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ARTICLE

The method for assessing the specificity of developing CAR therapies

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ABSTRACT The effectiveness of antitumor chimeric antigen receptor (CAR) therapy mainly dealt with an elevated sensitivity of CAR cells to target cells. However, CAR therapies are associated with nonspecific side effects: on-target off-tumor toxicity. Sensitivity and specificity of CAR cells are the most important properties of the recognition process of target cells among other cells. Current developments are mainly concentrated on exploring molecular biology methods for designing CAR cells with the highest sensitivity, while the problem of the CAR cell specificity is rarely considered. For the assessment of CAR cell specificity, we suggest that, in addition to an elevated level of CAR-antigen affinity, the ability of CARs for clustering should be taken into account. We assume that the CAR cell cytotoxicity is determined by CAR clustering. The latter is treated within the framework of nucleation theory. The master equation for the probability of CAR cell cytotoxicity is derived. The size of a critical CAR cluster is found to be one of two most essential parameters. The conditions for necessary sensitivity and sufficient specificity are explored. Relevant parametric diagrams are derived. Possible applications of the method for assessing the specificity of developing CAR therapies are discussed.

WHY IT MATTERS The sensitivity of antitumor therapies significantly increased with the advent of chimeric antigen receptor (CAR) technologies. Unfortunately, the specificity level is still a problem. The specificity of CAR cells is dealt with the accuracy of molecular pattern recognition. From the biophysical point of view, an ability for recognition is strictly determined by receptor clustering. This means that recognition can be treated in terms of nucleation theory. The critical size of receptor cluster on the surface of CAR cells is the main distinguishing factor between target and nontarget cells. The drawing of the state diagrams reflecting the CAR recognition probability is the main goal of the article. The mathematical expression for the specificity of CAR therapies is established.

INTRODUCTION

Recently, the attention of researchers and physicians has been focused on the capabilities of chimeric antigen receptor (CAR) technologies (1–3). With the advent of the first clinical results, enthusiasm among specialists has increased sharply (4–6). To a large extent, the effectiveness of tumor therapy is due to the fact that chimeric receptors have a higher affinity for the corresponding antigens than native T cell receptors. In other words, the higher sensitivity of CAR cells (CAR-C) to target cells is a consequence of the higher affinity of the corresponding receptors for the antigens present on the surface of target cells (7–9).

However, increased sensitivity to target cells, in some cases, is associated with nonspecific side effects (on-target off-tumor toxicity) (10–13). The latter is caused by the fact that some of the nontarget cells contain antigens on their surface (14–17). It is worth noting that the amount of antigens on nontarget cells can be noticeably less than on target cells (18,19); and yet, among tissues containing nontarget cells, it is customary to distinguish "critical tissue," damage to the cells of which by CAR-C leads to the most serious iatrogenic consequences (14,15).

In this regard, it is of great interest to create such CAR technologies that would allow the maximum damage to target cells with minimal damage to the cells of critical tissues (20,21). Ideally, the CAR-Cs being developed should not only have a high sensitivity to target cells, but also a low sensitivity to critical tissue cells; that is, be highly specific to target cells

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(22–24). Finding CAR-T and CAR-NK that meet these requirements is the goal of the most promising developments in this area (25–27).

Sensitivity and specificity of CAR-Cs are the most important properties of the recognition process by CAR-Cs of target cells among other cells, particularly among the cells of "critical tissue." Current developments are mainly concentrated on exploring molecular biology methods for designing a highly sensitive CAR-C system, while the problem of the specificity of the CAR-C approach seems to be beyond the scope of molecular biology (28,29). In the present article, the CAR-C system specificity is assessed with the aid of some additional ideas from the general theory of recognition (30–32).

It is well known, that the recognition of target cells by T lymphocytes crucially depends on the ability of the receptors on their surface to cluster (33–37). Receptor clustering was also shown for CAR cells (20,38–41). Receptor clustering may be considered from the point of view of the phase transition theory (42,43). Therefore, receptor clustering can be treated within the framework of the nucleation theory (44–46). The main idea of the presented approach is that the CAR-C cytotoxicity is directly related to CAR clustering. In other words, the relation between CAR-C cytotoxicity and CAR clustering is postulated. In this regard, the probability of specific recognition of target cells by CAR-C, as well as of nonspecific damage to cells of critical tissues must be assessed. In this work, we develop an approach for the calculation of CAR clustering probability, which we consider as a measure of specific and nonspecific recognition.

The master equation for the probability of cytotoxicity dependence on kinetic parameters characterizing recognition processes is derived. Corresponding parametric diagrams are constructed. It is demonstrated that a variation of the recognition and/or clustering parameters must be followed by a change in sensitivity and specificity of the relevant CAR-C system. Taking into account the parameters of the target and critical tissues, the conditions for sufficient sensitivity and specificity are established. These conditions are used to determine all types of the possible target-critical pair configurations. Relevant mapping of these configurations on the parametric plane results in assessing the specificity as well as the sensitivity of the CAR-C system. Possible applications for the development of new CAR-C systems are discussed.

METHODS

Within the framework of the developed approach, we will assume that, for any act of CAR-C cytotoxicity, the formation of a certain number of large CAR clusters M_c is required. The relevant differential equation that describes the dynamics of the number of antigen-containing cells (ACCs) $N_t(t)$ follows from the well-known generalized law of mass action (47,48) . It has the form:

$$\frac{dN_t(t)}{dt} = -\frac{J}{M_c N_h} N_{CAR} N_t(t) \tag{1}$$

where J denotes the number of large clusters assembling on one CAR-C per unit time, N_{CAR} denotes the amount of CAR-C in the system considered, N_h denotes the total number of cells in the system considered.

Currently it has been experimentally established that the formation of large CAR clusters can be of a threshold manner (49,50). In this regard, theoretical treatment of receptor clustering (45) revealed that the probability of the formation of supercritical receptor clusters significantly depends on the von Neumann entropy, H_L, of the cluster's connection graph (51). In particular, the value of parameter J (in Eq. 1) is determined by the properties of the nucleation process of receptor clustering. In these processes, antigens play a role of initiation sites for heterogenous nucleation (44,52).

