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TCR Vaccines against a Murine T Cell Lymphoma: A Primary Role for Antibodies of the IgG2c Class in Tumor Protection

Stacie L. Lambert,* Craig Y. Okada,† and Ronald Levy2*

Tumor-associated proteins can act as effective immunotherapeutic targets. Immunization with tumor TCR protein conjugated to the immunogenic protein keyhole limpet hemocyanin (KLH) protects mice from tumor challenge with the murine T cell lymphoma C6VL. The immune mechanisms responsible for this tumor protection are of interest for designing more effective vaccine strategies. Previous studies using depletion experiments had suggested a CD8-mediated component of protection induced by TCR–KLH vaccines. In this study we used CD8α knockout, μMT, and FcγR knockout mice to investigate the relative roles of CD8+ T cells and Ab in protective immunity induced by TCR-KLH immunization. We found that CD8+ T cells are not required for tumor protection, although they may contribute to protection. Vaccine-induced Abs are sufficient to mediate protection against this murine T cell lymphoma through an FcR-dependent mechanism. This was confirmed with Ab transfers, which protect challenged mice. Additionally, recombinase-activating gene 1−/− splenocytes can mediate Ab-dependent cellular cytotoxicity against this tumor in the presence of bound anti-TCR Abs. IFN-γ knockout mice demonstrated a requirement for IFN-γ, probably via generation of IgG2c Abs, in vaccine-induced tumor protection. IFN-γ knockout mice were not protected by immunization and had a severe impairment in IgG2c Ab production in response to immunization. Although mock-depleted anti-TCR Abs could transfer tumor protection, IgG2c-deficient anti-TCR Abs were unable to transfer tumor protection to wild-type mice. These results suggest that TCR-KLH vaccine-induced tumor protection in the C6VL system is primarily attributable to the induction of IgG2c Abs and humoral immunity. The Journal of Immunology, 2004, 172: 929–936.

The TCR expressed on T cells contains a variable region (Id) generated by V(D)J recombination during lymphocyte development. This portion of the surface-expressed protein is unique between different T cells and can therefore be used to identify a clonal population of T cells responsible for a disease phenotype such as a malignancy. The B cell Ag receptor (BCR) is expressed on B cells is generated in an analogous manner and can also be used to identify a clonal malignancy. Both TCR and BCR have been successfully used as targets for the immunotherapy of lymphocyte malignancies.

Original reports using Id-based immunotherapy demonstrated antigitumor effects after passive administration of Abs directed against the BCR of B cell lymphoma or the TCR of T cell lymphoma (1, 2). The limitations of passive immunotherapy led to the development of active immunization strategies that allow for the generation of a sustained polyclonal response involving both cellular and Ab-mediated responses against the targeted tumor Ag. In two separate murine B cell lymphoma models established to investigate BCR Id vaccination, both cellular and humoral responses could be detected after immunization, although the primary mechanism of tumor protection proved to be Ab mediated (3, 4).

We established a murine T cell lymphoma model to investigate TCR-targeted active immunotherapy of T cell lymphoma. Immunization with recombinant soluble tumor TCR protein conjugated to keyhole limpet hemocyanin (KLH) generated tumor-protective immunity to the murine T cell lymphoma C6VL (5). The use of either Q5-21 or IL-12 as adjuvants for TCR-KLH vaccines led to tumor protection and enhanced Th1-type Ab induction compared with nonprotective adjuvants (6). CD8 depletion experiments and serum transfers in this study suggested that there may be both a CD8+ cellular component and a humoral component to C6VL tumor protection, although the relative role of the humoral component was unclear (6).

In the current study we used mice genetically deficient in specific immune system components to investigate the effector mechanisms required for TCR-KLH vaccine-induced C6VL tumor protection. Experiments in μMT and FcγR knockout mice indicated that Abs in conjunction with FcR-mediated mechanisms were essential to immunization-induced tumor protection. Experiments in CD8α knockout mice and in wild-type mice depleted of CD8α+ cells indicated that CD8+ cells were not essential for tumor immunity. IFN-γ was required for the induction of protective antitumor immunity by immunization. IFN-γ knockout mice were found to be specifically deficient in the generation of IgG2c Abs in response to TCR-KLH immunization. Tumor protection could be mediated by passive transfer of Abs. This effect was dependent upon the presence of FcRs, and IgG2c-deficient immune Abs lacked tumor protective ability. This tumor protection is probably due to an Ab-dependent cellular cytotoxicity mechanism. These findings will guide the future development of optimal immunotherapeutic strategies against T cell lymphomas in humans.

