Dendritic Cell-Specific Antigen Delivery by Coronavirus Vaccine Vectors Induces Long-Lasting Protective Antiviral and Antitumor Immunity

Luisa Cervantes-Barragan,a,b Roland Züst,a Reinhard Maier,a Sophie Sierro,c Jozef Janda,c Frederic Levy,c Daniel Speiser,c Pedro Romero,a Pierre-Simon Rohrlich,d,e,f Burkhard Ludewig,a,g and Volker Thiel,h,i

Institute of Immunobiology, Kantonal Hospital St. Gallen, St. Gallen, Switzerland; Unidad de Investigación Médica en Inmunología, Hospital de Especialidades, Centro Médico Nacional Siglo XXI, IMSS, México City, México; Ludwig Institute for Cancer Research, Lausanne, Switzerland; INSERM U645-IFR133, Besançon, France; University of Besançon, Besançon, France; CHU Besançon, Service de Pédiatrie, Besançon, France; and VetSuisse Faculty, University of Zurich, Zurich, Switzerland

V.T. and B.L. share senior authorship.

ABSTRACT Efficient vaccination against infectious agents and tumors depends on specific antigen targeting to dendritic cells (DCs). We report here that biosafe coronavirus-based vaccine vectors facilitate delivery of multiple antigens and immunostimulatory cytokines to professional antigen-presenting cells in vitro and in vivo. Vaccine vectors based on heavily attenuated murine coronavirus genomes were generated to express epitopes from the lymphocytic choriomeningitis virus glycoprotein, or human Melan-A, in combination with the immunostimulatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). These vectors selectively targeted DCs in vitro and in vivo resulting in vector-mediated antigen expression and efficient maturation of DCs. Single application of only low vector doses elicited strong and long-lasting cytotoxic T-cell responses, providing protective antiviral and antitumor immunity. Furthermore, human DCs transduced with Melan-A-recombinant human coronavirus 229E efficiently activated tumor-specific CD8+ T cells. Taken together, this novel vaccine platform is well suited to deliver antigens and immunostimulatory cytokines to DCs and to initiate and maintain protective immunity.

IMPORTANCE Efficient vaccination against infectious agents and tumors depends on specific antigen targeting to dendritic cells (DCs). We report here that biosafe coronavirus-based vaccine vectors facilitate delivery of multiple antigens and immunostimulatory cytokines to professional antigen-presenting cells in vitro and in vivo. Vaccine vectors based on heavily attenuated murine coronavirus genomes were generated to express epitopes from the lymphocytic choriomeningitis virus glycoprotein, or human Melan-A, in combination with the immunostimulatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). These vectors selectively targeted DCs in vitro and in vivo resulting in vector-mediated antigen expression and efficient maturation of DCs. Single application of only low vector doses elicited strong and long-lasting cytotoxic T-cell responses, providing protective antiviral and antitumor immunity. Furthermore, human DCs transduced with Melan-A-recombinant human coronavirus 229E efficiently activated tumor-specific CD8+ T cells. Taken together, this novel vaccine platform is well suited to deliver antigens and immunostimulatory cytokines to DCs and to initiate and maintain protective immunity.

Vaccination against viral infections has saved millions of lives by protecting many individuals from diseases such as measles, rubella, mumps, and polio. However, there is a growing need not only to develop improved vaccines against such acute infections but also to generate therapeutic vaccines which can stimulate specific immune responses to persistent viruses such as the human immunodeficiency virus or the human hepatitis C virus (1, 2). Likewise, novel approaches for vaccination against tumors which counteract the immunosuppression associated with cancer are needed (3). There is compelling evidence that CD8+ cytotoxic T cells are crucial players in the protective immune response against viral infections and tumors (4). Novel vaccine approaches should thus be rigorously evaluated for their ability to maximally expand antigen-specific CD8+ T cells, to induce their optimal differentiation into effecter CD8+ T cells, and to elicit long-lasting protective memory (4). A major bottleneck in the development of new and effective vaccines is the delivery of antigens to dendritic cells (DCs) (5, 6), which sample the antigen, transport immunogenic components to secondary lymphoid organs, and initiate and maintain T and B cell responses. The excellent capacity of DCs to prime protective antiviral T cell responses has been shown in vivo (7–9). Likewise, several preclinical studies demonstrated that efficient antitumor immunity can be induced using adoptive transfer of DCs (10–12). Although individualized adoptive transfer of antigen-loaded DCs is feasible and, to a certain extent, efficient in clinical applications to humans (13), off-the-shelf vaccines that permit targeted delivery of antigens to DCs in vivo have become a major focus in vaccine development. Consequently, the description of cell surface molecules that, for example, exhibit a rather specific expression by DCs has fueled the development of antibody-based targeting
strategies (14–16). These protein-based vaccines generate CD4+ T cell and B cell responses against a range of different antigens. However, antigen coupling to antibodies is a major limitation for the induction of CD8+ T cell responses which are strictly dependent on cross-presentation (5, 17). In contrast, viral vectors encoding immunogenic antigens can deliver their genetic cargo directly into DCs, thus generating antigenic peptides in infected cells and allowing for efficient loading of major histocompatibility complex (MHC) class I molecules. Among the currently most exploited virus systems that facilitate antigen delivery to DCs are adenoviral (18, 19), lentiviral (20), arborviral (21), and alphaviral (22, 23) systems. However, major impediments of these vectors are frequent off-target transduction, resulting in antigen presentation by parenchymal cells outside secondary lymphoid organs, and limited cloning capacity for the insertion of multiple or large antigens. For example, the strong tropism of adenoviral vectors for hepatocytes, with >95% of the genetic material being deposited in the liver, leads to generation of functionally impaired CD8+ T cells (24, 25). Major efforts are thus required to engineer adenoviral vectors with improved specificity for the relevant antigen-presenting cells (12, 19). Likewise, lentiviral vectors preferentially infect cells other than DCs, and redirection of their target cell tropism is warranted (26). An additional potential impedi-
Viral challenge. Moreover, single application of these novel viral vectors generated prophylactic and therapeutic immunity against metastatic melanoma. Induction of cytotoxic T lymphocytes (CTL) directed against the human Melan-A (Mel-A) antigen in HLA-A2-transgenic mice and efficient human-DC-mediated stimulation of Mel-A-specific CTL through Mel-A-recombinant human coronavirus 229E (HCoV-229E) indicates that coronavirus-mediated gene transfer to DCs represents a versatile approach for murine and human immunization against both viral infection and cancer.