Analysis of nucleation processes revealed the dependence of J on ACC and CAR-C characteristics (see Eq. A1.21 in Appendix A1). The ratio of ACCs at time t_m to initial number of ACCs ($1 - N_t(t_m)/N_t(0)$) below will be associated with the elimination probability P of the ACCs. The corresponding mathematical equation is known in the stochastic theory as an exponential distribution with parameter $e^{-\lambda}/\lambda$ (53–55): It has the form (see Appendix A1):

$$P = 1 - \exp\left(-\tau \frac{e^{-\lambda}}{\lambda}\right) \tag{2}$$

where λ denotes normalized critical cluster size and τ is the dimensionless characteristic time of ACC death. Relevant values of λ and τ are found to be determined by the expressions:

$$\lambda = \frac{1}{d}\left(\frac{3\pi C_s}{C(1 + \mu_{Ar} K_{Ar}[A])}\right)^d \tag{3}$$

$$\tau = \frac{4\pi DSC^2}{M_c d \ln(SC_c)} \frac{N_{CAR}}{N_h} t_m \tag{4}$$

where d corresponds to the parameter that has the meaning of a "kinetic" dimension, depending on the shape and structure of clusters (45). Asymptotically, the value of parameter d is determined by:

$$\frac{d-1}{d} = \frac{H_L(\lambda d)}{\ln(\lambda d)} \tag{5}$$

where C is the CAR concentration on the CAR-C surface, C_s is the saturated concentration of CAR on the CAR-C surface, C_c is the surface concentration of CAR in a cluster, $[A]$ is the concentration of antigen on the ACC surface, K_{Ar} is the effective affinity constant of antigen from ACCs and CAR from CAR-C, μ_{Ar} is the relative increase in effective (oscillatory) CAR mass due to antigen association, D is the CAR diffusion coefficient on the CAR-C surface, S is the surface of CAR-C (see Appendix A1 for details).

The equations above can be used to assess both the sensitivity and specificity of the biological recognition processes of ACCs by CAR-C. It should be noted that, in cases where recognition receptor systems acquire increased sensitivity, the likelihood of detecting their "side" targets increases. Therefore, CAR-C needs to solve at least dual-objective problems: destruction of the target cells themselves, which represent a threat to the body, with minimal damage to the critical tissues of the body.

The primary goal of CAR-C therapy is to eliminate target cells. So, the rate at which target cells are killed due to CAR-C cytotoxicity must be at least higher than the rate at which the population of

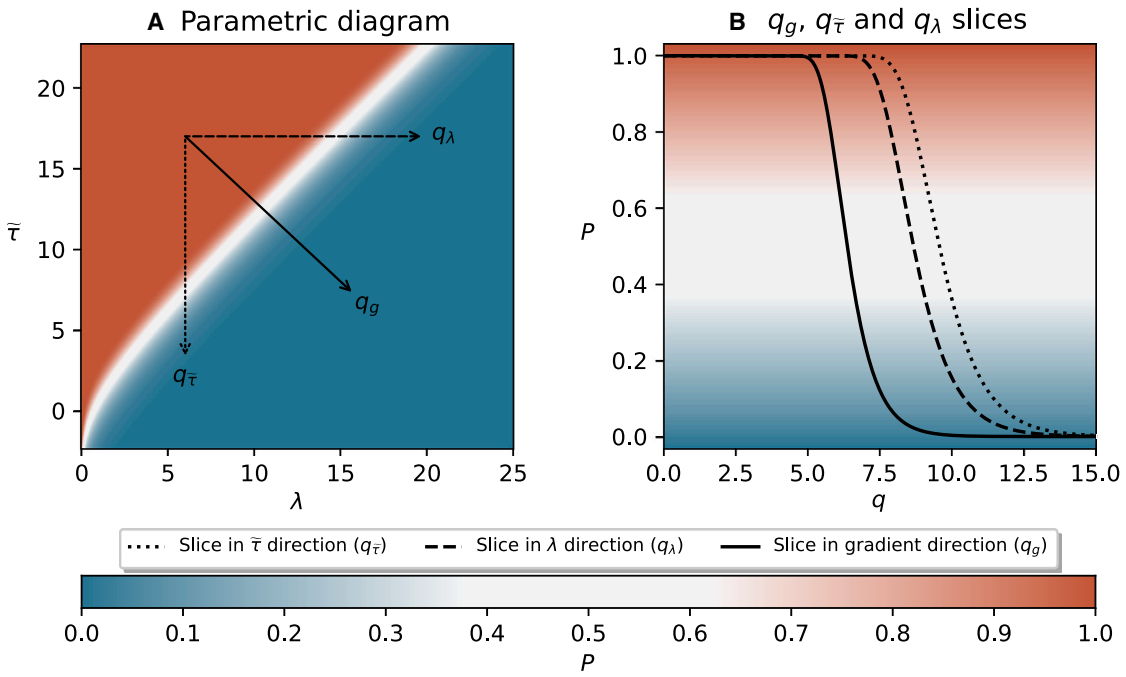

FIGURE 1 Parametric diagram of the probability of ACC elimination. (*A*) A parametric diagram in $\lambda, \tilde{\tau}$ axes, on which two zones are distinguished: logarithmic for small λ (dominated by $1/\lambda$ from Eq. 2) and linear for large λ (dominated by $e^{-\lambda}$ from Eq. 2). The color indicates the probability of ACC being destroyed *P*: red, close to 1; white, close to 0.5; blue, close to 0. Three arrows indicate the different directions of change in the parameters: the dotted line represents a change only in $\tilde{\tau}$, the dashed line represents a change only in λ and the solid line represents a change in the direction of the antigradient of probability. (*B*) The slices of the parametric diagram on (*A*) in the direction of different changes in parameters. The color indicates the probability of the ACC being destroyed *P*: red, close to 1; white, close to 0.5; blue, close to 0. Three curves indicate the different probabilities of ACC elimination dependence on change in the parameters: the dotted line represents a change only in $\tilde{\tau}$, the dashed line represents a change only in λ, and the solid line represents a change in the direction of the antigradient of probability. Ranges of the plot correspond to theoretical ranges pointed out in Appendix A2.

