Engineering Principles for Synthetic Biology Circuits in Cancer Immunotherapy

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ABSTRACT

Recent advances in biomolecular engineering have led to novel cancer immunotherapies with sophisticated programmed functions, including chimeric antigen receptor (CAR) T cells that bind tumor-associated antigens (TAA) to direct coordinated immune responses. Extensive engineering efforts have been made to program not only CAR specificity, but also downstream pathways that activate molecular responses. Collectively, these efforts can be conceptualized as an immunotherapy circuit: TAAs bind the CAR as input signals; intracellular signaling cascades process the binding interactions into transcriptional and translational events; and those events program effector output functions. More simply, this sequence may be abstracted as input, processing, and output. In this review, we discuss the increasingly complex scene of synthetic-biology solutions in cancer immunotherapy and summarize recent work within the framework of immunotherapy circuits. In doing so, a toolbox of basic modular circuits may be established as a foundation upon which sophisticated solutions can be constructed to meet more complex problems.

See related article on p. 5.

The Cancer Immunotherapy Circuit

Different immune cells have specialized roles, but the basic principles of an immunotherapy circuit are broadly applicable across cell types, with three distinct but linked stages: input, processing, and output. Understanding these stages and the interfaces between them can then yield insights regarding how a biological system can be rationally engineered to accomplish specific objectives. Herein, we use the endogenous T-cell response as an example to define and illustrate the interaction between each stage of the immunotherapy circuit (**Fig. 1**).

The input stage encompasses any chemical signal sensed by the cell. In an endogenous T-cell response, these inputs are three signals from an antigen-presenting cell (APC). These signals form the basis of the immunologic synapse: a tumor-associated antigen (TAA) bound by MHC, a costimulatory molecule (e.g., B7.1 or B7.2; also known as CD80 and CD86, respectively), and a set of soluble cytokines (1). Molecular binding events then initiate intracellular cascades that can trigger transcriptional and translational activity, constituting the processing stage of the circuit. For example, following T-cell receptor (TCR) engagement of the TAA–MHC complex, a signaling cascade involving ZAP70, LCK, LAT, and multiple downstream pathways ultimately leads to regulated gene expression (2). The altered transcriptional and translational landscape then provides the foundation for measurable functional changes in the cell, comprising the output stage. In an activated T cell, notable functional outputs include degranulation and release of cytotoxic compounds (e.g., granzyme B) for target-cell killing, metabolic remodeling for rapid proliferation, and T-cell subtype differentiation (3).

Knowledge of all elements in the T-cell immunotherapy circuit remains incomplete. However, identification of select mechanisms and pathways has enabled targeted design and engineering to accomplish specific goals. Adoptive cell therapy (ACT)—involving the isolation, and frequently the genetic modification, of immune cells to target TAAs, followed by their infusion into a patient in either autologous or allogeneic settings—has emerged as a promising cancer-treatment strategy. Several types of immune cells have been utilized for ACT, with T cells being the dominant effector-cell type at this time (4). Chimeric antigen receptor (CAR) T-cell therapy has remarkable efficacy against B-cell malignancies, but solid tumors remain challenging targets for ACT (5). In this review, we discuss recent T-cell engineering solutions according to the problem(s) they seek to address, and concordantly analyze patterns in these problem–solution pairings based on the principles of the immunotherapy circuit. This has the potential to provide a toolbox of solution patterns for engineering next-generation circuits so that multiple biological problems can be solved simultaneously.

Single-Solution Immunotherapy Circuits

Despite the simple abstraction of circuits as input, processing, and output, cellular systems remain complex with many unknown elements, and minimizing disruption to the system can help prevent unpredicted, undesirable effects. Accordingly, most engineering solutions for solid cancers to date have sought to address only one of the known challenges of T-cell ACT at a time.

