Chapter 10

Dynamic Nature of Tumour-Host Interactions Within the Tumor Microenvironment

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Abstract: The recent progress in tumor immunology exemplifies the successful application of modern biotechnology for the understanding of the complex natural or therapy-induced phenomenon of immune-mediated rejection of cancer. Tumor antigens recognized by T cells were identified and successfully utilized in active immunization trials for the induction of tumor-antigen specific T cells. This achievement has left, however, the clinicians and researchers perplexed by the paradoxical observation of the immunization-induced T cells can recognize tumor cells in standard assays but most often cannot induce tumor regression. In this presentation, we will argue that successful immunization is one of several steps required for tumor clearance but more work needs to be done to understand how T cells can localize and be effective at the receiving end within a tumor microenvironment in most cases not conducive to the execution of their effector function. In fact, metastatic melanoma stands out among human cancers because of its immune responsiveness. Yet, the reason(s) remain(s) unclear. We believe that the key to the understanding of this complex phenomenon relies on the real-time study of tumor/host interactions in the tumor microenvironment. Most likely, T cells induced by immunization can reach the tumor site but they are not capable of performing their effector function because they encounter a tumor microenvironment not conducive to T cell activation. In this chapter, we will review some of the basic approaches that may help solve this puzzle by studying directly human disease and its adaptation to treatment.

Key words: Immunotherapy, melanoma, functional genomics, microenvironment, chemokine receptor

1. IMMUNE SURVEILLANCE

The natural history of tumours is determined by interactions between four main components of the tumour microenvironment: tumour cells, stroma, blood vessels, and infiltrating immune cells. Relationships within this tetragon are responsible for development or containment of tumour cell growth. In the present review, we will focus on the interaction between tumour and immune system that are commonly covered by the general term: immune surveillance. Whether immune surveillance truly occurs in humans and the degree of its relevance in determining the final outcome of cancer remains a central focus of discussion (1, 2). The true role of immune surveillance, particularly in humans, is far from being validated although experimental models suggest that both the innate and the acquired immune response can control tumour growth. While the question remains, research has made progress recently by developing new methods that allow direct and accurate investigation of the interaction between host and tumour particularly in the context of human disease where pristine experimental

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conditions are often unachievable. The concept of immune surveillance against cancer is supported by several animal studies and various observations in humans (3,4). These include increased prevalence of tumour development following immune suppression and the observation that the extent of intra-tumoural T cells is correlated with improved clinical outcome in various solid tumours (3, 5-8). Therefore, we are increasingly induced to believe that tumour-host interactions occur along a two-way-road on which tumour and immune system shape each other. To emphasize the dual role of host-protection and tumour-sculpting immune surveillance processes have also been called "cancer immunoediting" (3).

2. TUMOUR ASSOCIATED ANTIGENS AND T CELLS

Since the identification of tumour associated antigens (TAA) in the early 1990s (9, 10), tumour immunology has become a rapidly growing field of research and clinical investigation. Within a decade, more than 60 major histocompatibility (MHC) class I-associated TAAs had been identified (11) and new ones are continuously described. The appearance of tumour-specific reactions against tumour cells is dependent on a complex system. As for all antigens recognized by T cells, TAA are cleaved within the tumour cells by proteasomes into short peptides of 8-12 amino acids. These peptides are transported through the transporter associated with antigen presentation (TAP) into the endoplasmatic reticulum. Here, peptides with good binding affinity for the MHC class I molecules (in humans called Human Leukocyte Antigens or HLA) naturally associate with them. The peptide/HLA complexes, stabilized by a further protein (β_2-microglobulin) migrate then to the cell surface where they become exposed to the potential interaction with HLA class I-restricted T cells. In an HLA-dependent fashion, TAA-derived epitopes are detected by T cells through HLA/epitope-T cell receptor (TCR) engagement. An effective T cell response can only be generated after the T cells have been primed by presentation of TAA by professional antigen-presenting cells (APC), such as dendritic cells. Therefore, APC within tumours (resident APC) may play a major role for the development of specific T cell responses.

