

Instant immunity through chemically programmable vaccination and covalent self-assembly

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The ability to instantly create a state of immunity as achieved in the passive transfer of hyperimmune globulin has had a tremendous impact on public health. Unlike passive immunization, active immunization, which is the foundation of vaccinology, is an anticipatory strategy with inherent limitations. Here we show that elements of active and passive immunization can be combined to create an effective chemistry-driven approach to vaccinology. Reactive immunization was used to create a reservoir of covalent polyclonal antibodies in 3 mouse strains that were subsequently engrafted with syngeneic CT26 colon or B16F10 melanoma tumors. Upon administration of designed integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ adapter ligands, the induced covalent polyclonal antibodies self-assembled with the adapter ligands and the animals mounted an instant, chemically programmed, polyclonal response against the implanted tumors. Significant therapeutic responses were observed without recourse to adjuvant therapy. The chemically programmed immune responses were driven by antibody-dependent cellular cytotoxicity and complement-directed cytotoxicity. We suggest that this type of chemistry-driven approach to vaccinology is underexplored and may provide routes to vaccines to protect against diseases that have proven intractable to biology-driven vaccine approaches.

aldolase | angiogenesis | colon cancer | melanoma | tolerance

Despite certain limitations, the time-honored tradition of vaccination has been extraordinarily successful. Typically, a disease relevant immune response is achieved following one or more immunizations, and the level of response in which a prophylactic or therapeutic effect is observed takes days or weeks to build. Thus, vaccination is anticipatory by nature and the kinetics of the immune response limits the efficacy of vaccine-based strategies against the aggressive pathogens or rapidly acting toxins against which one would want to instantly create an immune state. Ideally, immunity could be specifically and rapidly directed against a nonself antigen like a virus or bacterium or a self-antigen related to cancer or some other disease. The later class of antigens involves breaking tolerance and presents inherent challenges that have only recently begun to be addressed (1). Furthermore, the most commonly used vaccination strategies use whole proteins, viruses, or other complex immunogens and induce antibodies reactive against both nonfunctional and functional epitopes; the ideal approach would direct immunity only against functional or neutralizing epitopes, for example the conserved neutralizing epitopes on HIV-1. Finally, one would like to circumvent the age-related declines in immune function (2, 3).

To address these challenges in vaccine development, we have turned to reactive immunization as an approach for inducing a covalent-binding antibody response. Reactive immunization was originally developed for the generation of catalytic monoclonal antibodies and differs from the usual immunization approaches in that reactive chemicals designed to elicit covalent antibodies are used as immunogens (4–6). We have previously shown that immunization with β -diketone immunogens allows for the reproducible induction of covalent antibodies that can be used to

catalyze enamine- and iminium-based transformations like the aldol reaction (7, 8). We have also shown that covalent monoclonal antibodies can be programmed via their covalent reaction with designed ligands of a variety of specificities and that such chemically programmed antibodies possess potent biological activities in a variety of animal models of disease (9–14). Indeed, several human trials are ongoing to explore the efficacy of chemically programmed monoclonal antibodies in treatment of human disease. Given these successes, we hypothesized that covalent polyclonal responses might be efficiently induced *in vivo* to produce a therapeutic outcome. Herein, we demonstrate that the induced polyclonal response can be programmed by injection of a suitably designed programming compound to provide treated animals with “instant immunity.”

Results and Discussion

In previous studies, the covalent binding monoclonal antibody 38C2 was programmed with a variety of targeting agents and studied in multiple cancer models using human tumor xenografts in immune-deficient mice (9, 11, 12, 14). To explore the potential of an elicited covalent immune response, we have turned to immune competent mice and syngeneic cancer models. We aimed to determine if a high-titer covalent antibody response could be elicited in a variety of murine strains. We also sought to show that the induced responses could be programmed to bind murine (self) targets relevant to cancer and impact tumor growth in murine cancer models. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were chosen as target antigens since these surface proteins are expressed by a wide variety of tumor types and on angiogenic vasculature (15–23). Furthermore, we had previously validated these integrins as therapeutic targets using chemically programmed monoclonal antibody 38C2 (cp38C2). The targeting of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ with a chemically programmed immune response is illustrated in Fig. 1A. The compounds SCS-873 and cRGD-dk (Fig. 1B) serve as chemical adaptors that react with covalent antibodies through their diketone tags to redirect the binding of the immune response to the integrins expressed on cell surfaces. Because cell-bound antibodies can bind through their Fc regions to molecules of the complement cascade, (such as C1q) and to Fc receptors expressed on the surface of immune effector cells (such as natural killer cells), programmed immunity can potentially direct complement-directed cytotoxicity and antibody-dependent cellular cytotoxicity.

