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CUTTING EDGE

Cutting Edge: Cross-Presentation as a Mechanism for Efficient Recruitment of Tumor-Specific CTL to the Brain[1]

Thomas Calzascia, Wilma Di Berardino-Besson,* Rick Wilmotte,*† Frédérick Masson,* Nicolas de Tribolet,† Pierre-Yves Dietrich,* and Paul R. Walker[2]**

The number and localization of effector cells to the tumor site are crucial elements for immune rejection of solid tumors. However, for cerebral malignancies, antitumor responses need to be finely tuned to avoid neuropathologic consequences. In this study, we determine factors that regulate CTL localization and tumoricidal function after intracerebral implantation of tumors expressing model Ag. H-2^{bxd} mice implanted with a CW3$^+$ murine glioma lacking H-2K^d molecules necessary to present the CW3$_{170–179}$ epitope demonstrate cross-priming of H-2K^d-restricted CTL, and moreover, Ag-dependent accumulation of functional H-2K^d/CW3$_{170–179}$-specific CTL within the tumor bed. This implicates a role for cross-presentation not only in priming, but also in retention of fully differentiated CTL in the tumor stroma at the effector stage of the response. Modulating cross-presentation of Ag may be the key in regulating specific immune responses in the brain: either by augmenting protective responses or by down-modulating destructive autoimmune reactions. *The Journal of Immunology,* **2003, 171: 2187–2191.**

The brain parenchyma is a highly specialized site in which the anatomy, cellular composition, and the microenvironmental milieu of soluble factors have the potential to profoundly affect immune reactivity (1). These particularities are important for the rational design of immunotherapies for this delicate site. Indeed, there is a pressing clinical need to develop new treatments for incurable cerebral malignancies such as malignant astrocytomas, for which the best current therapies have obtained only limited success (2). Certain experimental cancer vaccines indicated that, despite the reputation of the brain parenchyma for being particularly hostile for immune function (3), peripherally induced CTL can play a role in destroying intracranial tumors (4, 5). It is undoubtedly necessary that sustained and high magnitude tumor-specific CTL responses will have to be elicited to observe measurable regression of tumor masses (6, 7). However, for solid cancers, the capacity of tumor rejection may soon be exhausted unless CTL are rapidly and efficiently recruited to the tumor bed (6). This is a major consideration for tumors growing in the CNS, because there is more limited trafficking of CTL to the CNS compared with other tissues (8). Increasing immune infiltration using nonspecific inflammatory mediators is too blunt a tool for the CNS and may result in neuropathology (9). An alternative approach for glioma therapy involves augmenting local immune function by transferring effector cells and IL-2 to the tumor cavity at the time of resection (10). However, although there have been isolated clinical responses, any clinical efficacy has not been confirmed, and there is frequently the induction of cerebral edema. Based on such data, it can be concluded that new approaches are required to maximize the therapeutic potential of antitumor T cells, while minimizing deleterious effects. We reasoned that two key factors will be the proportion of tumor-specific effector T cells, and their capacity to localize to the tumor stroma. Regarding localization of CTL, their accumulation within the CNS has been observed in neuropathologies other than cancer, such as demyelinating diseases and in paraneoplastic cerebellar degeneration (11). It has been shown that activated T cells can traffic through the brain, while only those recognizing locally expressed Ag are retained there (8). CNS resident APC participate in the CNS recruitment of autoreactive, MHC class II-restricted CD4 T cells in experimental demyelinating diseases (12), but whether CNS retention of tumor-specific, MHC class I-restricted CTL also implies recognition of local APC cross-presenting tumor-derived Ag is unknown.

In this study, using murine brain tumor models, we address this critical but unexplored issue and we show that APC present in the stroma of an intracranial tumor play a key role in recruiting and retaining CTL to the tumor site, even when the tumor itself is deficient in Ag presentation.