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Materials and Methods

Mice and cell lines
Female C57BL/6 (H-2b) mice (6–8 wk old) were purchased from Charles Rivers Laboratories (Wilmington, MA), The Jackson Laboratory (Bar Harbor, ME), or Taconic Farms (Germantown, NY). μMT, CD8α knockout, and (FAS-γ) knockout mice, and recombinase-activating gene 1 (RAG1) knockout mice on the C57BL/6 background were purchased from The Jackson Laboratory. FcγR knockout mice on the C57BL/6 background were purchased from Taconic Farms. All mice were housed at the Laboratory Animal Facility at Stanford University Medical Center (Stanford, CA). C6V_SL is an MHC I+, MHC II+ T cell lymphoma cell line of C57BL/6 origin (7). A subline of C6V_SL designated C6V_SL.L was obtained by continuous in vitro culture over several months. Flow cytometric analysis of C6V_SL.L indicated that this subline had a higher forward and side scatter than the original tumor, but a similar surface protein expression profile (data not shown). As C6V_SL retains a radioactive chromium label side scatter than the original tumor, but a similar surface protein expression profile (data not shown), this subline was used as the target cell in cytotoxicity assays. EL4 is an MHC I+ T cell lymphoma cell line of C57BL/6 origin used as a negative control in cytotoxicity assays. MC57G is an adenocarcinoma cell line of C57BL/6 origin that expresses MHC I molecules and was obtained from American Type Culture Collection (Manassas, VA).

Media
All cells were grown in rPMI-10 unless otherwise indicated. rPMI-10 consists of RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FCS (HyClone, Pittsburgh, PA), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), 50 U/ml penicillin, 50 μM streptomycin, and 2 mM glutamine (all from Life Technologies, Grand Island, NY).

Antibodies
mAb 124-40 (mouse IgG1) recognizes a C6V_TCR Vα domain and was purified from culture supernatant over a protein A-Sepharose column (Manassas, VA). C6V_SL.TCR genes were cloned into the expression vector pSRm (43) and designated C6V_SL.TCR. The transmembrane-coding region under the control of a CMV promoter and is sufficient to induce C6V_TCR protein concentration determined by ELISA were roughly 50 μg/ml. From Quillaja saponaria Molina extracts and was given at 10 μg/injection (10). Murine rIL-2 (R&D Systems, Minneapolis, MN) was given at 0.5 μg/injection. Control immunizations consisted of either PBS or 35 μg of self-conjugated KLH in adjuvant. All immunizations were given s.c. in 200 μl of PBS with a total of three immunizations performed, with 2 wk between injections. Serum samples were collected 10–12 days after the final immunization for analysis or passive transfer.

Serum anti-TCR ELISAs
Anti-TCR Abs in serum were quantified as previously described (5). Briefly, plates were coated overnight with 1 μg/ml mAb H57-597 (anti-TCR β) in carbonate buffer, blocked with 5% nonfat dry milk, then coated with purified soluble C6V_TCR protein at 0.5 μg/ml in PBS and 2% BSA. After washing, mouse serum was titrated over eight wells in 2-fold dilutions. After removal of unbound proteins, bound Abs were detected with a donkey anti-mouse IgG-HRP, followed by ABTS substrate (Roche, Indianapolis, IN). Quantification of anti-TCR Abs was achieved by comparison with a simultaneously generated standard curve of mouse anti-C6V_SL mAb (124-40). For isotype analysis of serum anti-TCR Abs, assays were performed as previously described with the following modifications (6). Briefly, plates were coated with H57-597 and C6V_SL at as described above and washed. Immune sera, each pooled from five identically immunized mice, were titrated over eight wells in 2-fold dilutions. Bound anti-TCR Abs were detected with HRP-conjugated donkey anti-mouse IgG (to detect all isotypes) or with isotype-specific HRP-conjugated goat anti-mouse IgG1, IgG2b, or IgG2c. Relative units were determined by comparison with a common pooled serum reference and used to compare anti-TCR isotype levels between different groups.