Toxicity

Engineering T cells to bind TAAs introduces the possibility of unintentional targeting of healthy tissues as a result of inadequate specificity. This may result in off-target toxicity (i.e., recognition of antigens other than the target TAA) or on-target/off-tumor toxicity (i.e., recognition of healthy cells that also express the target TAA). To address these specificity issues, efforts have gravitated toward either integrating multiple signals for increased target selectivity or implementing control mechanisms that can suppress effector function when

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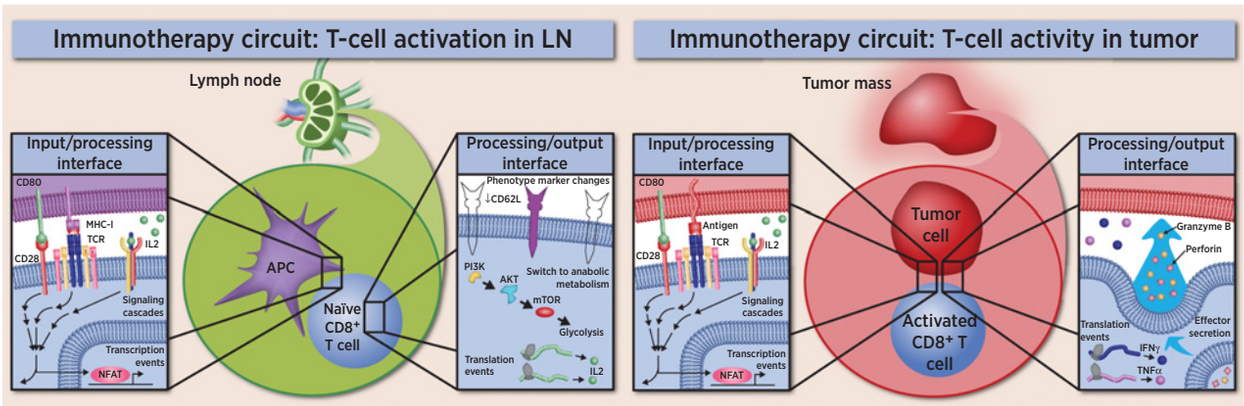

Figure 1.
Principles of the immunotherapy circuit in T cells. Schematic of input, processing, and output stages of the immunotherapy circuit in endogenous interactions of antigen-presenting cell (APC) and naïve T cell (left) or activated T cell and tumor cell (right). In both cases, the input stage involves some combination of receptor/ligand binding events, the processing stage involves intracellular signaling cascades that often lead to transcription, and the output stage involves functional changes that can manifest at the intracellular, membrane, or extracellular compartments. LN, lymph node; MHC-I, MHC class I; TCR, T-cell receptor.

necessary. Within the immunotherapy circuit framework, these strategies correspond to engineering at the input/processing and processing/output stage interfaces, respectively.

AND-gate Boolean logic at the level of input signals has become a common approach for integrating multiple signals for increased specificity (**Fig. 2A–C**). One such design functionally distributes the activation, costimulation, and cytokine-mediated signaling pathways into three separate receptors: (i) a first-generation CAR (i.e., one without costimulatory domains) targeting prostate stem cell antigen (PSCA), (ii) a hybrid receptor that fuses the ectodomain of the TGFβ receptor II (TGFβRII) with the cytoplasmic domain of the costimulatory receptor 4-1BB, and (iii) an IL4/IL7 inverted cytokine receptor (ICR; **Fig. 2A**; ref. 6). In theory, this system would require the simultaneous presence of PSCA, TGFβ, and IL4 to fully activate the T-cell response, thus increasing targeting specificity. Other signal integration approaches extend beyond surface markers to utilize intracellular antigens, broadening the range of targetable species. By engineering a synthetic granzyme B (GrB) molecule to become cytotoxic only upon intracellular exposure to the cancer-specific protease SENP1, Ho and colleagues demonstrated a serial AND gate that enables a two-step verification process for tumor specificity (**Fig. 2B**; ref. 7). However, true AND-gate responses can be challenging in biological systems as each system component can trigger nonzero levels of effector output described as "leakiness." For example, transient proliferation was observed in response to IL4 alone in the triple-receptor T cells described above, and cytotoxic mechanisms other than GrB could partially override the serial AND gate created through synthetic GrB. Nevertheless, Boolean logic has become a useful concept in immunotherapy circuit designs.