target cells increase due to division. This means that the probability of target cell elimination during the doubling time (τ_t) should be at least more than ½. This requirement for the sensitivity of pathogen recognition by the CAR-C system can be written as follows:

$$P(\lambda_t, \tau_t) > 1/2 \tag{6}$$

The secondary goal of therapy implies the minimizing of side effects. At least this means that the permitted probability of critical tissue cell damage during the time of CAR-C active circulation (τ_c) should be smaller than a certain critical level ω. The latter characterizes the specificity of the CAR-C system. This requirement for the specificity of the CAR-C system can be written as follows:

$$P(\lambda_c, \tau_c) < \omega \tag{7}$$

In this sense, the development of new generations of CAR-C systems supposes maximizing their sensitivity while simultaneously ensuring a guaranteed level of safety (minimal nonspecific harm). From this point of view, the tasks facing CAR-C developers seem related to the problems of finding a minimax (56,57).

RESULTS

Parametric diagram

Below we denote the value of τ logarithm as $\tilde{\tau} = \ln \tau$. The dependence of ACC elimination probability, *P*, as a function of the parameters $\tilde{\tau}$ and λ is graphically presented as the color plot diagram in Fig. 1.

It is easy to see that the parametric plane contains two large areas (a blue one and a red one) that correspond to the probability level close to zero and unity, respectively. At the same time, the margin area corresponding to probability approximately equal to ½ is very narrow. This means that the CAR-C recognition system considered demonstrates strong switch-on switch-off properties.

Target-critical pair

The diagram presented in Fig. 1 *A* allows one to compare the probabilities of receptor clustering under the influence of antigens present on target cells as well as on critical tissue cells. In both cases antigen influence manifests itself in the values of parameter λ. Moreover, the aim of creating CARs implies that the critical cluster size for target cells (λ_t) must be smaller than for the corresponding critical tissues (λ_c). In other words, the values plotted on Fig. 1 along the abscissa axis λ_t should always lie to the left of the corresponding λ_c values.

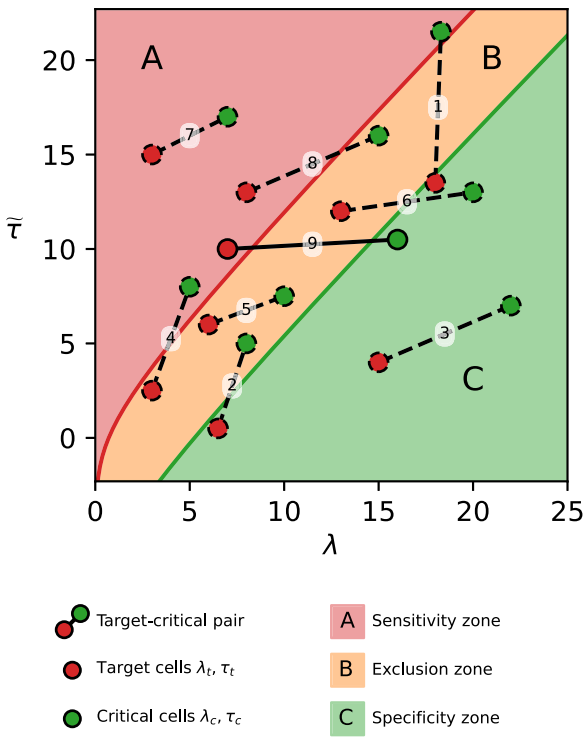

FIGURE 2 Target-critical pair configurations. Target-critical pairs are denoted as two circles connected with a line. Respective numbers are pointed out in the middle. Red circles correspond to the parameters of target tissue cells (λ_t,τ_t). Green circles correspond to the parameters of critical tissue cells (λ_c, τ_c). The light red zone corresponds to the parameters (λ, τ) where the CAR-C system has necessary sensitivity (see Eq. 6). The light green zone corresponds to the parameters (λ,τ) where the CAR-C system has sufficient specificity (in accordance with Eq. 7). The orange zone corresponds to all of the parameters (λ, τ) where the system is in transient state. Ranges of the plot correspond to theoretical ranges pointed out in Appendix A2.

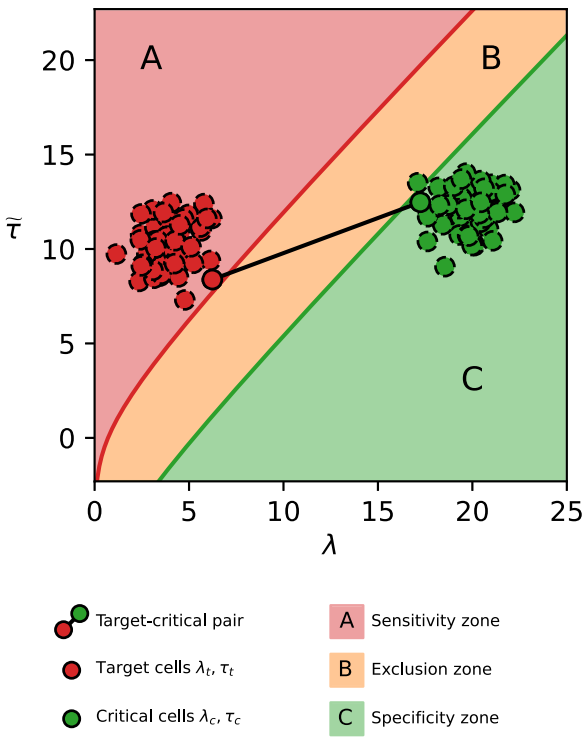

FIGURE 3 Target and critical tissue heterogeneity. Target cell parameters are shown as individual red circles. Critical cell parameters are shown as individual green circles. The indicative target-critical pair is shown with the solid line connecting cells with representative parameters. The light red zone corresponds to the parameters (λ,τ) where the CAR-C system has necessary sensitivity (see Eq. 6). The light green zone corresponds to the parameters (λ, τ) where the CAR-C system has sufficient specificity (in accordance with Eq. 7). The orange zone corresponds to all of the parameters (λ,τ) where the system is in transient state. Ranges of the plot correspond to theoretical ranges pointed out in Appendix A2.