3. CHANGES IN TUMOUR AND IMMUNE CELLS

It is likely that immune cells influence the tumour cell phenotype and changes in tumour cells shape in return the immune response. Through a selective process, tumour cells likely adapt to immune pressure by changing their phenotype and escaping immune surveillance. Due to genetic instability tumours continuously re-program their genotype and thus their phenotype. Several mechanisms may be involved. Tumour cells often are defective in TAAs (12) or HLA molecule expression (13, 14), and/or have malfunctioning antigen-processing (15). A loss of expression of the HLA–epitope complex on the surface of tumour cells renders impossible their recognition by TAA-specific T cells. Furthermore, HLA-epitope complex down-regulation on tumour cell membranes correlates with decreased T-cell-triggering capability (16). Dysfunction of antigen-processing machinery might have a strong role for the coexistence of TAA-specific T cells with cancer cells expressing the target components necessary for their recognition (17-19). Complete TAA loss obviously eliminates one of the most important pre-conditions for a targeted T cell function. All given escape mechanisms happen under an assumed pressure by the immune system. Tumor/immune cell interactions are bidirectional. Tumour cells can modulate T cell function. For example, systemic and intra-tumour T cell dysfunction including anergic T cells and T cells with down-regulated CD3-zeta chain has been described in tumour patients (20,21). This anergic status of T cells has been proposed as the cause of their ineffectiveness in containing tumour growth (22). The cause of malfunction of TAA-specific T cells is not known but it is known that tumour cells can secrete immune suppressive factors, such as TGF-β (23). Several additional variables might influence T cells effector function and, consequently, their clinical effectiveness (discussed in 24). These include the activation of regulatory T cells, the development of death resistance by tumour cells, involvement of natural killer cells and their receptors, and the potential contribution of Fas expression. Additionally, new data shed light on the role of transcription factors in tumour's defence against immune system. STAT3 expression in tumour cells leads to inhibition of production of pro-inflammatory cytokines and chemokines (25). These variables might have an important role in modulating the immune response at both the systemic level and the tumour site; and their significance for immune-system/tumour-interaction warrants further exploration.

4. THE SYSTEMIC IMMUNE RESPONSE

Several studies have demonstrated the presence of TAA-specific T cells in peripheral blood of immunized patients, proving that the primary goal of vaccination - inducing a systemic TAA-specific immune response - can reproducibly be achieved (26). Additionally, systemic tumour-directed T cell responses can evolve spontaneously in various malignant diseases without prior immunotherapy (27). Although these spontaneously occurring TAA-specific T cells have been characterized as CD3+CD8+IFNγ+CD45RA+ (28), a phenotype supposedly representative of cytotoxic effector T cells (29), their actual function in clinical settings remains unknown. Clinically, although rare clinical responses can be achieved using peptide vaccination (30), no conclusive correlation between systemic T cell response and clinical cancer regression has been convincingly demonstrated, so far. Thus, investigations of the systemic immune response do not provide sufficient information about the interaction between host and cancer cells at the actual site of "conflict" - in the tumour microenvironment.

5. METHODS TO ANALYZE THE TUMOUR MICROENVIRONMENT

Interactions between tumour and immune system lead to changes within the tumour microenvironment. These changes can affect different levels of biologic process, such as cell phenotypes, function, protein expression, and gene regulation; various methods have been utilized to investigate these alterations. Immunohistopathology remains a basic method for cell analysis in tumour immunology. Main applications for this method lie in the evaluation of antigen expression on tumour cells and in the detection/enumeration of tumour-infiltrating immune cells. From an immunological point of view, melanoma is one of the best investigated malignant diseases. Infiltration of melanomas with T cells occurs frequently and can be related to a favourable prognosis (31). In another example, we have shown that most melanoma lesions are infiltrated by CD14 positive mononuclear phagocytes (32). Taking a further example, for colorectal cancer, several independent studies have linked CD8+ infiltration of tumours or CD8+ infiltration patterns to a favourable prognosis (5,33-36). However, the antigen-specificity was not determined in those studies. Intralesional staining with fluorescence-labelled tetrameric HLA–peptide complexes (tHLA) represents an important extension of conventional antibody-based histopathology. tHLA enable direct enumeration and characterization of TAA-specific T cells (22,37,38). However, *in situ* staining with tetramers remains technically difficult (39). Culturing tumour cells and in particular tumour infiltrating cells, such as tumour infiltrating lymphocytes (TIL), is crucial for adoptive T cell transfer and for *ex vivo* and *in vitro* analyses. However, one always has to be aware of the fact that cells underlie complex changes during *in vitro* expansion. Tumour cells show an altered antigen repertoire and adhesion molecule profile. Cultured TAA-specific CD8+ T cells do not accurately reflect *in vivo* immune responsiveness because *in vitro* expansion leads to changes in the T cell function and characteristics (40). T cells can be further analyzed for their T cell receptor repertoire (spectratyping, immunoscope; 41, 42). These methods can provide important information about breadth and flexibility of TAA-specific T-cell responses. TCR analysis indicates, e.g., that the T-cell response in regressing melanoma lesions after cytokine therapy is dominated by T cells directed toward a limited number of epitopes and that epitope-specific T cells frequently use a highly restricted TCR repertoire (43). Protein-based assays include ELISA assays to investigate, e.g., cytokines or chemokines, and staining with specific antibodies against cell markers, such as tumour antigens or T cell subset characteristics. To investigate gene regulation within the tumour microenvironment, two main techniques are used: microarrays to analyze a broad variety of transcriptomal changes and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) to analyze single genes more detailed.