Materials and Methods

Cell lines and transfections

The MT539MG murine glioma line, hereafter referred to as MT, was provided by Dr. G. Y. Gillespie (University of Birmingham, Birmingham, AL). MT was

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doubly transfected (using FuGENE6; Roche, Rotkruz, Switzerland) with pZeoSV2$^+$ (Invitrogen, Basel, Switzerland) and pIRESHygro (Clontech Laboratories, Palo Alto, CA) expression vectors, either empty ("mock") or with cDNA encoding HLA-CW3 (kindly provided by Dr. C. Esslinger, Ludwig Institute for Cancer Research, Epalinges, Switzerland). P815-NP (H-2^d) and MC57-GP fibrosarcoma (H-2^b) were provided by R. M. Zinkernagel (Institute for Experimental Immunology, University Hospital, Zurich, Switzerland). For in vitro cytotoxicity tests, P815, EL-4 (H-2^b) or Renca (H-2^d) cells were used as targets.

Mice and tumor cell implantations

MT glioma was implanted in adult female VM × DBA/2 mice (VM from Institute for Animal Health (Newbury, U.K.); DBA/2 from IFFA Credo (L'Arbresle, France)) using 5×10^5 viable tumor cells in 125 and 5 μl of methylcellulose for s.c. and intracerebral (i.c.)[3]) implantations, respectively. Nucleoprotein (NP)-specific responses were analyzed in BALB/c mice (IFFA Credo) after i.p. (5×10^6 cells/500 μl PBS) or i.c. (5×10^5 cells/5 μl of methylcellulose) implantations of viable P815-NP tumor cells. gp-specific responses were analyzed in C57BL/6 mice (IFFA Credo) after i.c. (4×10^5 cells/5 μl of methylcellulose) implantations of viable MC57-gp tumor cells. Stereotaxic i.c. implantations were performed as described (13).

Cell preparations

PBMC were purified on Ficoll (Pharmacia, Upsalla, Sweden). Brain infiltrating leukocytes (BIL) were isolated from Ringer's perfused brains (13).

Flow cytometry

MT transfectants were phenotyped using anti-human class I mAb (B9.12.1 supernatant, provided by D. Rimoldi, Ludwig Institute for Cancer Research, Lausanne, Switzerland) and anti-murine MHC class I and II mAb (BD PharMingen, San Diego, CA). The CW3-specific response was assessed by double staining of PBMC or BIL with CD8 mAb (BD PharMingen) and H-2K^d/CW3$_{170-179}$ (RYLKNGKETL) tetramers (ProImmune, Oxford, U.K. and P. Guillaume, Ludwig Institute for Cancer Research, Lausanne, Switzerland) or by quantifying BV10$^+$CD62L$^-$CD8$^+$ cells (13). H-2K^d/CW3$_{170-179}$-specific CTL preferentially use a BV10 TCR. In nonimmune mice, 8–10% of naive T cells express a BV10 TCR and H-2K^d/CW3$_{170-179}$ tetramer$^+$CD8$^+$ cells are undetectable. After CW3 immunization there is a massive expansion of BV10$^+$CD62L$^-$CD8$^+$ cells, the vast majority of which are H-2K^d/CW3$_{170-179}$ tetramer$^+$ (see Fig. 1*B* and data not shown). H-2L^d/NP$_{118-126}$ (RPQASGVYM) and H-2D^b/gp$_{33-41}$ (KAVYNFATM) tetramers (provided by the Tetramer Core Facility, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, MD) were used with CD8 mAb. Cells were analyzed using a FACScan equipped with CellQuest software (BD Biosciences, Mountain View, CA).

Immunohistology

Coronal cryosections of perfused brains were stained for BV10 (B21.5) as described (13).

Cytolytic assay

Specific cytotoxicity of freshly isolated BIL was assessed using a standard ^{51}Cr-release assay (13).