CTL assay
Spleen and lymph node cell suspensions were prepared from two or three mice per group and were depleted of RBC using Easy Lyse buffer (Linco Research, St. Charles, MO). Lymphocytes (5 × 10^6/group) were restimulated for 6 days with 5 × 10^5 irradiated C6V_SL.L cells, with 30 U/ml human rIL-2 (Chiron, Emeryville, CA) added on the second day of stimulation. Restimulation to generate CTL effectors was conducted in 10 ml of RPMI-10 additionally supplemented with 10 ml HEPES (BioWhittaker, Walkersville, MD). 10 μM MEM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies, Grand Island, NY). C6V_SL.L, EL4, and MC57G targets were labeled with 150 μCi of 51Cr/10^6 cells for 1.5 h and washed extensively before use as targets in a chromium release assay. Targets were incubated in triplicate with washed effectors for 4 h at the indicated E:T cell ratios. Total lysis and background lysis controls were also included. Supernatant was harvested, mixed with scintillation fluid, and counted on a Microbeta 1450 scintillation counter (Wallac, Gaithersburg, MD). Percent specific lysis was calculated as: 100 × (experimental lysis – background lysis)/(total lysis – background lysis).

Generation of TCR<sup>+</sup> alternate target cell
E1/3-E3-deleted Ad5 adenoviral vectors Ad.C6V_SL and Ad.C6V_BL were generated and expanded in 293A cells as previously described (11). These vectors encode C6V_TCR V domains linked to human TCR constant regions under the control of a CMV promoter and are sufficient to induce C6V_TCR tumor protection when used as vaccines (11). Targets transiently expressing TCR Ags (designated MC57G.TCR) were constructed by infecting MC57G cells at 200 multiplicity of infection with each of the Ad.C6V_SL and Ad.C6V_BL. Controls were MC57G cells infected at 400 multiplicity of infection with an irrelevant control adenovirus (MC57G.irc). For each, 1.5 × 10^5 MC57G cells were washed with rPMI-2, mixed with virus in 1.5 ml of rPMI-2, and plated in a six-well plate for 5 h at 37°C. After 5 h, 4 ml of medium was added back for an additional 20 h of incubation to allow protein expression. MC57G.TCR cells were confirmed by flow cytometric analysis with mAb 124-40 to express TCR protein (data not shown) before being labeled with 250 μCi 51Cr as described above for use as targets in a chromium release assay.

In vivo depletion of CD8<sup>+</sup> cells
Vaccinated mice were injected i.p. with ascites fluid containing 250 μg of mAb 243 (anti-CD8.2) or control mAb SFR8-B6 in a total volume of 500 μl of PBS on days −6, −5, −4, and −3 before tumor challenge, with depletion maintained after tumor challenge by six weekly injections beginning on day +3. Flow cytometric analysis of peripheral blood, splenocytes, and lymph node cells from CD8-depleted mice using a nonblocking anti-CD8 mAb (53-5.8) confirmed >95% depletion of CD8 T cells in all compartments 1 day before tumor challenge and >95% depletion maintenance in PBL at 4 wk after tumor challenge (data not shown).

Soluble C6V_SL TCR protein generation
Briefly, the C6V_SL TCR α and β sequences were cloned from C6V_SL into DNA constructs and then modified such that the transmembrane-coding sequences of the receptor were replaced with sequences encoding a thrombin cleavage site, leucine zipper regions, and a terminal six histidine residues (8, 9). The modified C6V_SL TCR genes were cloned into the expression vector pSRm and transfected into BW5147 cells (5). A cell line was isolated that secreted soluble C6V_SL TCR into the surrounding media at 3 mg/liter in standard culture. For protein production, this cell line was maintained in the cell compartment of CL1000 flasks (Integra Biosciences, Jamisonville, MD) containing rPMI-15 supplemented with 3.6 mM methotrexate (Sigma-Aldrich). Under these culture conditions the cells produced TCR protein at 10 mg/liter. Cell supernatant was collected, cleared of cell debris by centrifugation at 500 × g, filtered through a 0.45-μm pore size filter, and applied to an Ni-NTA column (Qiagen, Valencia, CA). Bound histidine-tagged protein was eluted with 200 mM imidazole. The soluble TCR protein was concentrated by Centriplus ultrafiltration (Amicon, Beverly, MA), dialogized against PBS, and filter-sterilized. The total protein concentration determined by bicinchoninic acid (Pierce, Rockford, IL) and the TCR protein concentration determined by ELISA were roughly equivalent.