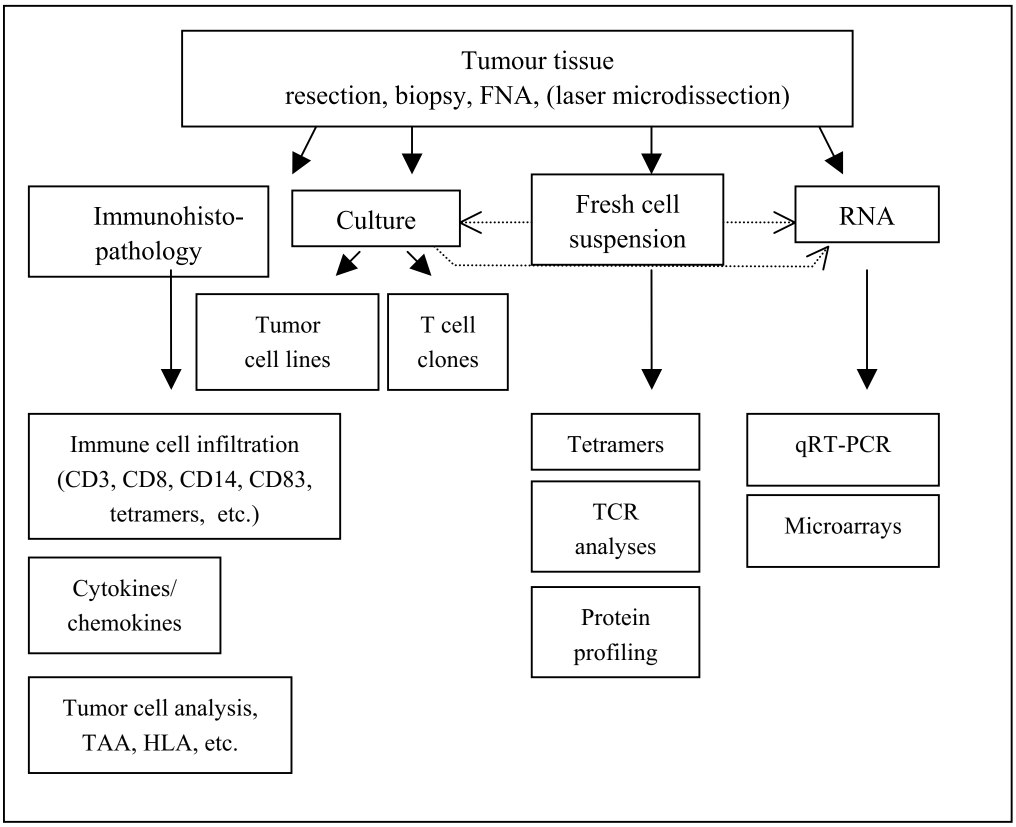

Figure 1. Several ways to analyze the tumour microenvironment.