Of note, signal integration through Boolean logic can apply to both the *presence* and the *absence* of specified signals. As an example, loss of heterozygosity (LOH) is a common characteristic in tumor progression, and Hwang and colleagues developed a T-cell system employing a conventional CAR targeting the product of one human leukocyte antigen (HLA) allele and an inhibitory CAR (iCAR) targeting the product of the other HLA allele (8). In the event that both alleles were

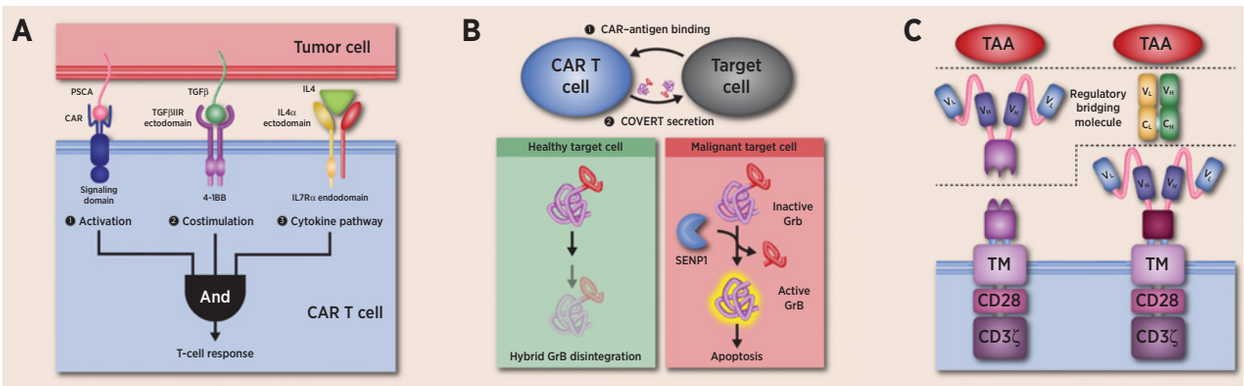

Figure 2.
Circuit examples employing combinatorial signal integration for enhanced specificity. **A,** Combinatorial antigen processing with distribution of three signals across different receptors to implement parallel Boolean AND-gate logic. **B,** Serial antigen recognition first at the target-cell surface and then in the cytoplasm to activate cytotoxic GrB. **C,** Safety strategies involving a regulatory bridging molecule that can reconstitute all CAR elements (left) or facilitate interaction (right) with an antigen of interest only when administered.

present in a probed cell, signaling through the iCAR would suppress the downstream pathways initiated by the conventional CAR. However, if LOH occurred for the iCAR target, suppression would be lifted and CAR signaling would trigger cytotoxic output.

In the absence of a perfectly tumor-exclusive antigen or combination of antigens, antigens that are overexpressed by tumor cells compared with healthy tissue become potential targets of interest. However, targeting such antigens requires the ability to quantitatively distinguish antigen expression levels. Aside from weakening the binding affinity of the CAR such that it preferentially targets antigen-high tumor cells, one could also modulate the availability of antigen-reactive CARs, such that CAR T cells can only trigger downstream signaling in the presence of antigen-high targets (**Fig. 2C**). One approach is to split the CAR protein into multiple parts that are reconstituted only upon the addition of a regulatory molecule, such that the CAR T cell would only be functional if the regulatory molecule were administered to the patient (9). Another approach is to engineer T cells to express a CAR that binds a peptide not normally present in the system; such a CAR can only recognize target cells in the presence of a separately administered, soluble fusion protein containing a TAA-binding antibody domain and a CAR-binding peptide (10). Despite the temporal and titratable control conferred by these approaches for toxicity management, they pose implementation challenges due to the requirement of intermittent dosing of the regulatory bridging molecule.

Antigen escape

The antithesis to insufficient tumor specificity is antigen escape, which occurs when malignant cells escape from therapy by downregulating or losing expression of the targeted antigen. Solutions to this problem typically involve combinatorial input processing employing OR-gate Boolean logic. One example is tandem CARs, which contain two single-chain variable fragments (scFv) that recognize two different antigens (11, 12). Phase I trials evaluating tandem CARs targeting CD19 and CD20 or CD19 and CD22 have shown promising efficacy against B-cell lymphoma as well as leukemia (13, 14). Moving beyond targeting two antigens, trispecific CD19/CD20/CD22 CAR T cells have employed coexpression of a bispecific CD19/CD20 CAR and a CD22 CAR (15). Similarly, "trivalent" CAR T cells with three separate CARs binding HER2, IL13Rα2, and EphA2 were shown to have superior tumor coverage over both 2-antigen and 1-antigen targeting systems for glioblastoma (GBM; ref. 16). In similar fashion to most signal-integration solutions for increased specificity, prevention of antigen escape typically involves engineering at the input/processing stage interface.