SCS-873 and cRGD-dk Program Antibody mAb 38C2 to Bind Human and Mouse Integrins. To validate the potential of SCS-873 and cRGD-dk to reprogram mAb 38C2 to bind integrins $\alpha_v\beta_3$ and

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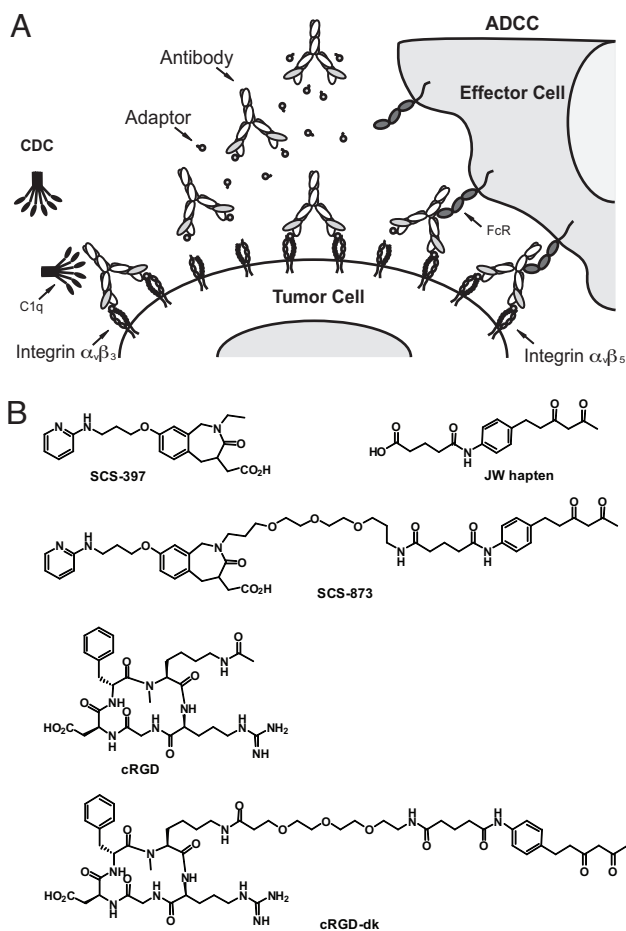


Fig. 1. Antibody redirection by chemical programming. (A) After programming with a chemical adaptor, the anti-JW hapten antibodies recognize $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on cancer cell surfaces. (B) Structure of the JW hapten, SCS-873 and cRGD-dk chemical adaptors, and SCS-397 and cRGD control ligands that lack diketone tags.

$\alpha_v\beta_5$ expressed on murine cancer cell lines, specific binding of cp38C2, formed following reaction with SCS-873 or cRGD-dk, was established in an ELISA using human integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Fig. 2A) (14). Both compounds were effective in directing 38C2 to bind $\alpha_v\beta_3$ and $\alpha_v\beta_5$; importantly, neither programmed antibody bound well to integrin $\alpha_{IIb}\beta_3$. Next, using flow cytometry, we showed that the cpAbs bound to integrin receptors expressed on murine cells, colon cancer line CT26, and melanoma line B16 (Fig. 2B). SCS-873 programmed antibody provided more robust binding to CT26 cells than the cRGD-dk programmed antibody. With B16 cells, no substantial binding of cRGD-dk programmed antibody was observed, whereas a substantial staining was observed using SCS-873 programmed antibody. As previously shown for SCS-873 (14), cRGD-dk effectively stained cells from the mouse endothelial cell line MS1 and from the human melanoma cell line M21 (see SI). In these and other studies, the corresponding targeting agents lacking diketone tags, SCS-397 and cRGD, served as negative controls; these molecules lack the diketone functionality required to bind to mAb 38C2 or to polyclonal antibodies induced through immunization with the β -diketone hapten JW.

Reactive Immunization. Immunization with keyhole limpet hemocyanin (KLH) coupled to JW hapten (JW-KLH) or to other diketone haptens has been used for the preparation of a variety of covalent-binding, monoclonal antibodies (4–6). Reactive