6. IMMUNOTHERAPY

Most studies analyzing the dynamic interaction between tumour and immune system in the microenvironment have been performed as part of an immune monitoring in association with immunotherapy. During the last years, various partially efficient, immunotherapeutic approaches including peptide vaccine, DC vaccine, cytokines, viral vectors, passive transfer of antibodies or adoptive antigen-specific T cell transfer have been developed (reviewed in 30, 44, 45). The aim of an immunotherapy is the initiation of an immune response at the tumour site or site of immunization that is followed by a stepwise expansion of the immune response to loco-regional, systemic and peripheral sites, which finally causes rejection of tumour cells by the immune system (46). Although some clinical success has been achieved by immunotherapy, the major break-through has not been reached yet. Nevertheless, valuable information about the dynamic processes within the tumour microenvironment and the systemic immune response has been obtained during these therapies.

7. ANALYSES OF THE TUMOUR MICROENVIRONMENT

For several malignancies, it has been shown that a T cell infiltration or a specific pattern of T cell infiltration of the tumour is correlated with better survival (5-8). However, in these studies, the Ag-specificity of infiltrating T cells was not analyzed. For most tumours it is unknown whether TAA-specific T cells – spontaneously or vaccine induced– do migrate to the tumour site. A possible mechanism causing the variable behaviour of tumours of individual patients or lesions might be found in defects in the localization of TAA-specific T cells at the tumour site. Localization at a tumour site of adoptively transferred tumour-infiltrating lymphocytes (TILs) is a prerequisite for a clinical response (47). By analogy, we can assume that immune responses elicited by immunization might not work because they do not migrate to the tumour site. Few studies have addressed this question in humans because of technical difficulties to analyze TAA-specific responses *in situ* within tumours (39). Comparison of pre- and post-immunization samples obtained from melanoma lesions through fine-needle aspiration (FNA) biopsies suggests that immune responses elicited by vaccination can localize at a tumour site. Expansion of tumour–TIL pairs from repeated FNA biopsies of identical lesions in patients undergoing epitope-specific immunotherapy demonstrated that immunotherapy-induced T cells can be expanded more readily from melanoma metastases after treatment (18). Furthermore, quantitative real-time PCR of cytokine gene expression in FNA samples before and after vaccination demonstrated a post-immunization enhancement of IFN-γ transcripts in lesions that maintained expression of the targeted TAA and of the restricting HLA class I allospecificity (19). The relationship between IFN-γ and TAA expression suggests that vaccine-induced T cells interacted with tumour cells and/or APCs in the tumour microenvironment. Thus, vaccine-induced T cells can migrate to melanoma metastasis. Furthermore, the lack of clinical responses in this study demonstrate that tumour localization of T cells and TAA expression are not the sole factors required for an effective immunotherapy.

Cancer–host interactions within the tumour microenvironment might not be sufficient to elicit and maintain an effective T cell response because the danger signal required for full activation is not present (48). This might enable tumours to survive and grow in an "ignorant" immune environment, which is not sustaining and promoting the function of immunization-induced T cells that have localized within the tumour (49, 50). Local cytokines might promote such activation and proliferation of TAA-specific T cells. However, the required levels for interleukin-2 (IL-2) and other cytokines are relatively high (51, 52). Furthermore, CD4+ helper T cells might be necessary to provide an additional stimulus, which could lead to survival and amplification of CTL responses at the tumour site. Interestingly, for vaccination purposes, an additional foreign helper protein aiming at the support by CD4+ T cells increases the frequency of CD8+ T cell responses (53). On the other hand, e.g., in colorectal cancer, about 15% of tumour-infiltrating T cells express CD25 (36). These CD4+ CD25+ regulatory T cells play an important role for the suppression of tumour-directed T cell responses (54, 55). It is not understood to which degree these regulatory T cells influence the immunological "ignorance" systemically and at the tumour site.

Slowly, we might begin to understand homing processes of T cells. Apparently, one important component is the interaction between chemokine expression within a tissue, such as the tumour, and expression of chemokine receptors on the surface of antigen-specific T cells. First experience has been gained from analysis of chemokine receptor expression in antigen-specific T cells in viral diseases and melanoma. Expression of CCR7, a chemokine receptor important for homing into lymphatic tissue, is associated with memory subsets of antigen-specific T-cells (56), CXCR4, the receptor mediating migration to its ligand SDF-1 (highly expressed in bone marrow), is functionally expressed on melanoma-specific T-cells in peripheral blood and in bone marrow (57). The importance of chemokine receptors for cellular homing is also supported by data from tumour cells. Only tumour cells from the intestine were activated by CCR9 ligand Teck, a chemokine which is only expressed in thymus and intestine (58).