On the other hand, times τ should be calculated for target and critical tissues in different ways. Target cell time is determined by their doubling time τ_t, while for critical cells time is determined by the active circulation of the CAR-C in the patient τ_c. In general, for aggressive tumors τ_t is always smaller than τ_c.

Below we consider several presumably interesting situations. First of all, let us start from the simplest situation, when we restrict ourselves to one target and one critical tissue. In Fig. 2 all of the principally possible target-critical pair configurations are shown. It is easy to see that only one of them (denoted with a number 9) corresponds to CAR-C systems that are both sensitive and specific.

Target and critical heterogeneity

In a practical setting, no cells of any tissue are characterized by exactly the same parameters. For example, the surface concentration of any antigen may vary significantly (58). In accordance with Eq. 3, which leads to a change in the λ value. Another example concerns cells that are less or more accessible by CAR-C (59,60). This leads to a change in the τ value. In a general case, the corresponding diagram for heterogenous tissues is shown in Fig. 3.

Selection of the indicative target-critical pair

Taking into consideration that even one clone can survive CAR-C therapy and give rise to a relapse (61), it is imperative that all of the target tissue parameters (*all red circles*) must lie in the light red zone of sufficient sensitivity. On the contrary, constraint for the specificity of CAR-C systems is determined by the overall elimination ratio ω. Therefore, even if some small subset of critical tissue gets completely eliminated, the overall elimination ratio could still be less than ω. Thus, small outlier subsets for critical tissue may be ignored (e.g., one *green circle* in exclusion zone B in Fig. 3 should not be taken into account). After that,

the closest to the exclusion zone circles may always be selected both in target and in critical tissues. These two circles represent the most indicative target-critical pair, which characterize the sensitivity as well as the specificity of CAR-C systems with respect to heterogeneous tissues.

DISCUSSION

Numerous publications are devoted to the problem of creating highly sensitive CAR systems (62–64). At the same time, the question of the specificity of CAR systems being developed remains out of sight. However the actual magnitude of side effects is clarified at the stages of in vivo biological tests and clinical trials (12,65–67). This means that, currently, the degree of specificity of CAR systems being developed is estimated only by a posteriori complications.

It should be noted that the problem of finding the least "traumatic" CAR systems is a rather complex and costly one (68,69). The costs of developing systems with appropriate specificity are higher the more side effects are revealed at later stages of testing (70). As several research groups demonstrated, detection of side effects at the stage of in vivo biological testing, rather than at later stages of clinical trials, can significantly reduce overall development costs (28,29). Moreover, it is clear that any assessment of side effects at prebiological stages of CAR-C system development should reduce the costs by orders of magnitude. Thus, many studies are currently being conducted aimed at reducing the side effects of CAR-C therapy (28,58,71–74).

In this regard, the probability of specific recognition of target cells by CAR-C, as well as of nonspecific damage to cells of critical tissues must be assessed as early as possible. Our approach is substantially based on the fact that the processes of recognition of target cells by CAR-C crucially depend on the ability of the receptors on their surface to cluster (20,39–41). Receptor clustering is often treated from the point of view of the phase transition theory in accordance with the ideas of Rosen (43,75,76). For this reason, we considered receptor clustering in CAR-C systems within the framework of the nucleation theory (44,45). As a result, we obtained the method for the calculation of CAR clustering probability, which we consider as a measure of specific and nonspecific recognition.

The analysis performed is based on the assumption that the processes of CAR-C recognition of ACCs as targets are significantly determined by nucleation in receptor clustering. This assumption seems reasonable since receptor clustering during the recognition process is well established (33,35,37,77,78). From a thermodynamic point of view, receptor clusters were previously considered as a separate phase. Corresponding thermodynamic conditions for the equilibrium are studied (43,79,80). The coexistence of two separate phases (free receptors and clustered receptors) for a long time implies that the transition between phases is of the first order (52,81,82). As a consequence, the transition from free receptors to the clustered receptors phase becomes possible to be treated in terms of nucleation theory (45,46).

As far as the authors know, the approaches of the general nucleation theory were never applied to CAR-C systems. Using the concepts of the general nucleation theory we derive several expressions that establish a connection between CAR-C system parameters and the probabilities of CAR-C cytotoxicity to target tissue and to nonspecifically affected critical tissues (Eqs. 2, 3, 4, and 5).

Several limitations to the present study deserve mention. Within the framework of the model no spatial variation of CAR or target cells was taken into account. Probably this would be rather limiting, for instance, in the context of metastatic and solid cancers (83,84). The relevant spatiotemporal extensions to this model seem to be the next stage of the developed approach.

Cluster formation is a rather complex biological process. Underlying mechanisms of clustering are still under investigation (85,86). Not only the values of parameters but also the relevant impact of different biological processes should be determined. Particularly, several known underlying biological processes that impact CAR cluster formation remained outside the scope of the model. At least, intracellular proteins (such as membrane actin-myosin or the cytoskeleton) (87–89), contractile mechanisms of dynamic polar filaments (90,91), electrostatic interactions between proteins and lipids (92,93), obstruction by ligands, and variation in membrane lipid order (94,95), and precise nanoscale spatial organization of individual receptors and receptor clusters (96,97) are worth further theoretical analysis.

Within the framework of the developed approach, we consider the simplest situations when movement of receptors occurs in an essentially 2D membrane system. However, membrane curvature can play an important role in initiating an immune response (e.g., during the formation of the immunological synapse) (98,99). Analysis of membrane curvature influence on receptor-receptor interactions is a question of some current theoretical descriptions (100,101). It seems that, on curved membranes, receptor clustering should take place easier than on flat ones. In terms of the parameters of the model considered it should be reflected as a decrease of λ value.