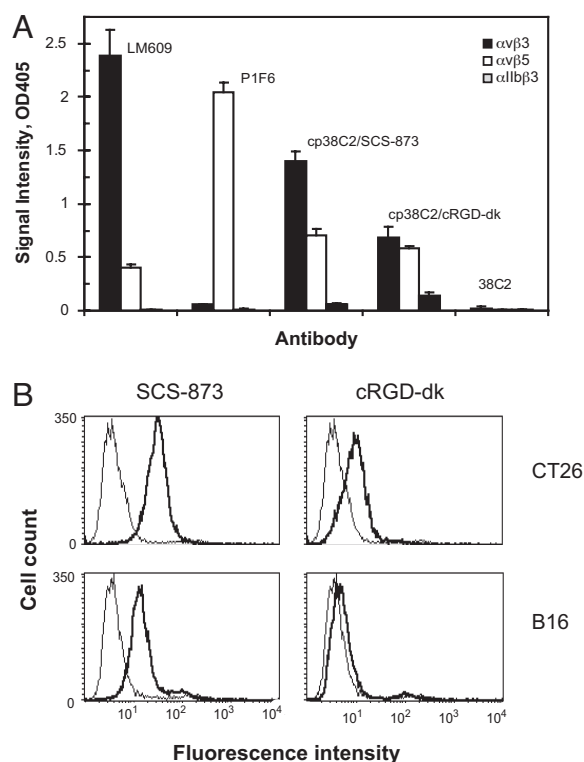


Fig. 2. Adaptor validation through cp38C2 binding to integrins and integrin-expressing cells. (A) Specific binding of cp38C2 to human integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ was measured by ELISA as described in the *Materials and Methods*. LM609 (anti- $\alpha_v\beta_3$) and P1F6 (anti- $\alpha_v\beta_5$) and mouse mAb 38C2 were also tested. Data shown represent the mean \pm SD of triplicate samples. (B) Flow cytometry analysis of cp38C2 binding to mouse B16 melanoma, mouse colon carcinoma CT26. For studies on human M21 melanoma and mouse endothelial MS1 cell lines, see SI, all of which express both integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on their surface, was performed as described in the *Materials and Methods*. Cells were stained with cp38C2 mAb programmed with indicated ligand (bold line) and unprogrammed 38C2 mAb (thin line). Bound antibodies were detected with FITC-conjugated donkey antimouse IgG.

immunization differs from classical immunization approaches in that reactive chemicals, in this case β -diketones, are used as immunogens and provide the immune system with the opportunity to select for the formation of a covalent bond between the antibody and the reactive antigen during the maturation of the immune response. Here, to elicit a covalent polyclonal antibody response through immunization, 3 mouse strains [BALB/C, C57BL6, and -Fc γ RIII (receptor knockout mice)] were immunized with JW-KLH and subsequently boosted with 2 additional injections of JW-KLH. Immune serum was pooled from immunized animals of each strain and examined by ELISA for induction of covalent binding antibody responses. Using a panel of 20 monoclonal antibodies (4), we have demonstrated that monoclonal antibodies that bind covalently to the JW hapten through enaminone formation are not released following treatment with acid (0.05M citric acid, pH 2.5), whereas noncovalent, albeit high-affinity, JW-binding antibodies are readily released following an acid wash (see SI). Non-covalent complexes are readily disrupted at low pH, the buffer type typically used as an eluant in antibody affinity chromatography. Therefore, acid-wash ELISA allowed us to approximate the covalent polyclonal JW titer. Substantial covalent antibody titers were found for all 3 mouse strains (Fig. 3) and, with the exception of IgA, a variety of antibody isotypes were found to constitute the anti-JW response (Table 1). Upon addition of SCS-873 to immune sera, the serum was efficiently programmed to bind integrin $\alpha_v\beta_3$ as