Immunosuppression

A particular challenge associated with solid tumors is the immunosuppressive tumor microenvironment (TME), leading to several engineering efforts at the input/processing stage focused on blocking inhibitory signals or converting them into stimulatory signals. TGFβ is one of the most well-established immunosuppressants associated with the TME (17). Kloss and colleagues demonstrated that coexpression of a CAR targeting the prostate-specific membrane antigen (PSMA) and the dominant negative TGFβ receptor increases antitumor effect by abrogating the immunosuppressive effects of TGFβ (18). Going one step further, immunomodulatory fusion proteins consisting of an inhibitory receptor ectodomain coupled to a costimulatory endodomain can not only block inhibitory signals but actively convert them into stimulatory signals for immune cells. One example is a Fas/4-1BB fusion (19), and another is the IL4/IL7 ICR previously mentioned (6). Furthermore, a CAR engineered to target soluble TGFβ can activate T cells in response to TGFβ, triggering both costimulatory signals embedded in the CAR as well as multiple downstream effector outputs such as cytokine production and T-cell proliferation (20, 21). By necessity, blockage and inversion of inhibitory signals requires engineering at the input/processing stage interface, usually utilizing truncated or fused receptors. However, recent developments of fourth-generation "armored" CAR T cells have demonstrated that immunosuppression may also be addressed at the processing/output stage through programmed secretion of proinflammatory factors such as IL12, resulting in enhanced proliferation and cytotoxicity as well as reduction in exhaustion markers (22).

T-cell exclusion and exhaustion

In addition to immunosuppressive factors such as TGFβ, physical barriers around solid tumors and T-cell exhaustion in the TME further limit the efficacy of ACT against solid malignancies. Given that extracellular matrix (ECM) components like hyaluronic acid are often overproduced by tumors, CAR T cells have been engineered to coexpress hyaluronidase to better access gastric cancer cells (23). In similar fashion, CAR T cells have also been designed to target fibroblast activation protein (FAP), which is an antigen on both the stromal and parenchymal compartments of malignant pleural mesothelioma (24). Such strategies aim to undermine the tumor niche that nourishes tumor growth, thereby enhancing T-cell infiltration and antitumor efficacy. It has also been shown that CARs that strongly tonically signal (i.e., signal in the absence of antigen stimulation) can drive T cells toward exhaustion (25, 26). As a counter measure, T cells have been engineered to enable CAR signaling in a regulated fashion, such that CAR proteins are stably expressed (27) or rendered signaling competent (28) only in the presence of a small-molecule drug.

Multi-Solution Immunotherapy Circuits

Circuits aimed at solving single problems have demonstrated considerable benefits, but effective tumor clearance is often thwarted by multiple hurdles, frequently with overlapping or even synergistic effect. Thus, single-solution immunotherapy circuits are often inadequate for long-term efficacy against aggressive, heterogeneous, or complex cancers. As a result, recent immunotherapy circuits have been designed with multiple engineered elements with the intent of addressing multiple problems simultaneously.

GBM is a classic example of diseases that pose simultaneous challenges in antigen heterogeneity and lack of tumor specificity, as no antigen is known to be both uniquely and uniformly expressed on GBM cells. One strategy to address both nonspecific toxicity and antigen escape in GBM is CART.BiTE T cells, which constitutively coexpress a CAR targeting EGFRvIII, a mutated form of EGFR specific to GBM, together with a bispecific T-cell engager (BiTE) that redirects T cells to wild-type EGFR (29). EGFR is expressed by a variety of healthy tissues, but constitutive BiTE expression did not lead to systemic toxicity, possibly due to the fact that the BiTE-producing CAR T cells could locally expand near GBM lesions through EGFRvIII engagement. The therapeutic window of BiTE secretion allowed for multi-antigen targeting that mitigated antigen escape but also conferred added benefit of engaging bystander immune cells against the tumor.