TCR immunizations
Purified heterodimeric C6V_SL TCR was chemically conjugated to KLH (Pierce) at a 1:1 ratio with glutaraldehyde and dialyzed against PBS as previously described (5). Each TCR immunization contained 35 μg of TCR, an equal amount of conjugated KLH, and adjuvant. QS-21 (provided by Antigenics, Framingham, MA) is a purified saponin-based adjuvant

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Mice were challenged with tumor 2 wk after final immunization. Two to 3 days before tumor challenge, an aliquot from a single batch of frozen C6VL tumor cells was thawed and cultured in RPMI-10. On the day of tumor challenge, cells were collected by centrifugation and washed three times in ice-cold HBSS. Two thousand cells were injected i.p. in a volume of 500 μl of ice-cold HBSS, and animals were then followed for survival for 70 days. Groups of 10 mice were used for survival analysis. Survival curves were plotted by the Kaplan-Meier method, and statistical significance was determined by log-rank using PRISM software (GraphPad, San Diego, CA).

Passive γ-globulin cotransfer

Serum was harvested 10–12 days after final immunization from C57BL/6 mice immunized three times with TCR-KLH or with control KLH vaccines. The γ-globulin fraction of the serum was obtained by precipitation at 4°C with ammonium sulfate at a final saturation of 45%. Precipitated proteins were collected by centrifugation at 18,000 g for 60 min at 4°C, redissolved, and dialyzed extensively against PBS. Anti-TCR IgG in the γ-globulin was quantified by ELISA as described above. Total IgG (of all target specificities) in the γ-globulin was quantified by a modified ELISA. Briefly, γ-globulin was titrated over plates coated with anti-mouse IgG (H+L), bound total IgG Abs were detected with donkey anti-mouse IgG-HRP, followed by ABTS substrate, and quantities were determined by comparison with a mouse IgG1 standard curve. Hyperimmune γ-globulin containing 3 μg of anti-TCR Abs (in 50–80 μg of total IgG) or control γ-globulin matched for total IgG was mixed with 2000 C6VL cells in a total volume of 500 μl of HBSS on ice. Mice were challenged as described above within 30 min of cell preparation.

Ab-dependent cellular cytotoxicity (ADCC) assay

Splenocytes harvested from RAG1 knockout mice were used as ADCC effector cells. Spleens were harvested, made into single-cell suspensions, and depleted of RBC using Easy Lyse lysis buffer (Linco Research). These splenocytes were confirmed by flow cytometric analysis to lack T cells and to contain ~30% DX5+ (NK) cells and ~60% CD11b+ (macrophage) cells (data not shown). C6VLSL was labeled with 100 μCi of 3HCl/100 cells in minimal volume for 1.5 h at 37°C, washed twice, allowed to postleak 15 min at 37°C, and washed again. C6VLSL cells were then incubated at 1 × 10⁶ cells/ml with anti-CD4, anti-TCR, or control Abs at 10 μg/ml on ice for 30–45 min, washed, and resuspended with effector cells at different E:T cell ratios for 4-h coincubation in 96-well plates. Total lysis and background lysis controls were also included. Percent specific lysis from radioactive counts released into the supernatant was determined as described for CTL assays.

Depletion of IgG2c Abs from immune γ-globulin

Goat anti-mouse IgG2c Abs (Southern Biotechnology Associates) were coupled to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ), and the resulting beads were dialedyzed against PBS. IgG2c was then specifically depleted from an aliquot of immune γ-globulin by successive rounds of exposure to the anti-IgG2c beads. Mock-depleted immune γ-globulin was simultaneously generated by exposure of a separate aliquot of immune γ-globulin to control BSA-conjugated Sepharose beads. Total IgG, anti-TCR IgG, anti-TCR IgG1, and anti-TCR IgG2c in the depleted γ-globulin was determined by ELISA following the final round of bead incubation. IgG2c-depleted immune γ-globulin was confirmed to lack detectable IgG2c Abs, whereas mock-depleted immune γ-globulin contained a ratio of IgG2c to IgG1 Abs equivalent to that of unimunized immune γ-globulin. IgG2c-depleted and mock-depleted immune γ-globulins were then used in passive γ-globulin cotransfer experiments as described above.