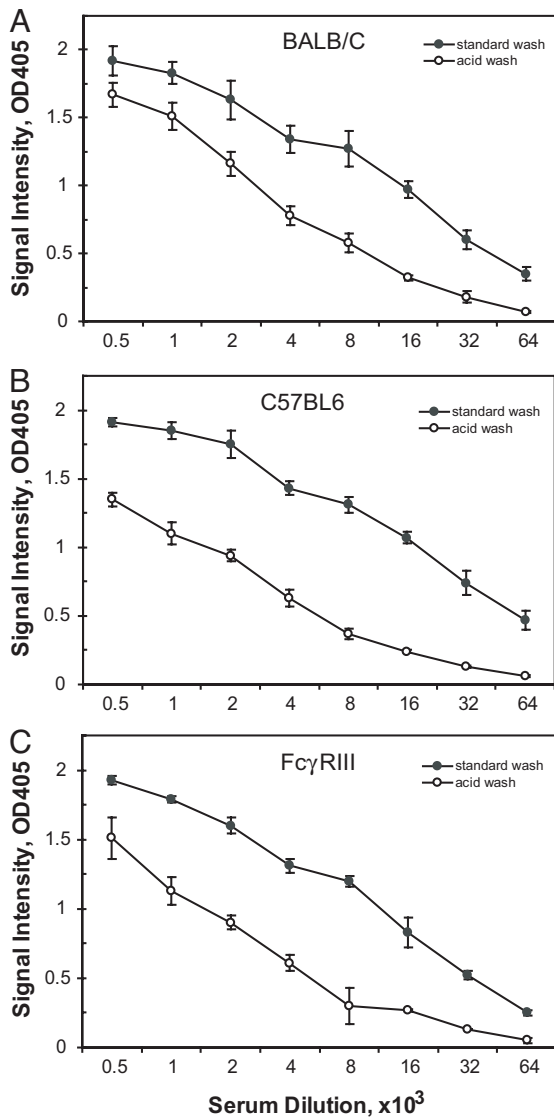


Fig. 3. Induction of high-titer covalent antibody responses. Mice were immunized with JW-KLH and subsequently boosted with 2 additional injections of JW-KLH. Direct binding of indicated dilutions of pooled immune serum from (A) BALB/C, (B) C57BL6, and (C) Fc γ RIII knockout mice to immobilized JW-BSA was measured by ELISA as described in *Materials and Methods*. This acid-insensitive binding provided an indirect measure of covalent antibody titer.

demonstrated by ELISA (Fig. 4). It should be noted that while the programmed immunoglobulins all display the same antigen specificity as programmed by the adapter ligand, antibodies of the various isotypes will display the adapter ligands with different display valancies; IgM will display 10 adapter ligands and very avidly bind to cell surfaces while IgG classes will display 2 adapter ligands and induce a different spectrum of immune

Table 1. Serum titers and anti-hapten isotypes

Mice	Serum titer	Anti-JW Ab isotypes (%)					IgA
		IgG1	IgG2a	IgG2b	IgG3	IgM	
BALBC	1:3,000	21	16	21	18	23.5	0.5
C57BL6	1:6,000	22	5	20	34	18.5	0
Fc γ RIII	1:6,000	23	3.5	20	25	28	0.5

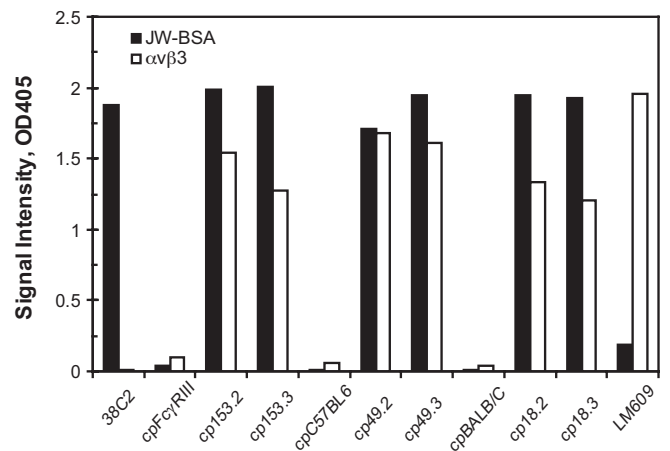


Fig. 4. Adaptor-mediated redirection of anti-JW hapten antibody binding. Specific binding of anti-JW hapten mouse sera to human integrins $\alpha_v\beta_3$ in the presence of SCS-873 was measured by ELISA as described in *Materials and Methods*. Mouse LM609 (anti- $\alpha_v\beta_3$) and 38C2 (anti-JW) mAbs were also tested. Sera were tested at days 0, 22, and 50 postimmunization.

effector functions. Preimmune serum did not react with SCS-873 and did not bind to integrin following addition of SCS-873.

Programmed Polyclonal Immune Responses Have Therapeutic Effects in Cancer Models. We evaluated the therapeutic potential of this approach in 2 syngeneic cancer models, the murine CT26 colon tumor model (24) and the B16 syngeneic melanoma model (25). In the first model, tumor induction was performed by s.c. injection of CT26 cells into the right flank of JW-KLH-immunized BALB/C mice. Three different groups of 6 mice with similar anti-JW titers were treated between days 2 and 17 after tumor induction in 2 independent experiments. In the first experiment (Fig. 5A), mice were given 200- μ L i.p. (i.p.) injections of PBS alone, 60 μ g/ml SCS-873 in PBS, or 27.5 μ g/ml SCS-397 (equimolar to the SCS-873 dosage) in PBS according to the schedule described in *Materials and Methods*. In the second experiment (Fig. 5B), mice were given 200 μ L i.p. injections of PBS alone, 77 μ g/ml cRGD-dk in PBS, or 42.5 μ g/ml cRGD in PBS. Tumor volumes were measured at 3-day intervals from 12 to 30 days postgraftment and removed and weighed at the end of the experiment. Profound and statistically significant reduction in tumor growth was observed in animals treated with targeting agents designed to covalently program the immune response: Treatment with SCS-873 resulted in approximately 75% reduction ($P < 0.003$) and treatment with the cRGD-dk resulted in approximately 90% reduction ($P < 0.0002$) in tumor weight relative to treatment with PBS at 30 days. Treatment of mice with ligands that lack the diketone tags necessary for binding to anti-JW immunoglobulins (SCS-397 and cRGD) was no more effective than treatment with PBS.