Results

Induction of glioma-specific CTL by cross-presenting APC

To address the issue of cross-presentation in the CNS, we investigated CTL responses to a defined tumor Ag. It has previously been established that CW3-transfected tumor cells (P815-CW3) spontaneously elicit H-2K^d-restricted CTL responses directed against the CW3$_{170-179}$ epitope after i.c. implantation in DBA/2 mice (13). In this study, we transfected the MT glioma (syngeneic to H-2^b VM mice) with a vector encoding the whole CW3 protein (Fig. 1*A*). Because of their H-2^b origin, MT-CW3 transfected tumor cells do not express H-2K^d molecules and are thus unable to endogenously present the CW3$_{170-179}$ decapeptide. Moreover, the absence of MHC class II molecules on MT-CW3 transfectants (Fig. 1*A*) avoids all direct recognition by the CD4 arm of the immune system. As H-2K^d/CW3$_{170-179}$-specific CTL do not cross-react with CW3$_{170-179}$-pulsed H-2^b targets (data not shown), we reasoned that after implantation of the MT-CW3 tumor in semisyngeneic VM × DBA/2 F$_1$ mice (H-2^{bxd}) any expansion of H-2K^d-restricted CW3-specific CTL would be dependent on cross-presentation of CW3$_{170-179}$ by host APC expressing H-2K^d molecules. Indeed, after i.c. implantation of the MT-CW3 tumor, we measured an important H-2K^d-restricted CW3-specific CTL population (detectable either using H-2K^d/CW3$_{170-179}$ fluorescent tetramers or as an expansion of BV10$^+$CD62L$^-$CD8$^+$ T cells) among the BIL (Figs. 1*B* and 2*A*) suggesting that, beside their unequivocal role in the priming phase of the CW3-specific response, host APC were also involved in the local retention of the effector CTL in the brain.

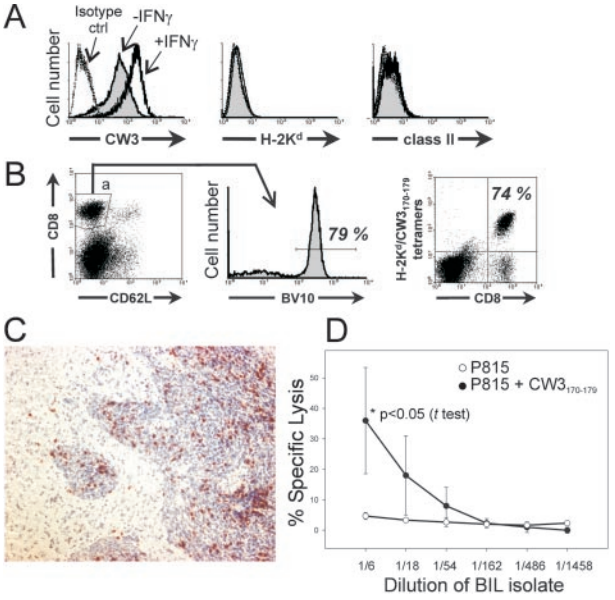

FIGURE 1. Induction of functional tumor-specific CTL occurs through cross-presentation. *A*, Surface phenotype of MT-CW3 transfectants analyzed by flow cytometry. *B*, H-2K^d/CW3$_{170-179}$-specific CTL were detected in BIL derived from H-2^{bxd} mice implanted i.c. with MT-CW3, by measuring either BV10$^+$ cells (histogram, *center*) in CD62L$^-$CD8$^+$ T cells (gate a, dot plot, *left*), or by tetramer$^+$ cells in CD8$^+$ cells (dot plot, *right*). Staining profiles are representative of results obtained in 32 of 36 implanted mice tested with BV10 mAb and in 12 of 12 implanted mice tested with tetramers. *C*, BV10 staining of CTL infiltrating MT-CW3 glioma growing i.c. in H-2^{bxd} mice. *D*, Ex vivo cytotoxicity of BIL isolated from MT-CW3-implanted mice (mean values ± SD, $n = 3$). Results in *B–D* are from mice sacrificed 6–7 wk after i.c. implantation.

CNS retention of functional glioma-specific CTL by cross-presenting APC

Immunohistochemical analyses revealed that brain infiltrating CW3-specific CTL preferentially localized within the MT-CW3 tumor bed, clearly identifiable as a densely counterstained infiltrative mass (Fig. 1*C*). There were no BV10$^+$ T cells detectable in the normal brain parenchyma of the contralateral hemisphere (data not shown), consistent with the notion that Ag-specific interactions between CTL and cross-presenting APC were occurring at the tumor site. Moreover, i.c. CTL exhibited significant CW3$_{170-179}$-specific cytotoxicity when

[3] Abbreviations used in this paper: i.c., intracerebral; NP, nucleoprotein; BIL, brain infiltrating leukocyte; DC, dendritic cell.