Also, it should be mentioned that in derivation of the mathematical expressions, we limited ourselves to the "linear approximation" of CAR-C cytotoxicity on the number of CAR clusters (see Eq. 1). This means that we left aside the effects that dealt with the interaction of different nucleation clusters in the processes of "focusing" CAR-C on the corresponding targets (40). Despite the apparent simplicity of the developed approach, it has a number of advantages. In particular, it opened new possibilities for obtaining a priori estimates for both sensitivity and specificity at the early stages of CAR systems development. Furthermore, the approach seems to be extendable for additional effects in the future, including the effects of multicenter nucleation during "focusing."

The developed approach makes it possible to evaluate specificity already at the in vitro cellular stage of CAR system development. Conditions (6) and (7) contain only five parameters: λ_t, λ_c, τ_t, τ_c, and ω, among which ω is determined by therapeutic reasons. In many cases the probable toxicity effects on critical tissues is known to clinical professionals before any molecular engineering procedures of CAR-C system start (102). This means that, in the developed approach, only four parameters (λ_t, λ_c, τ_t, and τ_c) are essential for the assessment of sensitivity and specificity.

In essence, these four parameters (λ_t, λ_c, τ_t, and τ_c) may be considered as true indicative parameters of any CAR-C system. The value of λ characterizes the critical nucleation size of receptor clusters while the value of τ denotes the dimensionless exposition time. In accordance with Eq. 6, the value τ_t reflects the target cell doubling time. The wide spectra of inhibitors of a cell cycle increase the doubling time (103). In terms of the suggested approach, this means that the corresponding τ_t should also be increased by the influence of all known inhibitors. Using the diagram presented in Fig. 1, it is easy to see that the probability of damage of target cells is increased when the value of τ_t increases.

The suggested approach incorporates the idea of receptor clustering. In particular, the value of λ reflects the critical nucleation receptor size. Considering the diagram presented in Fig. 1, it is easy to conclude that diminishing of λ leads to an increase of the elimination probability. On the contrary, the growth of λ is followed by the decreasing of the elimination probability. This is true for the CAR clusters initiated both by target cells and by critical tissue cells. Thus, for the achievement of sufficient specificity, as follows from Fig. 2, it is necessary to realize the condition $\lambda_c > \lambda_t$. This means that the clustering of CARs is at least as important as the temporal characteristics of cell cycle (doubling and circulation times).

It is easy to see from Eqs. 3 and 4 that the values of relevant parameters depend on several molecular and kinetic characteristics of receptors. Some of these dependencies are shown qualitatively in Fig. 4. Molecular and kinetic characteristics can be routinely determined based on traditional cell biology procedures. Indeed, one of these widely used procedures is

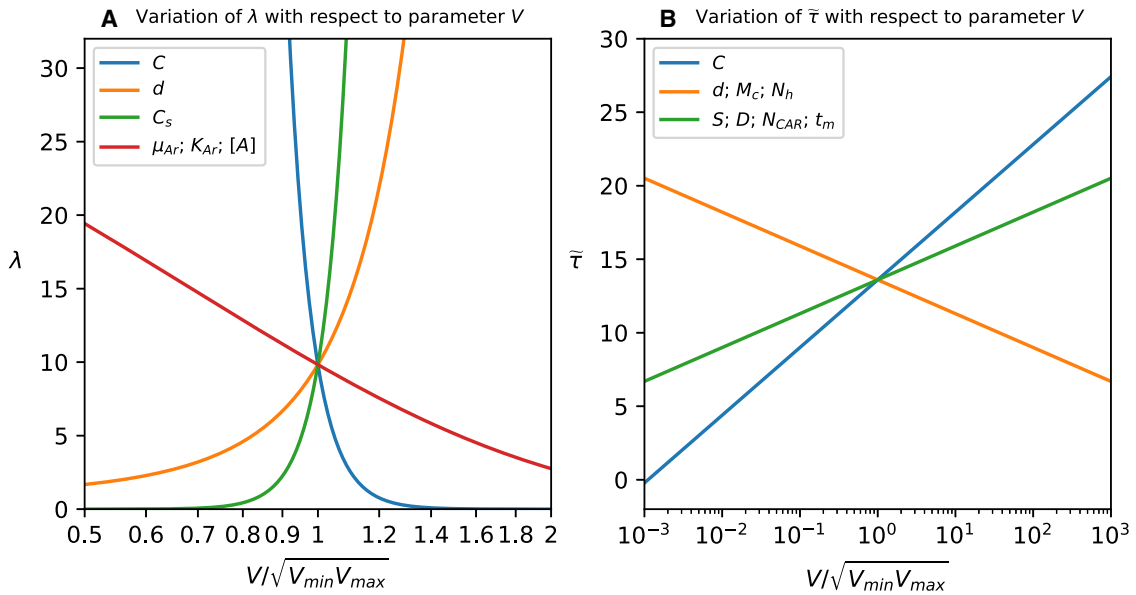

FIGURE 4 Variation of critical cluster size λ (*A*) and cell death time $\tilde{\tau}$ (*B*) as a function of the model parameters. The variations of critical cluster size λ and cell death time $\tilde{\tau}$ are shown as a function of the model parameter *V*. Different colors are used for the cases, when *V* denotes certain model parameters (*C*, C_s, μ_{Ar}, etc.). The values of λ and $\tilde{\tau}$ are shown alongside the *y* axis, while the values of $V/\sqrt{V_{min}V_{max}}$ are shown alongside the *x* axis. The minimal (V_{min}) and maximal (V_{max}) values of the parameter are pointed out in Appendix A2.

the assessment of cytotoxicity (104–106), which implies that labeled target cells are mixed with the developed CAR-C. Then, after a period of time, t_m, the concentration of the label substance is measured, determining the proportion of target cells destroyed. The experimental points are possible to be fitted with the aid of the Eq. 2 describing a probability of cytotoxicity.