We next evaluated our approach in the B16 syngeneic melanoma model in C57BL6 mice. Previous studies have demonstrated the efficacy of treating xenografted human melanoma in immunocompromised mice using SCS-873 and mAb38C2. Since flow cytometry studies indicated strong binding of SCS-873 to B16 cells and insignificant binding of cRGD-dk to these cells (Fig. 2), only SCS-873 therapy was studied in this model. Tumor induction was performed by s.c. (s.c.) injection of 2×10^5 B16 cells into the right flank of C57BL6 mice previously immunized with JW-KLH. Three groups of 6 mice were treated between days 2 and 17 after tumor induction. Mice were treated with 200- μ L i.p. injections of PBS alone, 60 μ g/ml SCS-873 in PBS, or 27.5 μ g/ml SCS-397 in PBS according to the schedule described in *Materials and Methods*. As shown in Fig. 5C, growth

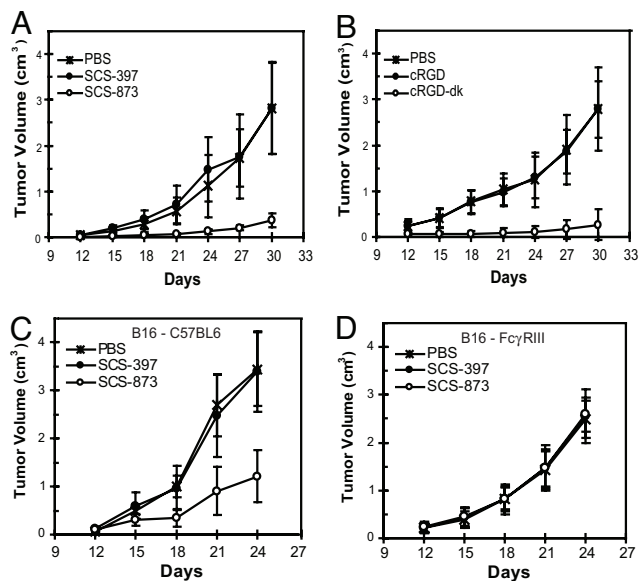


Fig. 5. Inhibition of tumor growth in syngenic mice mediated by adaptor-targeted antibodies. (A) Treatment with SCS effectively inhibited growth of CT26 tumors in JW-KLH-immunized BALB/C mice. Mice (6 per group) were treated between days 2 and 17 with 200 μ l i.p. injections of PBS alone, 60 μ g/ml SCS-873 in PBS, or 27.5 μ g/ml SCS-397 in PBS. Mean tumor volumes \pm SD were determined at 3-day intervals from 12 to 30 days postgrafting. (B) Treatment with cRGD-dk effectively inhibited growth of CT26 tumors in JW-KLH-immunized BALB/C mice. Mice (6 per group) were treated between days 2 and 17 with 200 μ l i.p. injections of PBS alone, 77 μ g/ml cRGD-dk in PBS, or 42.5 μ g/ml cRGD in PBS. Mean tumor volumes \pm SD were determined at 3-day intervals from 12 to 30 days postgrafting. (C) Treatment with SCS-873 effectively inhibited growth of B16 tumors in JW-BSA-immunized C57BL6 mice. Mice (6 per group) were treated between days 2 and 17 after tumor induction with 200 μ l i.p. injections of PBS alone, 60 μ g/ml SCS-873 in PBS, or 27.5 μ g/ml SCS-397 in PBS. Mean tumor volumes \pm SD were determined at 3-day intervals from 12 to 24 days postgrafting. (D) Treatment with SCS-873 effectively inhibited growth of B16 tumors in JW-BSA-immunized Fc γ RIII knockout mice. Mice (6 per group) were treated between days 2 and 17 after tumor induction with 200 μ l i.p. injections of PBS alone, 60 μ g/ml SCS-873 in PBS, or 27.5 μ g/ml SCS-397 in PBS. Mean tumor volumes \pm SD were determined at 3-day intervals from 12 to 24 days postgrafting.

of this very aggressive tumor was significantly inhibited in mice treated with SCS-873 (78% growth inhibition, $P < 0.004$, relative to treatment with PBS); tumor volumes were similar in mice treated with SCS-373 and PBS buffer.