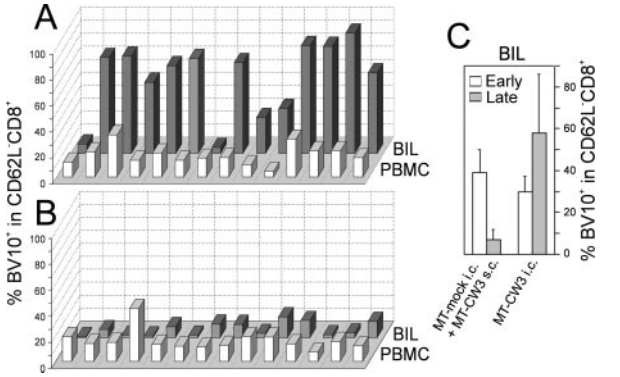

FIGURE 2. Retention of CW3-specific, H-2K^d-restricted CTL in the brain parenchyma of mice only occurs when a CW3-expressing tumor is implanted i.c. *A* and *B*, CW3-specific CTL (BV10$^+$ T cells in CD62L$^-$CD8$^+$ lymphocytes) in PBMC or BIL of individual mice implanted 6–7 wk previously with (*A*) MT-CW3 i.c. or (*B*) MT-CW3 s.c. together with MT-mock i.c. There was a significant accumulation of CW3-specific CTL in brains of group *A* compared with group *B* (Mann-Whitney, $p < 0.001$). All mice of group *B* had elevated proportions of CW3-specific CTL in PBMC at earlier time points (mean: 27.8 ± 10.4% BV10$^+$ in CD62L$^-$CD8$^+$). *C*, Proportion of CW3-specific CTL in BIL, 10 days (early, $n = 15$) or 6–7 wk (late, mean data of mice shown in *A* and *B*) after tumor implantation as indicated.

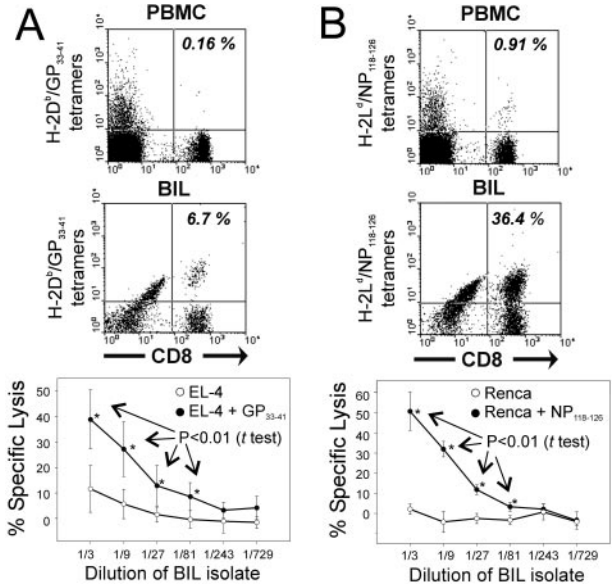

FIGURE 3. Ag-specific accumulation of functional CTL after i.c. implantation of gp- or NP-expressing tumors. *A*, PBMC and BIL from MC57-gp-implanted mice analyzed by flow cytometry 21 days after i.c. implantation. Percent tetramer$^+$ cells in CD8$^+$ T cells is indicated in dot plots (representative results of six individual mice). gp_{33-41}-specific ex vivo cytotoxicity of BIL is also shown (mean values ± SD, $n = 6$). *B*, BALB/c mice immunized i.p with P815-NP tumors 29 days previously were challenged i.c. with the same tumor. H-2L^d/$NP_{118-126}$ tetramer staining of PBMC and BIL purified 13 days after boost are shown in dot plots, with percent tetramer$^+$ cells in CD8 T cells indicated (representative results of four individual mice). $NP_{118-126}$-specific ex vivo cytotoxicity of BIL is also shown (mean values ± SD, $n = 4$).

tested ex vivo (Fig. 1*D*), indicating that cross-presentation of CW3 by local APC can maintain CTL function in the brain.