Results

IL-12 addition to TCR-KLH/QS-21 vaccines enhances anti-TCR IgG2c Abs

Our previous studies in the C6VL system demonstrated that the best individual adjuvants for use with TCR-KLH immunizations to induce a protective immune response were QS-21 or IL-12. Use of QS-21 adjuvant had been shown to generate higher anti-TCR Ab titers, whereas use of IL-12 adjuvant had been shown to generate low anti-TCR Ab titers, but an enhanced Th1 skewing of the induced Abs, and both resulted in significant protection of challenged animals (6). We compared the individual use of QS-21 and IL-12 adjuvants with TCR-KLH vaccines to the combined use of QS-21 and IL-12. We show that the combination of QS-21 and IL-12 adjuvants with TCR-KLH vaccines induced both high titer anti-TCR Abs and a high IgG2c/IgG1 Ab ratio and additionally provided a high level of tumor protection (Fig. 1 and Table I). Although the absolute levels of tumor protection observed in this system can vary between experiments, the groups within a given experiment are comparable (being from the same vendor, born at the same time, given the same handling, and given tumor grown the same). In this experiment all TCR-KLH-immunized groups were protected from tumor challenge. Because the combination of QS-21 and IL-12 adjuvants induced the most robust immune response, it was chosen for subsequent immunization experiments in this study.

CD8⁺ T cells are not essential for vaccine-induced tumor protection

The role of CD8⁺ T cells in C6VL tumor immunity was investigated by immunizing and tumor-challenging both CD8α knockout animals and wild-type C57BL/6 animals depleted of CD8α cells by anti-CD8α Ab injections. CD8α knockout mice have virtually no functional CTL population due to defective thymic maturation of the CD8⁺ T cell compartment (12). CD8α knockout mice were equivalently protected to wild-type C57BL/6 mice by TCR-KLH immunization (Fig. 2A) and generated anti-TCR Abs in equivalent titers to wild-type mice (Fig. 8). This suggests that cytotoxic CD8⁺ T cells are not required for C6VL tumor protection. Depletion of CD8α-expressing cells by Ab injections partially decreased tumor protection in TCR-KLH-immunized, wild-type animals, but did not abrogate protection (Fig. 2B). This result again suggested that cytotoxic CD8⁺ T cells are not required for C6VL tumor protection, as some protection exists in the absence of CD8⁺ T cells. The observed decrease in protection after Ab-mediated depletion indicated a possible contribution by CD8⁺ T cells to tumor protection in wild-type mice.

CD8⁺ CTL activity is not detectable in immunized mice

As no absolute in vivo CD8⁺ T cell requirement could be demonstrated, we wanted to determine whether CD8-dependent CTL...
activity against C6VL lymphoma could be detected in vitro before or after tumor challenge. Lymphocytes were harvested 10 days after final vaccination from mice immunized three times with either PBS or TCR-KLH/QS-21 plus IL-12. Lymphocytes were concurrently harvested from mice that had been immunized previously with TCR-KLH/QS-21 plus IL-12 and survived C6VL tumor challenge to day 70. No CTL activity was detectable from restimulated lymphocytes from PBS- or TCR-KLH-immunized mice that did not see tumor, but specific anti-C6VL CTL activity could be detected from restimulated lymphocytes harvested from tumor survivors (Fig. 3A). This demonstrates that CTL against C6VL are not induced in significant numbers by TCR-KLH immunization, although they can develop as a result of tumor exposure. To determine whether this tumor-specific CTL activity was directed against epitopes within the TCR variable region, as initially hypothesized, a TCR-transfected MC57G target cell was generated. This TCR-transfected target was not lysed by specific anti-C6VL CTLs, suggesting that CTL induction was not the result of TCR-KLH immunization, but was more likely a result of in vivo cross-priming to non-TCR Ags expressed by the tumor after tumor exposure (Fig. 3B). The target Ag that is recognized by these anti-C6VL CD8+ CTL is currently unknown.

Vaccine-induced tumor protection is dependent upon a humoral immune response and FcRs