Synthetic Notch (synNotch)-CAR T cells represent another class of multi-solution circuits that have been applied to GBM. Combining input/processing and processing/output interface engineering, the

synNotch-CAR platform uses a synthetic receptor (synNotch) that triggers the release of a transcription factor upon ligand binding, leading to the expression of a CAR. The CAR subsequently recognizes a second antigen and triggers T-cell activation and effector function. Alternatively, the transcription factor can be engineered to trigger other transgenic outputs, such as inflammatory cytokines, that directly program effector functions (30). Additional synNotch-CAR systems have been developed, highlighting the inherent IF-THEN logic of receptor-induced receptor expression as an approach for implementing multi-antigen prime-and-kill switches (31). The synNotch receptor can "prime" the CAR T cell to express a tandem CAR upon binding the GBM-specific antigen EGFRvIII; the tandem CAR then targets either EphA2 or IL13Rα2, which are not restricted to GBM. This design simultaneously tackles issues of specificity and antigen escape by using a tumor-specific antigen as a first-pass filter before broadening the targetable scope to include less-specific antigens (31).

Another approach called the split, universal, and programmable (SUPRA) CAR system employs a soluble molecule (zipFv) with an antigen-binding domain connected to a zipper domain that may mediate interactions with an immune cell engineered to express a complimentary zipper domain (32). With this design, complex intra- and intercellular circuits were developed through engineering different zipFvs and their receptors within different cell types, representing a novel step forward toward coordination within an immune cell consortium. However, as systems become increasingly complex, design considerations must also be made to ensure robustness and practicality. Multicomponent designs such as the SUPRA CAR system require soluble proteins that must colocalize and engage with both engineered T cells and tumor cells, raising pharmacokinetics challenges. Similarly, the synNotch system requires stable integration of a large number of transgenes, some with strong immunogenic potential. Further engineering will be required to ensure the biocompatibility of such higher-order systems for safe clinical implementation.

Although receptor engineering at the input stage is one of the most popular circuit modifications, precise functional control over processing and output stages can also be achieved with engineering at the promoter level. One such circuit employs a thermal gene switch to mitigate antigen escape and improve specificity (33): intravenously injected gold nanorods that passively accumulate within tumors are utilized to convert spatially focused near-infrared light to heat. This thermal signal then activates designer promoters governing CD19 CAR and NKG2DL BiTE expression in CAR T cells (33). Another promoter-based circuit uses a feed-forward "self-driving" CD19 CAR T-cell system (34). This design was motivated by the observation that constitutive, high-level expression of some CARs can lead to tonic, ligand-independent signaling that may predispose CAR T cells to exhaustion. To overcome this challenge, T cells were engineered to coexpress a CD19 CAR and a dominant-negative TGFβ receptor from an AP1–NF-κB promoter. This promoter drives low-level basal expression in the absence of antigen, but upregulates transgene expression in response to antigen-triggered activation of basally expressed CAR molecules. This extension of the self-driving system enables continued function of the CAR T cells in immunosuppressive conditions (34). **Table 1** summarizes the single- and multi-solution works discussed in this review based on their design features and problem–solution categories.

Table 1. Summary of solutions discussed in the context of the immunotherapy circuit.

Problem(s)	Number of engineered elements	Immunotherapy circuit design implementation	Refs.
Specificity	Single	Integration of three surface input signals (AND logic)	6
	Single	Integration of surface and intracellular input signals (IF-THEN AND logic)	7
	Single	Integration of present and absent surface input signals (AND-NOT logic)	8
	Single	Exogenous administration of a CAR fragment to form the full CAR	9
	Single	Exogenous administration of a peptide bridging molecule to direct CAR	10
Antigen escape	Single	Integration of two surface input signals (OR logic)	11–14
	Single	Integration of three surface input signals (OR logic)	15, 16
Immunosuppression	Single	Blocking of immunosuppressive input signal	18
	Single	Conversion of immunosuppressive input to immunostimulatory signal	19–21
	Single	Release of stimulatory outputs to oppose chemical immunosuppression	22
Spatial exclusion, TME	Single	Release of functional outputs to remove structural barriers	23
	Single	Targeting aberrant, nonmalignant tumor-supporting tissues	24
Exhaustion	Single	Customization of costimulatory input signal processing to reduce exhaustion	25, 26
	Single	Transient modification of activation status to facilitate recovery from exhaustion	27, 28
Specificity, antigen escape	Multiple	Targeting tumor-specific input signals for spatial localization Secretion of bispecific antibodies to target less-specific antigen to oppose antigen escape	29
	Multiple	Receptor-induced receptor expression for enhanced targeting to ensure specificity Customization of multiple effector output programs to target multiple cells	30
	Multiple	Sequential targeting of specific and then less-specific input signals to enhance specificity and prevent antigen escape	31
	Multiple	Integration of multiple surface input signals (AND, NOT, AND-NOT logic) Modular targeting of input signals to oppose antigen escape	32
	Multiple	Spatial control over CAR expression, cell activation, and other outputs	33
Exhaustion, immunosuppression	Multiple	Self-driving promoters for control of CAR and other receptor expression levels	34