Polyclonal Antibody Effector Functions Can Be Chemically Programmed. The antibody effector functions, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), are believed to be key mechanisms underlying the tumor-growth inhibiting activities of therapeutic antibodies (25). ADCC is mediated by the activation Fc γ receptor, Fc γ RIII, and modulated by its inhibitory counterpart, Fc γ RIIB (25). Natural killer cells, which express Fc γ RIII but not Fc γ RIIB, are a principal cell type involved in ADCC. We hypothesized that a significant component of the therapeutic effect that we observe using the programmable immunization strategy results from antibody-mediated cellular cytotoxicity. We, therefore, evaluated the growth of B16 tumors in C57BL6 mice lacking Fc γ RIII (strain B6.129P2-Fcgr3^{tm1Sjv}/J from Jackson Laboratory). In these animals, the Fcgr3^{tm1Sjv} targeted mutation eliminates the ligand-binding α chain of Fc γ RIII and the mice lack NK cell-mediated antibody-dependent cytotoxicity. Fc γ RIII knockout mice produced similar levels of covalent diketone binding antibody induced by immunization (Fig. 3). In

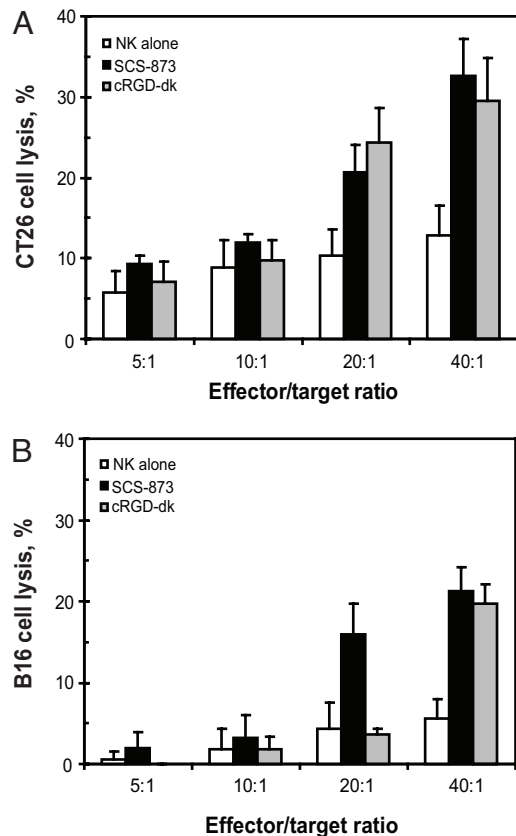


Fig. 6. NK cell-mediated ADCC activity of chemically programmed antibodies. (A) Radiolabeled CT26 tumor cells were mixed with SCS-873- or cRGD-dk-programmed BALB/C mouse sera, and lysis was measured in the presence of BALB/C spleen-isolated NK cells as effectors. (B) Radiolabeled B16 tumor cells were mixed with SCS-873- or cRGD-dk-programmed C57BL6 mouse sera, and lysis was measured in the presence of C57BL6 spleen-isolated NK cells as effectors. The values shown are means of triplicate samples (\pm SD).

Fc γ RIII knockout mice, however, treatment with SCS-873 did not inhibit tumor growth (Fig. 5D), clearly indicating that ADCC is a major mechanism of therapeutic action in this model.

To further confirm our hypothesis that ADCC mediates the activity of our programmed antibodies, we isolated NK cells from the spleens of C57BL6 and BALB/C mice and assessed their ADCC capacity in vitro using B16 melanoma and CT26 colon cancer lines as targets. Sera derived from immunized animals and programmed with SCS-873 and cRGD-dk clearly potentiated NK cell killing of CT26 and B16 cells (Fig. 6). These results were similar to those reported earlier using mAb 38C2 and the human melanoma line M21. To examine the potential of the polyclonal response to direct complement-directed cytotoxicity, we studied the lysis of radiolabeled CT26 and MS1 cells in the presence of SCS-873 programmed polyclonal sera and rabbit complement using a standard [⁵¹Cr]-release assay (14). This experiment demonstrated significant CDC-based killing of CT26 cells in the presence of SCS-873-treated immune sera supporting the potential of this immunization strategy and chemical programming to direct CDC (see SI).