CNS retention of glioma-specific CTL is Ag-dependent

To establish whether nonspecific factors related to the i.c. tumor outgrowth and consequent local inflammatory response could substitute for Ag-specific interactions in the CNS retention of CW3-specific CTL, we combined a peripheral MT-CW3 implantation of VM × DBA/2 mice with an i.c. implantation of a MT-mock tumor lacking the CW3 Ag (Fig. 2, *B* and *C*). This experiment allowed us to evaluate whether H-2K^d-restricted CW3-specific CTL generated in the periphery were recruited nonspecifically to the brain. Although at early time points (day 10 after implantation, Fig. 2*C*) CW3-specific CTL were detectable at similar levels in BIL of mice implanted i.c. with either MT-mock or MT-CW3, a long term CW3-specific CTL accumulation was observed only in mice where a CW3-expressing tumor had been implanted i.c. (Fig. 2*A*), substantiating the hypothesis that local cross-presentation of CW3 is the main factor responsible for the retention of specific CTL in the CNS.

The CNS microenvironment allows efficient primary and secondary CTL responses

The recruitment of high numbers of effector CTL at the tumor site is critical for efficient antitumor function (14), therefore we checked whether vigorous brain CTL responses where also measurable in other defined systems based on tumors expressing Ag (i.e., lymphocytic choriomeningitis virus gp or NP proteins) inducing well-characterized CTL responses in different mouse strains (15). We used gp-transfected (MC57-gp) or NP-transfected (P815-NP) tumors and H-2D^b/gp_{33-41} and H-2L^d/$NP_{118-126}$ tetramers to follow the expansion of specific CTL. Primary CTL responses, based on the activation and expansion of naive CTL precursors, were initially monitored. The analysis of BIL isolated from mice implanted i.c. with MC57-gp tumor cells revealed that gp-specific functional primary effector CTL were recruited to the CNS (Fig. 3*A*). The CNS persistence of gp-specific CTL after tumor rejection suggests that cross-presenting APC play a role in the maintenance of effector CTL at the site of initial Ag exposure. We then broaden our observations to secondary CTL responses defined by the rapid and massive expansion of memory CTL. Indeed, high numbers of functional Ag-specific CTL persist in the brain of previously i.p. immunized mice that rejected an i.c. challenge of tumor cells. We obtained similar results in C57BL/6 mice implanted with MC57-gp (data not shown) and in BALB/c mice boosted with P815-NP (Fig. 3*B*).

Discussion

To investigate the involvement of cross-presenting APC in the recruitment and retention of tumor-specific CTL in the brain parenchyma, we monitored H-2K^d-restricted CW3-specific CTL responses in H-2^{bxd} mice implanted i.c. with an H-2^b glioma transfected with the CW3 Ag. In view of the lack of H-2K^d on glioma cells (Fig. 1*A*), Ag-specific interaction could only occur between H-2K^d/$CW3_{170-179}$-restricted CTL and host APC (H-2^{bxd}). The mechanism of cross-presentation in antitumor responses, whereby APC take-up exogenous tumor-derived Ag and present them to CD8 T cells has been previously documented (16), although the in vivo significance of this is controversial (17). Nevertheless, recent data are clarifying how cross-presentation may be influenced by the identity, localization, and quantity of the target Ag (18, 19). However, such issues have never been addressed for tumors located in the brain. After i.c. implantation of MT-CW3 glioma, the expansion of H-2K^d/$CW3_{170-179}$-specific CTL unequivocally indicated that cross-presenting APC were mediating CTL priming, most

likely in cervical lymph nodes (based on data from another brain tumor model, not shown). Moreover, the significant and preferential retention of specific CTL within $CW3^+$ tumors growing i.c. (Fig. 1*C* and 2, *A* and *C*) implies that cross-presentation of tumor Ag by host APC in the tumor stroma is the main mechanism contributing to this process. In support of this conclusion, we have preliminary evidence showing that after simultaneously generating similar levels of CTL specific for CW3 or an irrelevant peptide (by i.p. immunization), only CW3-specific CTL significantly accumulate in the MT-CW3-implanted brain (data not shown).