The Immunotherapy Circuit Toolbox

The potential and challenges of immunotherapy design discussed above highlight the need to identify a toolbox of evidence-based immunotherapy circuits that can be assembled rationally and rapidly to address higher-order problems. Understanding the specific stages of the circuit (input, processing, or output) in which each tool can be most effectively implemented further provides a set of design principles for effective circuit construction.

The CAR is a prime example of engineering at the interface of input/processing stages, and it dictates which antigens serve to activate the cell. Problems related to identifying and processing appropriate input signals are thus often best solved by CAR or cytokine-receptor engineering strategies. In contrast, endogenous and inducible promoters with engineered genetic programs are prominent examples of modifications to the processing/output stages, and they confer significant control of effector functions and behavior. Thus, problems related to effector output intensity and T-cell exhaustion or differentiation status are often best solved by output-focused solutions. Basic immunotherapy circuit principles are outlined below to serve as a toolbox for next-generation therapies. Their combination in modular fashion may provide a useful starting point for addressing complex combinations of problems.

- Input/processing interface:
  - Multi-antigen signal integration:
    - AND-gate logic addresses specificity and toxicity management.
    - OR-gate logic addresses antigen escape and heterogeneous tumor marker expression.
  - Signal inversion (e.g., IL4/IL7 ICR or Fas/4-1BB fusion receptor) addresses immunosuppression.
  - TME modulation (e.g., TGFβ CAR) addresses immunosuppression and tumor infiltration.
  - Engineering at receptor and downstream signal-processing levels addresses control over activated functions.
- Processing/output interface:
  - Spatial or temporal control of transcription, translation, and posttranslational processing may address problems of toxicity and immune-cell exhaustion.
  - Expression of transgenic effector molecules can armor immune cells with enhanced antitumor function or program new input/processing interfaces.

In addition to recognizing basic circuit design principles, it is also important to consider how such designs may be implemented and for what purpose. The engineering strategies described above are typically executed by stably modifying the T-cell genome—either by virus-mediated construct integration or by more targeted gene-editing techniques such as clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) systems (35). Virus-directed gene delivery confers additive function by supplementing endogenous cellular programs with synthetic ones and is thus best suited for enhancing existing functions or introducing new functions outright. On the other hand, gene-editing platforms permit "redesign" of endogenous programs, enabling direct rewiring of program machinery. Risks of oncogenic insertion by viral integration and off-target gene modification by gene editing are important factors for consideration, but transient delivery modalities that avoid such concerns (e.g., mRNA electroporation) have also been demonstrated (35).

Discussion of how circuits may be implemented would be remiss without acknowledging their inherent design limitations. Complexity often comes at the price of robustness as more modifications often require larger genetic constructs and correspondingly reduced delivery efficiency, and each additional element in the circuit introduces more opportunities for unintended functions due to imperfect orthogonality or loss of function due to component malfunction. Additionally, the underlying mechanisms for many solutions demonstrated to date are not fully understood, and there may be functional nuances that only emerge as new combinations are attempted. The T-cell therapy space is ripe for innovation, but care must be taken to characterize and optimize each design accordingly. With these tools, more effective treatments may be developed, and the benefits of CAR T-cell therapy in the liquid cancer space may begin to translate to the solid tumor space.

Authors' Disclosures

Y.Y. Chen reports patents and consulting activities related to CAR T cells. No disclosures were reported by the other author.

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