Among the molecular and kinetic characteristics of the receptors involved in Eqs. 3 and 4 there are some that seem to be more easily varied in relevant experimental settings. Let us consider the exposition time, t_m. To change the t_m value it is sufficient to measure the concentration of the label substance in the cytotoxicity assessment at several moments (106). After that, the sequence received should be used for the fitting of Eqs. 2 and 3 and finding the dependence of τ on t_m. Substituting the value of the target cell doubling time as the exposition time, t_m, the calculation of the value of τ_t becomes possible. In a similar way the value of τ_c could be calculated based on CAR-C active circulation time.

Subsequently, one can use fitting for Eqs. 2, 3, and 4 for finding how λ depends on surface antigen concentration $[A]$. To change $[A]$ it is sufficient to sort labeled target cells using a cell sorter, and several cytotoxicity assessments can be performed on the resulting subsets (58,74,107). The concentrations $[A]_c$ and $[A]_t$ for critical and target tissues can be measured using a flow fluorimeter (108,109). As a result, it becomes possible to calculate the values of λ_t, λ_c. This means that all the necessary parameters for assessing the CAR-C sensitivity, as well as specificity, can be obtained at the earlier stages of CAR-C system development. That is, before any in vivo testing.

Currently, several studies are being conducted aimed at reducing the toxicity side effects of CAR-C therapy (28,58,71–74). Within the framework of the developed method, almost all of the research studies deal with reducing $\mu_{Ar}K_{Ar}$ in the expression for λ (see Eq. 3). One of the most promising is the choice of low-affinity antibodies for creating CARs (28,71), corresponding to a decrease of affinity constant K_{Ar} in Eq. 3. New generations of CARs (third and higher) have as their main goal a reduction in τ_c, allowing CARs to be "turned off" in the case of side effects or total elimination of target cells (62,110,111). In addition to this, adding new domains to CAR leads to a decrease in μ_{Ar}, which has a similar effect to a decrease in K_{Ar}. Noticeably, greater sensitivity to K_{Ar} compared with τ in expression (2) gives a reason to believe that the main advances in the specificity of new generations of CARs are associated precisely with the latter.

The main drawback of current efforts to improve specificity by reducing $\mu_{Ar}K_{Ar}$ is that it greatly reduces sensitivity. From expression (3) it is clear that this leads to a decrease in λ_c/λ_t, and therefore makes it more difficult to separate critical and target tissue (see Fig. 2). That is, achieving the required specificity in this way will lead to a catastrophic drop in sensitivity.

Thus, it is clear that, in principle, there are several different ways for influencing the critical characteristics of the CAR-C recognition processes. In accordance with Eqs. 2, 3, 4, and 5, most of them might lead to necessary sensitivity and sufficient specificity of the developing CAR-C system. The presented mathematical equations precisely express the relative influence of different critical tissue and target cell parameters on the values of specificity of the developing CAR-C system. It should be noted that "the cost" of variation of each parameter is known only to researchers involved in a certain experimental situation. So, the practical selection of the effective way for increasing the specificity should be based on theoretical, experimental and commercial aspects.

Based on the developed approach we can give minimal recommendations to bioengineers.

1) In addition to determination of doubling times for the target cells and exposition times for nontarget cells, it is strongly recommended to take into account the ability of CARs to cluster (20,38–41).
2) Determine the most vulnerable (critical) tissue for the CARs investigated.
3) Calculate the size of the CAR critical nucleus for target and critical tissue cells (λ_t, λ_c) using Eq. 3 (for details, see Appendix A2).
4) Select those CARs among developing samples that have the largest margin in λ values between critical and target cells.

We hope that the suggested approach may be effectively used by bioengineers for a significant reduction of the overall CAR-C developing cost.

APPENDIX A1. DERIVATION OF THE FORMULA FOR THE FLOW OF SUPERCRITICAL CLUSTERS

The growth of a receptor cluster consists of elementary acts of formation and destruction of bonds between receptors. The probability of breaking each connection per unit time only requires the overcoming of a potential barrier; that is, it does not depend on the size and configuration of the cluster. The probability of the formation of a new bond requires overcoming a kinetic barrier; that is, on the contrary, it depends on the size and configuration of the cluster.

In a quantitative sense, this means that at a minimum to add each new receptor to the cluster a kinetic energy barrier ($\varepsilon_k k_B T$) must be overcome (overcoming the kinetic barrier is a necessary condition for cluster growth) (45):

$$\varepsilon_k k_B T = \frac{6\pi}{1+\mu_{Ar}K_{Ar}[A]} \frac{\exp H_L}{D_m N} \varepsilon_p k_B T \qquad \text{(A1.1)}$$

where D_m is the dimension of the CAR motion area CAR, H_L is the von Neumann entropy of the receptor connection graph in the cluster, N is the number of receptors in the cluster, μ_{Ar} is relative increase in effective (oscillatory) CAR mass due to antigen association, K_{Ar} is the antigen-receptor association constant, $[A]$ is the concentration of the antigen, $\varepsilon_p k_B T$ is the bond breaking energy, k_B is Boltzmann's constant, T is the absolute temperature.

The value of $\mu_{Ar}K_{Ar}[A]$ reflects the transversal contribution of antigens to the kinetic barrier. In this work it will be assumed that the movement of receptors occurs on a flat membrane, that is $D_m = 2$. Let us introduce the value ρ inversely proportional to the average increase in receptor mass due to association with the antigen (45):

$$\rho = 3\pi / (1 + \mu_{Ar}K_{Ar}[A]) \tag{A1.2}$$

Association of antigens with CARs reduces the latter's ability to move, which, from the point of view of energy redistribution across degrees of freedom, should lead to a decrease in ρ, and therefore to a decrease in ε_k.

The value of the von Neumann entropy of the graph of receptor connections in a cluster included in expression Eq. A1.1 at $5 < N < 300$ can be approximated as follows (45):

$$H_L = \frac{d-1}{d} \ln N \tag{A1.3}$$

where d is the parameter that has the meaning of a "kinetic" dimension, depending on the shape of the clusters (45).