As CD8+ T cells were not shown to play an essential role in tumor protection in this system, the role of Abs in TCR-vaccine-induced tumor protection was next investigated using μMT and FcγR knockout animals. μMT animals lack the transmembrane portion of the Ig μ H chain. They have a severe block in the development of mature B cells and lack circulating IgM and IgG Abs, although T cell development is unaffected (13). C6VL tumor grew more
slowly in μMT mice than in wild-type mice, perhaps due to minor residual allogeneic differences in this backcrossed C57BL/6 strain. In these μMT mice, TCR-KLH immunization did not provide any protection from tumor challenge in comparison with control immunization (Fig. 4A). Wild-type mice immunized and challenged concurrently were protected (data not shown). As μMT mice lacked anti-TCR Ab responses (Fig. 8), this suggests a requirement for either B cells or Ab in antitumor protection. FcγR knockout animals lack the common γ-chain required by the two murine activating FcRs, FcγRI and FcγRIII, for surface assembly and signal transduction (14, 15). As a result, these mice have specific defects in Ab-dependent cellular responses, such as NK-mediated ADCC and FcR-dependent uptake of Ab-Ag immune complexes by macrophage and dendritic cells. FcγR knockout mice generated humoral responses to immunization equivalent to wild-type mice (Fig. 8). In our experiments, FcγR knockout mice were not protected from C6VL tumor challenge by TCR-KLH immunization, although wild-type mice immunized and challenged concurrently were protected (Fig. 4B and data not shown). This indicates that activating FcRs, possibly in conjunction with interactions with Abs, are required either during the induction of a protective immune response or during the effector stage of tumor protection in this system.

Abs can transfer tumor protection in an FcγR-dependent manner

The lack of TCR-KLH vaccine-induced tumor protection in μMT and FcγR knockout mice combined with vaccine-induced tumor protection in CD8α knockout and depleted animals strongly suggested that immune Abs might be the primary mechanism of immune protection. However, it was possible that Abs and FcRs were required for optimal immunization. B cell Ag presentation and FcγR-mediated enhancement of Ag presentation are known to contribute to the priming of cellular immune responses, and T cells from μMT mice and FcγR knockout mice demonstrate diminished secondary in vitro proliferative responses compared with T cells from wild-type mice (16, 17). We therefore wished to investigate whether immune Ab transfers at the time of tumor challenge would be sufficient to protect animals. We found that tumor protection could be achieved by simultaneous passive transfer of immune γ-globulin with tumor into naive wild-type mice (Fig. 5A). As expected, transfer of immune γ-globulin into FcγR knockout mice could not mediate tumor protection (Fig. 5B), and transfer of control γ-globulin could not protect either wild-type or FcγR knockout mice (Fig. 5).

C6VL cells can be in vitro targets of ADCC with anti-TCR Abs

The mechanism suggested by this Ab- and FcγR-dependent tumor protection was that of ADCC. NK cells and macrophage can mediate ADCC through their activating FcRs. These cells are usually a minor population in murine splenocytes, so splenocytes from RAG1 knockout mice lacking mature peripheral T or B cells were used as a source of ADCC effector cells. These cells, confirmed by flow cytometric analysis to contain both NK and macrophage, but a minor population in murine splenocytes, so splenocytes from RAG1 knockout mice lacking mature peripheral T or B cells were used as a source of ADCC effector cells. These cells, confirmed by flow cytometric analysis to contain both NK and macrophage, but a minor population in murine splenocytes, so splenocytes from RAG1 knockout mice lacking mature peripheral T or B cells were used as a source of ADCC effector cells. These cells, confirmed by flow cytometric analysis to contain both NK and macrophage, but a minor population in murine splenocytes, so splenocytes from RAG1 knockout mice lacking mature peripheral T or B cells were used as a source of ADCC effector cells. These cells, confirmed by flow cytometric analysis to contain both NK and macrophage.
IFN-γ is required for tumor protection induced by TCR vaccines

IFN-γ is a key Th1-biasing cytokine that can be induced by the adjuvants QS-21 and IL-12 (18). IFN-γ knockout mice have been reported to have deficient IgG2a responses, impaired macrophage function, and reduced NK activity (19). As any of these functions might be required for Ab and FcR-dependent protection, we investigated whether IFN-γ knockout mice could be protected from tumor challenge by TCR-KLH vaccines. Immunization failed to protect these mice, indicating that some role of IFN-γ is required for TCR-KLH vaccine-induced tumor protection (Fig. 7). Although a strong humoral anti-TCR response was induced by immunization in IFN-γ knockout mice, isotype analysis of the induced anti-TCR Abs revealed that these mice had severely compromised IgG2c responses in comparison with wild-type mice (Fig. 8). This is in accordance with compromised IgG2c responses in comparison with wild-type mice (Fig. 9). Transfer of immune γ-globulin harvested from wild-type mice to IFN-γ knockout mice conferred a degree of tumor protection (data not shown) despite their reported macrophage and NK deficiencies, suggesting that IgG2c anti-TCR Abs might be the relevant immune component lacking in IFN-γ mice.

