapy also has improved efficacy against tumor cells. However–in patients with high tumor burden–not only did ssCART19 cells have limited efficacy on tumors, but a significant portion of patients developed sCRS despite knockdown of IL-6 [11].

Although more aggressive expansion of CAR T-cells is often correlated with higher tumor burden within patients, there is no significant difference in the count of ssCART19 cells between low-tumor-burden and high-tumor-burden patients [11]. Regardless–because tumor burden is indicative of the total amount of cancer in the body–higher tumor burden combined with higher counts of ssCART19 cells may lead to more pyroptosis. In turn, DAMPs become more crucial in the progression of CRS.

In specific studies, although ssCART19 did decrease the concentration of IL-6, it was not enough to lower the risk of CRS.

Therefore, another potential outlet for consideration is to deplete the CAR T-cells should any onset of CRS or other toxicities be detected. Although this avenue faced limitations in that the destruction of target CAR T-cells was rather slow, new research has greatly mitigated the problem through incorporation of CRISPR-Cas technology. However—even with CAR T-cell administration—long term survival in patients is already rare. In turn, inducing CAR T-cell death during early CRS will make long-term cancer remission an impossibility. Even with administration of suicide agents during the onset of grade 3 or grade 4 CRS symptoms, the signal cascades responsible for CRS may already be set in motion. As a result, patients that undergo CAR T-cell depletion could experience cancer relapse simultaneously to severe symptoms of CRS (Table 1).

Table 1. Overview of current treatment options for CRS prevention and their statuses in clinical development

Treatment	**Description**	**Status**
Tocilizumab	Prevention of CRS by inhibiting either bound or soluble forms of IL-6 receptor	FDA Approved Notable side effect: can exacerbate ICANS
Siltuximab	Directly inhibits IL-6 expression	In progress Shown results in individual cases, widespread applicability still need research
ssCART cells	Knockdown of proinflammatory cytokines in CAR T-cells using shRNA interaction with mRNA Cytokines include: ● IL-6 ● IL-1 ● INF-	Under review ssCART19 cells currently in clinical trials
CAR T-cell suicide	Inducing CAR T-cell death at the onset of any symptoms of CRS or other side effects such as ICANS	In progress Efficiency of technology has improved significantly using CRISPR-Cas systems

Overall, the prevention of CRS in CAR T-cell therapy remains difficult. However, treatment options such as ssCART19 cells are not only recent, but have already proven to be safer than tocilizumab. Furthermore, although incidence of CRS in CAR T-cell patients is still common, recent treatments have reduced the frequency significantly from 42-100 percent. Ideally, the future will only be marked by more research to make CRS in CAR T-cell therapy the exception.

6. Conclusion

In general, CRS is triggered by either the presence of pathogens or the byproducts of tissue damage and cell pyroptosis. Elevated levels of proinflammatory cytokines can either upregulate the function of white-blood cells or trigger further cytokine release. However, it is unclear whether CAR T-cell-produced cytokines or DAMPs from tumor cell pyroptosis are responsible for the onset of CRS in patients undergoing CAR T-cell therapy. This ambiguity is one of the prominent reasons why treatment options of CRS are so varied in their targets. The most effective example of treating CRS is the FDA approved drug tocilizumab. Yet, patients taking tocilizumab are at risk of experiencing

severe symptoms of ICANS. Meanwhile, the knockdown of cytokine production in CAR T-cells is effective in individual cases. However, this avenue displays limited efficacy for patients with higher tumor burden. Finally, options such as corticosteroids or CAR T-cell suicide can prevent CRS at the cost of the modified T-cells losing their efficacy. Still, with further research into the specific mechanisms of CRS progression induced by CAR T-cells, it can be predicted that only better treatments are to come through new avenues or a combination of current treatments.

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