Corresponding rates of receptor detachment and attachment to cluster v^- and v^+ are given by expressions:

$$v_N^- = k_- s_N^- \exp(-\varepsilon_p) \tag{A1.4}$$

$$v_N^+ = k_+ s_N^+ \exp(-\varepsilon_k(N) + \varepsilon_f) \tag{A1.5}$$

where $\varepsilon_f k_B T$ is the energy released during the formation of a bond (energy difference between bound and free states of receptors), $s_{\bar{N}}$ and s_N^+ are the cross-sectional areas of the reactions of detachment and addition of individual receptors in a cluster consisting of N receptors. In what follows, unless otherwise stated, the cross sections s_{N+1}^- and s_N^+ will be assumed to be equal.

To analyze the degree of supersaturation of the receptor system, we will find the saturated concentration of receptors. Let us consider a fairly large receptor cluster whose kinetic barrier is lower than the potential one. In this case, the potential barrier acts as a limiting factor in the process of adding a new receptor to the cluster. The expression for the rate of this process has the form Eq. A1.6:

$$v_N^+ = k_+ s_N^+ \exp(-\varepsilon_p + \varepsilon_f) \tag{A1.6}$$

Saturated concentration C_s determined from the balance condition $v^- = C_s v^+$. Taking into account expressions Eqs. A1.4 and A1.6 for the value of C_s we have:

$$C_s = \frac{k_-}{k_+} \exp(-\varepsilon_f) \tag{A1.7}$$

Let us consider a quasistatic approximation of the size distribution of subcritical clusters. For this purpose, let us introduce the critical cluster size, N_c, which separates subcritical and overcritical clusters. Then for each cluster size $1 \le N \le N_c$ we can write down the dynamic equilibrium condition n clusters $n_{N+1}v_{N+1}^- = Cn_N v_N^+$, after substitution Eqs. A1.1, A1.3, A1.4, and A1.5 we obtain:

$$\frac{n_{N+1}}{n_N} = \frac{C}{\rho C_s} \sqrt[d]{N} \tag{A1.8}$$

where C is the concentration of receptors on the membrane of CAR cells.

For values $N < N_c$ the expression on the right side of Eq. A1.8 is less than 1; that is, the larger their size, the smaller the number of clusters. The value N_c essentially plays the role of the required number of receptors in the cluster that is necessary to induce the formation of a stable cluster capable of downstream activation signaling. It can be represented in the form:

$$N_c = (\rho C_s / C)^d \tag{A1.9}$$

By multiplying the ratios (Eq. A1.8) for all $1 \le n \le N$ and the number of free receptors SC_r (where S is the CAR-C surface area) we can obtain a quasistatic distribution of cluster sizes:

$$\begin{aligned} f_0(N) = SC_r \prod_{n=1}^{N} \frac{C_r}{\rho C_s} \sqrt[d]{n} &= SC_r \prod_{n=1}^{N} \sqrt[d]{\frac{n}{N_c}} \\ &= SC_r \sqrt[d]{\frac{N!}{N_c^N}} \end{aligned} \tag{A1.10}$$

Substituting the Stirling approximation into Eq. A1.10, we obtain the final expression:

$$f_0(N) \approx SC_r \exp\left(-\frac{N}{d}\left(1 + \ln\frac{N_c}{N}\right)\right) \tag{A1.11}$$

Using this quasistationary distribution, we will estimate the flow of supercritical clusters using the procedure described by Frolov et al. (82). To do this, let us move on to the continuous number of receptors in a cluster and write the Fokker-Planck equation (81) for the kinetic distribution of cluster sizes $f(N,t)$:

$$\frac{\partial f}{\partial t} = -\frac{\partial j}{\partial N} = -\frac{\partial}{\partial N}\left(-B\frac{\partial f}{\partial N} + Uf\right) \tag{A1.12}$$

where j is the flow of supercritical clusters, B is the diffusion coefficient of the cluster size, U is the speed of movement of clusters in the size space. The relationship between B and U can be found in equilibrium at $j = 0$:

$$U = \frac{B}{f_0}\frac{\partial f}{\partial N} \tag{A1.13}$$

For a quasistationary flow of supercritical clusters ($j = const$) we obtain from Eqs. A1.12 and A1.13:

$$j = -Bf_0 \frac{\partial}{\partial N}\frac{f}{f_0} \tag{A1.14}$$

By integrating the expression Eq. A1.13 and using the limit values $f/f_0 \to 1$ at $N \to 1$ (equilibrium) and $f/f_0 \to 0$ at $N \to \infty$ (since f is finite, and $f_0 \to \infty$) we obtain:

$$\frac{1}{j} = \int_1^{\infty} \frac{dN}{Bf_0} \approx \sum_{N=0}^{\infty} \frac{1}{Bf_0} \tag{A1.15}$$

The function under the sum has a sharp peak near the critical size, using expression Eq. A1.11 we obtain:

$$j = \frac{B(N_c)}{N_c} f_0(N_c) \tag{A1.16}$$

Using the equilibrium diffusion rate, we obtain the rate of increase in cluster size depending on the size:

$$U(N) = 2\pi D \frac{C_r - C_s(N)}{\ln(r_*/r(N))} \quad \text{(A1.17)}$$

where D is the diffusion coefficient of receptors on the membrane, $C_s(N)$ is the saturated concentration of receptors near a cluster of size N, r_* is the cutoff radius (comparable with the cell size), $r(N)$ is the characteristic radius of a cluster of size N.