Anti-TCR Abs of the IgG2c isotype play a role in tumor protection

To test our hypothesis that IgG2c anti-TCR Abs play a key role in TCR-KLH vaccine-induced tumor protection, we set up two approaches to transfer IgG2c-deficient immune Abs to wild-type mice. First, γ-globulin harvested from immunized IFN-γ knockout mice (which was deficient in IgG2c anti-TCR Abs) was transferred with tumor to naive wild-type mice (Fig. 9A). In the second approach, immune γ-globulin harvested from wild-type mice and depleted of IgG2c Abs was transferred with tumor to naive wild-type mice (Fig. 9B). In both experiments, animals that received immune γ-globulin deficient in IgG2c Abs were significantly less protected than animals that received immune γ-globulin complete in IgG2c Abs. More importantly, these groups were not protected compared with animals that received control nonimmune γ-globulin.

Discussion

These data demonstrate that immunization with TCR-KLH/QS21 plus IL-12 generates protection against a murine T cell lymphoma in an Ab- and activating FcγR-dependent manner consistent with an ADCC mechanism. CD8+ T cells are not directly induced by immunization and are not required for tumor protection. IFN-γ knockout mice, which were deficient in IgG2c Abs, demonstrated an absence of vaccine-induced protection. Although transfer of immune Abs to naive wild-type animals could protect against tumor challenge, IgG2c deficiency in these transferred Abs was nonprotective, suggesting a critical role for Abs of the IgG2c subclass in tumor protection and thus a critical role for IFN-γ in generating these Abs.

Adjuvants may be essential to the form of protective immunity observed after immunization. QS-21 and IL-12 augment cellular immune responses, in part by inducing production of IFN-γ and in combination increase vaccine potency (23, 24). IL-12 enhances both NK and CTL activity (25), whereas IFN-γ additionally enhances B cell IgG2a Ab production (26). In this study TCR-KLH vaccines were given with the adjuvant combination of QS-21 plus IL-12, whereas our previous studies have used one or the other adjuvant (6). This difference in adjuvant usage may contribute to the difference in findings. Although the adjuvant combination used in this study should optimally induce CTL responses in addition to enhancing innate immunity and Th1-type Ab responses, we were unable to demonstrate any immunization-induced CTL responses.

In this study experiments in both CD8α-depleted animals and CD8α knockout animals demonstrate the lack of a requirement for CD8+ T cells in C6VL tumor protection. Additionally, we demonstrate that TCR-specific CTL were not present even in surviving immunized animals. Data in our earlier report that suggested a role for CD8+ T cells in C6VL tumor protection was based on the abrogation of tumor protection following in vivo Ab depletion of CD8α+ cells (6). Ab-mediated anti-CD8α depletions remove the targeted CD8αβ T cell subset, but can also remove CD8αα populations of dendritic cells (27). The CD8αα dendritic cells are a major producer of IL-12 and IFN-γ in vivo, and their loss may contribute to the decreased protection seen in CD8α-depleted animals (28, 29). We have in this study confirmed a reduction in vaccine-induced tumor protection after in vivo depletion of CD8α+ cells in wild-type mice, although significant protection remained. CD8α knockout animals exhibit a near-complete lack of CTL function, but retain the functional presence of dendritic cell subsets affected by Ab depletion, thus making them a better model to test the role of CD8+ T cells in vaccine-induced protection (12,
immunization with C6VL TCR Vα141 Vβ14-4.1 and compared for isotype-specific anti-TCR Abs by ELISA. Plates were coated with soluble recombinant TCR as described in Materials and Methods. Serum was tetanized over the plates and detected with HRP-conjugated anti-IgG, anti-IgG1, anti-IgG2b, or anti-IgG2c. A pooled diluted immune serum was used as the standard to compute relative units. Bars indicate the SD within groups of 10–20 mice.

CD8α knockout animals showed full vaccine-induced protection from tumor challenge. These results are consistent with the recent report of CD8-independent protection against C6VL following DNA immunization with C6VL TCR VαVββBF fused to the immunogenic fragment C of tetanus toxin (31).

Our prior study showed a minor protective effect of hyperimmune serum transfer (6). In this study we found that Abs induced by TCR-KLH/QS21 plus IL-12 and fragment C of tetanus toxin (31).