Leading cells into a specific tissue based on chemokine receptor expression, such as T cells into tumour, would require that corresponding chemokines are expressed within the target tissue. Tumour cells, stroma, and infiltrating immune cells create a complex chemokine milieu within the tumour microenvironment. In murine models, it has been shown, that expression of certain chemokines leads to a hampered tumour growth (59, 60). For human ovarian carcinoma, it was shown that T cell infiltration is an important survival marker. Furthermore, T cell infiltration is – in a preliminary study - closely correlated to mRNA levels of monokine induced by interferon-gamma (MIG) (61). Using radiolabelling several cytokines and chemokines were analyzed *in vivo* and found in several tumours. This technique is also very promising, however beyond the scope of this review (more information on this methodology can be found in the excellent review 62).

Besides changes in the immune response within the tumour microenvironment, changes in the tumour cells themselves influence the relationship between host and malignant tumour. Malignant cells are genetically unstable. The resulting tumour-cell heterogeneity during the course of disease represents a major challenge for cancer immunotherapy (24,63). Comparison of the antigenic profile of autologous melanoma lesions surgically removed at different time points of the natural course of the disease or following immunotherapy has demonstrated that temporal changes often are associated with the specificity of the immunization (16). In particular, antigen expression by tumour cells can be lost after TAA-specific immunization (12, 64). Such a loss of antigen is an important component of tumour immune escape mechanisms (16) and represents a possible explanation for the paradoxical co-existence of cancer cells with TAA-specific immune cells in the same host (65).

However, the antigenic heterogeneity of synchronous metastases raises the question whether differences in the expression of various markers among distinct lesions might simply reflect the intrinsic heterogeneity of metastases rather than time- or treatment-induced changes (15, 66). This possibility can be tested using a series of FNA biopsies (17, 67), which allow the study of the kinetics of gene expression within the same tumour lesion at several time points relevant to the disease process or its treatment. In FNAs serially obtained from 52 melanoma metastases before and after immunization with a gp100-derived peptide showed a rapid decrease in gp100 expression in metastases that regressed following immunization but detected no change in lesions that did not regress (68). This finding suggests that a successful immunization primarily induces killing of cells expressing the target TAA. It is not known whether this process is able to initiate a broader immune response. Frequently, however, the immune selection induced by the originally successful localization of TAA-specific T cells might lead to immune escape in recurring lesions by loss of complexes of HLA with TAA epitope from cancer cells (12). Thus, it is likely that tumour escape variants will emerge most frequently during or after effective immunotherapy (24).

Tumour cells potentially can revert to a stage in which they can function like stem cells with strong modulatory effects on the surrounding environment. A growing number of mechanisms that might mediate tumour–host interactions are known (summarized in 16). The identification of individual mechanisms capable of modulating tumour–host interactions has reached its limits because of genetic polymorphism of humans and heterogeneity of their diseases. The complexity of the several molecular pathways responsible for the natural and/or treatment-induced behaviour of tumour cells can be analyzed with the microarray or genechip technology, which can portray a whole gene expression pattern by measuring the expression of thousands of genes (69). Combining FNA and microarray technique, two subsets of molecular phenotypes that underlie the extent of instability of cancer over time were found in serial analysis of melanoma lesions (70). One subset of metastases containing mainly early tumour samples showed a transcriptional repertoire associated with normal human melanocytes, whereas a second subset portrayed a distinct, late-progression expression profile. Ranking of individual genes identified 30 transcripts whose gene transcription pattern was predictive of responsiveness to immunotherapy in malignant melanoma (70). Approximately half of these genes were related to T cell regulation suggesting that responsiveness of melanoma metastases to systemic immune stimulation is pre-determined within an environment conducive to immune recognition. In a further microarray study, a specific pattern of cytokines and chemokines, including T cell attracting chemokines PARC and MIG, was shown to be up-regulated during systemic immune therapy with interleukin-2 (71). Molecular methods cannot identify the source cells of the found cytokines; they could have been expressed by tumour cells as well as immune cells. Subsequent protein profiling data suggest that DC maturation at the tumour site might possibly play a role in mediating the systemic immunotherapy at the tumour site (72).