The saturated concentration of receptors near a cluster of size N is easily found from expression Eq. A1.9 with $N_c = N$ and $C_r = C_s(N)$:

$$C_s(N) = C_r \sqrt[d]{N_c/N} \quad \text{(A1.18)}$$

The cutoff radius can be estimated as the root of the cell area $\sqrt{S}$, and the characteristic cluster radius as $\sqrt{N/C_c}$, where C_c is the concentration of receptors in the cluster. Substituting these estimates together with Eq. A1.18 into Eq. A1.17 we obtain:

$$U(N) = 4\pi D C_r \frac{1 - \sqrt[d]{N_c/N}}{\ln(SC_c/N)} \quad \text{(A1.19)}$$

Substituting this expression into Eq. A1.13 and taking the limit at $N \to N_c$ we obtain an expression for the diffusion coefficient by size near the critical size:

$$B(N_c) = \frac{4\pi D C_r}{\ln(SC_c/N_c)} \quad \text{(A1.20)}$$

The expression under the logarithm SC_c means the number of receptors in a cluster covering the entire surface, obviously many orders of magnitude greater than the critical cluster size N_c for cases where clustering is, in principle, possible. Because of this, $\ln(SC_c/N_c) = \ln(SC_c) - \ln(N_c) \approx \ln(SC_c)$.

Substituting expressions Eqs. A1.11 and A1.20 into A1.16 we obtain the final expression for the flow of supercritical clusters:

$$J = \frac{4\pi D S C^2}{N_c \ln(SC_c)} \exp\left(-\frac{N_c}{d}\right) \quad \text{(A1.21)}$$

Let us write a differential equation describing the dynamics of the number of target cells $N_t(t)$:

$$\frac{dN_t(t)}{dt} = -\frac{j}{M_c N_h} N_{CAR} N_t(t) \quad \text{(A1.22)}$$

where M_c is the average number of clusters required to trigger CAR-C cytotoxicity, N_{CAR} is the number of CAR-C in the body, N_h is the number of cells in the body.

Having solved the differential equation(Eq. A1.13), we write down the probability of each individual cell to be eliminated $P = 1 - N_t(t_m)/N_t(0)$ for time t_m:

$$P = 1 - \exp\left(-\frac{J}{M_c}\frac{N_{CAR}}{N_h} t_m\right) \quad \text{(A1.23)}$$

Let us introduce the dimensionless characteristic time of cell death τ and the reduced size of the critical cluster $\lambda = N_c/d$:

$$\tau = \frac{4\pi D S C^2 C_r^2}{M_c d \ln(SC_c)} \frac{N_{CAR}}{N_h} t_m \quad \text{(A1.24)}$$

Substituting Eqs. A1.12 and A1.15 into A1.14 we obtain the final equation for the elimination probability from two parameters:

$$P = 1 - \exp\left(-\tau \frac{e^{-\lambda}}{\lambda}\right) \quad \text{(A1.25)}$$

APPENDIX A2. THEORETICAL RANGES OF THE MODEL PARAMETERS

TABLE A1 Parameter values from the literature

Parameter	Value range $V_{min} \sim V_{max}$	Description	Data sources
C	$30 \sim 200\ \mu m^{-2}$	the concentration of CAR on the surface of CAR-C in rest	see S value and the overall number of CARs found in (74)
C/C_s	$2 \sim 10$	oversaturation of CAR on the CAR-C	this could be estimated as the part of receptors in the cluster after activation (97)
C_c	$10^3 \sim 10^5\ \mu m^{-2}$	the concentration of CAR in the cluster	Al-Aghbar et al. (98)
$[A]_t$	$50 \sim 300\ \mu m^{-2}$	the concentration of antigens on target cells surface	the surface value is taken from (112), while the amount of antigens from (58)
$[A]_c$	$20 \sim 150\ \mu m^{-2}$	the concentration of antigens on critical tissue cells surface	the surface value is taken from (112), while the amount of antigens from (113)
d	$13 \sim 15$	kinetic dimension of CAR oscillations on the surface of CAR-C	Prikhodko and Guria (45)
K_{Ar}	$10^{-5} \sim 10^{-1}\ \mu m^2$	affinity constant between CAR and antigen	He et al. (114)
μ_{Ar}	$0.2 \sim 4$	relative increase in effective (oscillatory) CAR mass due to antigen association	real masses from protein database were taken from (115)
D	$3 \sim 30\ \mu m^2/s$	2D CAR diffusion coefficient on the CAR-C surface	for similarly sized membrane bound protein (116)
M_c	$10^2 \sim 10^5$	the number of overcritical CAR clusters required to induce cytotoxicity of CAR-C	estimated from T cell clusters (117)
N_{CAR}	$10^6 \sim 10^8$	the amount of CAR-C in the system considered	Li and Yu (118)

(*Continued on next page*)

Table A1. ***Continued***

Parameter	Value range $V_{min} \sim V_{max}$	Description	Data sources
N_h	$10^9 \sim 10^{11}$	the total amount of cells in the system considered	Sender and Milo (119)
S	$300 \sim 800,\ \mu m^2$	the surface of CAR-C	Grakoui et al. (120)
t_{mt}	$10^5 \sim 10^7$ s	the doubling time of target cells	Roesch et al. (121)
t_{mc}	$10^6 \sim 10^8$ s	the time of active CAR-C circulation in the system	Chaudhury et al. (122)
λ_t	$0 \sim 20$	normalized critical cluster size for target cells	Eq. 3 with geometric mean and variance
$\tilde{\tau}_t$	$-2 \sim 20$	dimensionless time for target cells elimination	logarithm of Eq. 4 with geometric mean and variance
λ_c	$0 \sim 25$	normalized critical cluster size for critical tissue cells	Eq. 3 with geometric mean and variance
$\tilde{\tau}_c$	$0 \sim 23$	dimensionless time for critical tissue elimination	logarithm of Eq. 3 with geometric mean and variance

AUTHOR CONTRIBUTIONS

Conceptualization, I.V.P. and G.Th.G.; investigation, I.V.P. and G.Th.G.; writing – original draft, I.V.P.; visualization, I.V.P.; methodology, G.Th.G.; resources, G.Th.G.; writing – review & editing, G.Th.G.; supervision, G.Th.G.; project administration, G.Th.G.; funding acquisition, G.Th.G. Both authors have read and agreed to the published version of the manuscript.

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DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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