Presentation of exogenous Ag on MHC class I molecules to CD8 T cells by cells of hemopoietic and nonhemopoietic origin can have immunogenic or tolerogenic outcomes (20, 21). Because the outcome of cross-presentation seems to be organ-related and dependent on the particular cell type involved, we assessed the functional status of CW3-specific CTL retained in the brain by local cross-presenting APC. BIL isolated from the brain of MT-CW3-implanted mice demonstrated a significant ex vivo $CW3_{170-179}$ specific cytotoxicity (Fig. 1*D*) indicating that the interaction between cross-presenting APC and CW3-specific CTL maintains CTL function in the brain, rather than inducing tolerance. Moreover, these CTL had comparable avidities to other well-characterized CW3-specific CTL populations (i.e., an established CTL clone and ex vivo CTL from PBMC of P815-CW3 immunized mice), based on H-$2K^d$/$CW3_{170-179}$ tetramer titration experiments (data not shown).

The efficient retention of functional immune effectors by cross-presenting APC may be of particular relevance for the immune-privileged brain, where low expression of MHC class I molecules by resident cells is believed to limit direct Ag presentation (1). However, the complex cellular composition of the brain and the additional recruitment of peripheral accessory cells sharing surface markers with CNS resident cells complicate the precise identification of cross-presenting cells (22). The principal candidates identified by immunohistochemistry are $F4/80^+$ cells (activated macrophages/microglia) that abundantly infiltrate the tumor, as well as cells expressing dendritic cell (DC)-associated Ag (CD11c) that are detectable in BIL by flow cytometry (data not shown). As bone marrow-derived DC are absent from the normal brain parenchyma, it is possible that the CD11c-positive cells represent DC recruited from the periphery, but this remains to be clarified experimentally. Overall, our results clearly show that Ag-specific CD8 T lymphocytes accumulate in the CNS of mice implanted i.c. with experimental tumors, even in the absence of direct tumor recognition by the CTL. To our knowledge, this is the first direct demonstration that local cross-presentation of CNS Ag determines the retention in the brain of functional, MHC class I-restricted CTL of defined specificity. Future studies using brain tumor rejection models will be necessary to determine whether this process significantly impacts on tumor clearance.

In view of the widespread defects in Ag presentation and HLA expression by tumors, including glioma (23), indirect presentation of tumor Ag by local APC may open possibilities for Ag-specific therapies independent of direct tumor recognition by the CTL. For example, CTL secretion of cytokines (such as IFN-γ or TNF-α) after interaction with intratumoral APC may provide a feedback loop enhancing the recruitment and local activation of additional effector cells (e.g., tumoricidal macrophages) able to kill tumor cells in an Ag-independent manner (24). Alternatively, genetic engineering of tumor-specific CTL may allow the delivery of therapeutic molecules or cytokines specifically to the tumor mass as reported for tumor-tracking neural stem cells (25).

The relevance of our findings may not be limited to brain tumor immunology. Indeed, several spontaneous and experimental neurological disorders are characterized by the CNS persistence of CTL considered to actively participate to the pathological process (11, 26). However, with the exception of in vitro data (11), a direct recognition of neural or glial cells by brain infiltrating CTL has never been demonstrated in these disorders.

In conclusion, the local cellular interactions occurring in the brain must be taken into consideration for any immunomodulatory treatment destined for the CNS, because inappropriate immune responses in this site have been the pitfall of many clinical and experimental immunotherapies. In view of the limited immunotherapeutic window characterizing malignant tumors (6), a rapid and intense lymphocytic infiltration of the tumor bed is necessary to achieve tumor regression. In this study, we show that a substantial CNS infiltration by CTL can be tolerated when a high proportion of these cells are tumor-specific (Fig. 3). Furthermore, we show that local cross-presenting APC can direct a preferential CTL localization at the tumor site (Figs. 1 and 2), thus limiting risk for normal tissue from immune effector molecules. However, the same immunological process of cross-presentation may explain the persistence of pathologic CTL in certain CNS disorders, meriting consideration as a therapeutic target for modulating inappropriate immune reactivity.

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