For now, these transcriptional profiling analyses may raise more questions than they answer. Most importantly, as already pointed out, it remains unclear which cell population produces which molecules, and what is their bioactivity and its functional consequence. Nevertheless, transcriptional profiling gives an important impression on the whole picture of the tumour microenvironment and will stay an important hypothesis-generating tool in the future. Gene profiling technology has already entered clinical oncology as tool for prediction of clinical outcome of breast cancer patients (73, 74).

We have developed a hypothesis which suggests that there are some tumours more `immunogenic' than others, such as melanoma and renal-cell cancer. They spontaneously express and secrete cytokines and chemokines which induce an inflammatory reaction within the tumour microenvironment (46). When this local inflammatory reaction is strong enough, tumours regress spontaneously. However, most commonly, this inflammatory reaction is not sufficient to induce tumour regression (see figure 2). A response to therapy might occur in these cases when an additional inflammatory stimulus is brought to the tumour site by antigen-specific and/or non-specific therapy, such as IL-2 therapy. By contrast, response to therapy does not occur when a certain threshold of inflammation is not reached by the sum of therapeutic and spontaneous response. Another possible way getting an inflammatory response to the tumour site might possibly be a bacterial infection in few cases (75).

8. CONCLUSION

Tumours represent complex, individual microenvironments with four main components: tumour cells, stroma cells, blood vessels, and infiltrating immune cells. Tumour and immune system underlie permanent changes during the course of a malignant disease or during immunotherapy of cancer. Tumour cells change their antigen repertoire or develop sophisticated defence mechanisms; over time of disease T cells are primed and altered in their characteristics, and various cytokines/chemokines are produced within the tumour environment. All these mechanisms seem to be insufficient to cause tumour regression in more than a vast minority of patients. The immune system is usually only capable of keeping the tumour at bay for a limited time. An external trigger, like a systemic immunotherapy, might change the balance in favour of an effective immune response in some cases by causing a pro-inflammatory environment at the tumour site.

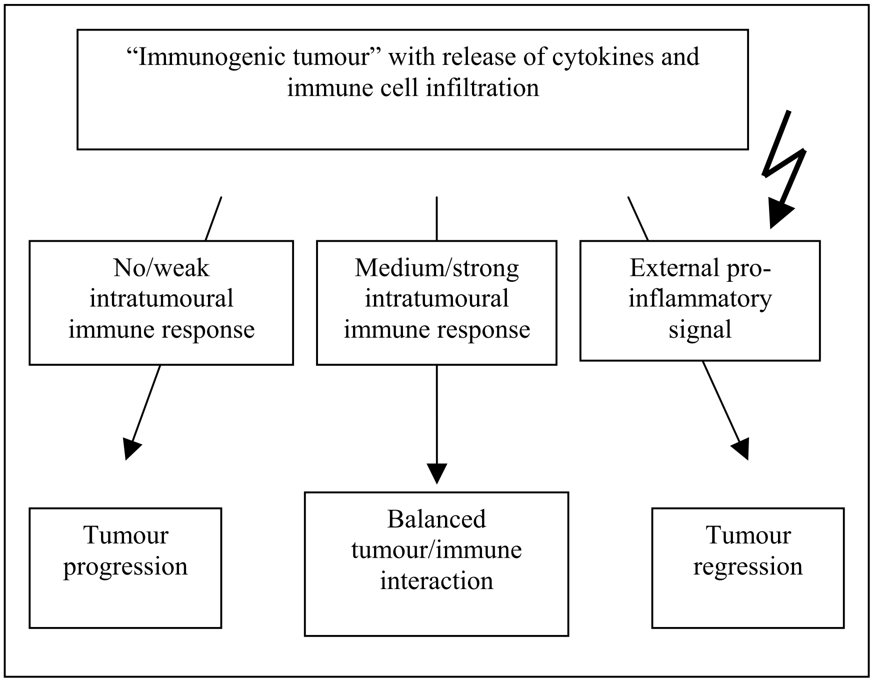

Figure 2. Balance between tumour and immune system within the tumour microenvironment.

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