FIGURE 8. Isotype analysis of the anti-TCR humoral response to TCR-KLH immunization in serum from C57BL/6 knockout mice. Serum was obtained from wild-type and knockout mice 10 days after final immunization with TCR-KLH/QS21 plus IL-12 and compared for isotype-specific anti-TCR Abs by ELISA. Plates were coated with soluble recombinant TCR as described in Materials and Methods. Serum was tetanized over the plates and detected with HRP-conjugated anti-IgG, anti-IgG1, anti-IgG2b, or anti-IgG2c. A pooled diluted immune serum was used as the standard to compute relative units. Bars indicate the SD within groups of 10–20 mice.

CD8α knockout animals showed full vaccine-induced protection from tumor challenge. These results are consistent with the recent report of CD8-independent protection against C6VL following DNA immunization with C6VL TCR VαVββBF fused to the immunogenic fragment C of tetanus toxin (31).

Our prior study showed a minor protective effect of hyperimmune serum transfer (6). In this study we found that Abs induced by TCR-KLH/QS21 plus IL-12 and fragment C of tetanus toxin (31).

FIGURE 9. IgG2c-deicient immune γ-globulin does not protect mice from tumor in cotransfer assay. Immune γ-globulin containing 3 μg of anti-TCR Abs or control γ-globulin matched for total IgG content was mixed with 2000 C6VL tumor cells and cotransferred into naive recipients. A, Immune γ-globulin from wild-type mice (●), IgG2c-deficient immune γ-globulin from IFN-γ knockout mice (▲), or control nonimmune γ-globulin from wild-type mice (▲) was cotransferred with tumor into C57BL/6 wild-type mice. Mice were then followed for survival differences (● vs ▲, p = 0.0002; ▲ vs ●, p = 0.023; ▲ vs ●, p = 0.0611). B, IgG2c Abs were depleted from immune γ-globulin by passage over Sepharose-conjugated anti-IgG2c Abs. Immune γ-globulin from wild-type mice (●), mock-depleted immune γ-globulin from wild-type mice (▲), IgG2c-depleted immune γ-globulin from wild-type mice (▲), or control nonimmune γ-globulin from wild-type mice (▲) was cotransferred with tumor into C57BL/6 wild-type mice. Mice were then followed for survival differences (● vs ▲, p = 0.0041; ▲ vs ●, p = 0.0039; ▲ vs ●, p = 0.5391; ▲ vs ▲, p = 0.5445; ▲ vs ●, p = 0.0145; ▲ vs ●, p = 0.0352).

Activating FcRs are also expressed on APCs, such as macrophage and dendritic cells, to facilitate APC uptake of Ab-opsonized cells and cell fragments for cross-presentation to T cells. Ab-mediated Ag uptake has been reported to induce dendritic cell maturation (35), whereas coating a tumor with Abs has been demonstrated to enhance CTL induction, presumably through APC cross-presentation of cellular tumor Ags to CD8+ T cells (36).

Cross-presentation may thus play a role in the induction of a non-TCR-directed CD8+ CTL response against C6VL in animals after tumor exposure. Anti-C6VL CD8+ T cells induced by cross-presentation may also contribute to the tumor protection observed in this system, as CD8 depletion did decrease the level of tumor protection seen in immunized mice.

Although IFN-γ plays a key role in inducing Th1-type responses, of which CD8+ CTL are one component, IFN-γ knockout mice are capable of generating functional CTL in response to immunizations (37, 38). In our studies of TCR vaccination against C6VL lymphoma, IFN-γ knockout mice were not protected. The
Ab-dependent, CD8-independent nature of tumor protection in our system combined with the low IgG2c Ab levels induced in IFN-γ knockouts by vaccination suggested that IgG2c Ab induction and not CTL induction might be the IFN-γ-dependent mechanism involved. This supposition is supported by the finding that IgG2c-deficient Ab transfers were unable to transfer tumor protection. It may be worthwhile to compare passive immunotherapy with anti-TCR Abs (such as those against Vβ chains) to active TCR vaccination of human T cell malignancies. Passive immunotherapy with therapeutic Abs, either monoclonal or polyclonal, would be easier to apply to a broad patient population than the individualized vaccine formulations needed for active vaccination. Our results also suggest that clinical trials of active vaccination against human T cell lymphoma should attempt to generate Abs of the IgG1 subtype, the human analog of murine IgG2a/IgG2c.

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References