Conclusions

The development of new and more effective vaccine strategies is critical for public health. Despite decades of effort, no effective vaccines are available for diseases such as HIV-1 and malaria. We believe that chemistry-based vaccine approaches have been underexplored and may provide opportunities to make inroads

into intractable areas of vaccinology. In contrast to biology-based vaccine approaches, which aim to educate the immune system to create immunoglobulins of a defined specificity, the chemistry-based vaccine approach described here provides the immune system with defined specificities by coupling the biology-based induction of programmable immunoglobulins with ligand design and covalent self-assembly. The earliest related chemistry-based vaccine strategies were aimed at redirecting common natural antibody specificities such as anti-dinitrophenyl and anti- α -galactosyl antibodies to targets by decorating them with highly immunogenic antigens like dinitrobenzene and galactosyl- α (1–3)galactose (26–29). Such natural antibody specificities are typically of low affinity and to the best of our knowledge no such study has reported efficacy in a disease model. More recently, fluorescein-hapten-based immunizations were proposed as an alternative to the ineffective low affinity natural antibody approach; in this strategy, induced high-affinity anti-fluorescein immunoglobulins are programmed with fluorescein conjugates (30, 31). This strategy was effective in animal models of cancer but only when combined with cytokine or radiation adjuvant therapy and may suggest therapeutic limitations inherent with noncovalent approaches.

The efficacy of our chemically programmed, covalent monoclonal antibody approach has been proven in multiple animal models of disease and chemically programmed antibodies are now being evaluated in multiple clinical studies. Here, we demonstrate the efficacy of this approach as a covalent vaccine strategy. We showed that high-titer covalent antibody responses were induced in 3 mouse strains and that the resulting polyclonal antibody responses could be reprogrammed to target the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ with therapeutic effect. Unlike earlier noncovalent approaches based on natural antibodies or anti-fluorescein responses, no adjuvant therapy was required. The integrins we have targeted are of significant interest because the malignant progression of melanoma, glioma, ovarian, cervical, and breast cancer have all been strongly correlated with the level of expression of the integrin $\alpha_v\beta_3$ and in some cases with $\alpha_v\beta_5$ (16–23). Additionally, these integrins are expressed on the surface of angiogenic endothelial cells and are thus targets of antiangiogenic therapy (32, 33). The studies presented here further validate the potential of targeting these receptors in melanoma and colon cancer therapy.

While we focused our efforts toward programming an induced covalent antibody response against self receptors, we believe that this approach can be broadly applied to a wide variety of diseases (Fig. 7). Development of effective vaccines using this approach is limited only by our ability to develop high affinity neutralizing small molecule or peptide ligands. We do not believe this to be a severe limitation given that many such ligands are now available and molecular diversity approaches based on phage display and synthetic strategies can be used. The development of several ligands directed against different epitopes on a virus like HIV-1, for example, might produce programmed immunity that reduces the potential for viral escape or broadens prophylactic efficacy. A universal covalent vaccine approach might have other advantages. It should be possible to develop orally available programming agents to direct an immune response. These compounds could be stockpiled and then administered en masse in response to a biological threat or pandemic. This approach should have considerable economic advantages as compared with classic monoclonal antibody therapy. Because this approach induces a wide variety of antibody isotypes, the full range of effector functions and valencies available to the immune system would be tapped, and the potency of the vaccine should be greater than that achieved with monoclonal therapy. Recent studies have highlighted the long-lived nature of the circulating B memory cell in response to influenza (34). Such a long-lived covalent vaccine response might circumvent the age-related

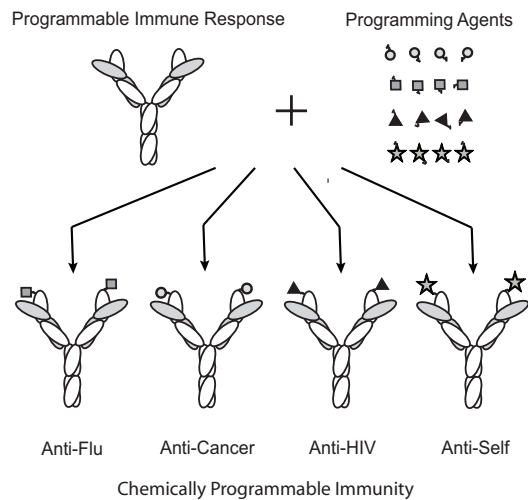


Fig. 7. The broad potential of the chemically programmable covalent vaccine strategy. With the development of a wide variety of targeting molecules (different geometric shapes as shown), chemically programmed vaccines can be created to address a number of diseases and biological threats.

decline in humoral immunity if given early in life (2, 3). Furthermore, if widely adopted, universal programmable covalent polyclonal antibodies would be readily available for passive transfer to nonimmunized individuals, who could be provided with “instant immunity” following administration of a designed ligand. Finally, programmable covalent human polyclonal antibodies could be produced in transgenic organisms for passive transfer and programming (35). We believe that these potential advantages support the further development of chemistry-based approaches to vaccinology.

Materials and Methods

Antibodies, Reagents, Targeting Agents. mAb 38C2 was prepared as described and is commercially available from Sigma-Aldrich. Antibodies mAb LM609, mAb P1F6, and purified integrin proteins were obtained from Chemicon. FITC-conjugated donkey antimouse IgG polyclonal antibodies and HRP-conjugated goat antimouse IgG polyclonal antibodies were from Jackson ImmunoResearch Laboratories. JW-KLH and JW-BSA were prepared as described (4). Targeting agents SCS-873, SCS-397, and cRGD-dk were prepared in accord with published methodologies (10, 13, 36). cRGD peptide [cyclo(Arg-Gly-Asp-D-Phe-Lys)] was obtained from Peptides International, Inc.

Cell Lines, Cells, and Animals. Mouse colon carcinoma cell line CT26 (syngeneic with BALB/C mice) were purchased from American Type Culture Collection (ATCC) and were maintained in DMEM supplemented with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1 mM sodium pyruvate, 10% FCS, and antibiotics. B16F10 mouse melanoma cell line (syngeneic with C57BL6) was purchased from ATCC and maintained in RPMI medium 1640 containing 10% FCS and antibiotics. Female (5–6 weeks of age) BALB/C, C57BL6, and Fcgr3^{tm15jv/j} knockout mice in the C57BL6 background, strain name B6.129P2-Fcgr3^{tm15jv/j}, were obtained from Jackson Labs. NK cells were isolated from spleen of BALB/C and C57BL6 mice using the MACS system according to the manufacturer’s recommendations (Miltenyi Biotech). Non-NK cells (i.e., B cells, T cells, dendritic cells, macrophages, granulocytes, and erythroid cells) were depleted with a mixture of biotin-conjugated antibodies against CD19, CD4 (L3T4), CD8a (Ly-2), CD5 (Ly-1), Ly-6G (Gr-1), Ter-119, and antibiotin MicroBeads. Purity of NK fractions was >95% as determined by FACS analysis.

Reactive Immunization and ELISA Titering. Mice were immunized with JW-KLH according to the published method with antigen boosting on days 15 and 43 (4). Individual JW-antiserum from JW-KLH-immunized mice was collected on days 22, 50, and 85 and used for *in vitro* assays. For ELISA, Costar 96-well ELISA plates (Corning) were coated with 100 ng of JW-BSA in 25 μ l PBS and incubated overnight at 4 $^{\circ}$ C. After blocking with 150 μ l of TBS/3% BSA for 2 h at 37 $^{\circ}$ C, 50 μ l of different dilutions (from 1:500 to 1:64000) of pooled (5 mice of each strain) sera was added into each well and the plates were incubated for

2 h at 37 °C. Washing and detection were performed essentially as described (14) using HRP-conjugated goat antimouse IgG antibody (diluted 1:3000 in TBS/1% BSA). In some experiments, additional incubation with 50 μ l of 0.05M citric acid, pH 2.5 (acid wash) for 15 min at RT was performed after the initial washing step. The quantitation of anti-JW IgG1, IgG2a, IgG2b, IgG3, IgG4, and IgM antibodies by ELISA was performed using biotin-conjugated goat-antimouse Ig-specific antibodies and Streptavidin-conjugated HRP (Caltag).

Chemical programming, evaluation of binding to integrins in ELISA and on cells, complement-dependent cytotoxicity, and antibody-dependent cellular cytotoxicity assays were performed as previously described (14).

Syngeneic Colon Cancer Model. On day 65, JW-KLH-immunized BALB/C mice were sorted (6 groups with 6 animals each) to form matched anti-JW titer groups and were inoculated s.c. into right flanks with 0.1 ml (2×10^5 cells/mouse) of CT26 cell suspension in PBS (day 0 for tumor model). Animals were further injected i.p. with identical amounts of the targeting compound in 200- μ L of PBS on days 2, 5, 8, 11, 14, and 17. Tumor volumes of

treated animals were measured over the skin in 2 dimensions using a slide caliper every third day starting on day 12, and the tumor volume was calculated according to the following formula, $1/2(\text{width})^2 \times \text{length}$. Toxicity was monitored by determining the body weight of mice once a week. On day 30, all mice were euthanized and the tumors dissected and weighed. Results are reported as means \pm SD for each group. Differences were considered statistically significant at $P < 0.05$ using unpaired two-tailed Student's *t* test. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute before the experiments were started.

Syngeneic Melanoma Model. B16 melanoma tumor model using C57BL6 and Fc γ RIII knockout mice immunized with JW-KLH was performed as described above, except all mice were euthanized on day 24.

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