RESULTS

Design and propagation of biosafe coronavirus-based vaccine vectors. To assess immunogenicity and to ensure maximal safety of coronavirus-based multigene vaccine vectors, we have rationally designed a series of prototype vectors based on the mouse hepatitis virus strain A59 (MHV-A59) (Fig. 1A). With regard to safety, we introduced three basic modifications to obtain replication-competent, but attenuated and propagation-deficient, MHV-based vectors. First, we deleted all MHV accessory genes (NS2, HE, gene4, gene5a), a strategy that attenuates MHV in the natural host (29) and provides space for the introduction of heterologous genes. Second, we removed 99 nucleotides within the replicase gene-encoded sequence of nonstructural protein 1 (nspl), because this deletion has been shown to greatly reduce MHV pathogenicity while retaining immunogenicity (28). Finally, we deleted structural gene E to restrict proper particle formation (33). Indeed, although replication of wild-type MHV to maximal titers occurred around day 5 postinfection, in vivo replication of MHV vectors could not be detected (not shown). Consequently, there was also no MHV vector replication detectable in livers or brains (not shown). The high safety profile of MHV-based vectors is further illustrated by the fact that the health status of C57BL/6 (B6) or different immunodeficient mice was not affected, even if the vectors were applied at high doses (see Fig. S1 in the supplemental material).

In order to test the coronavirus-based vaccine concept and to develop a vaccine that provides strong CTL responses, in terms of both magnitude and longevity, we used the CTL epitope gp33-41 (the region from residues 33 to 41), derived from the lymphocytic choriomeningitis virus (LCMV) glycoprotein, and the Mel-A26-35 analog peptide, derived from the human Melan-A/melanoma antigen recognized by T cells 1 (MART1) protein (Fig. 1A). The CTL epitopes were cloned as fusion proteins with the enhanced green fluorescent protein (EGFP) (34, 35), and the corresponding genes, designated GP-EGFP and EGFP-Mel-A, were cloned between the MHV vector-borne spike and membrane genes. Since appropriate stimulation of DCs is critical for the generation of efficient T cell response for the use of DNA-based viral vectors in clinics is their potential to integrate genomic material into the host genome (27). Coronavirus vectors display a number of features that clearly overcome these limitations. First, replication of these positive-stranded RNA viruses is restricted to the cytoplasm, without a DNA intermediate, making insertion of viral sequences into the host cell genome unlikely. Second, there is accumulating knowledge on how to attenuate coronaviruses in order to provide biosafe vectors (28, 29). Third, coronavirus genomes with sizes of 27 to 31 kb represent the largest autonomously replicating RNAs known to date and thus offer a cloning capacity of more than 6 kb. Fourth, the unique transcription process generates 6 to 8 subgenomic mRNAs encoding the four canonical structural proteins and various numbers of accessory proteins, which can be replaced to encode multiple heterologous proteins (30). Finally, and certainly most intriguing, cell surface receptors of human and murine coronaviruses are expressed on human and murine DCs, respectively (31, 32).

The present study describes the generation and evaluation of rationally designed coronavirus-based vectors that efficiently target antigens and immunostimulatory molecules to DCs. We show that murine-coronavirus-based vectors can deliver multiple antigens and immunostimulatory cytokines almost exclusively to CD11c+ DCs within secondary lymphoid organs. Immunization with only low numbers of particles elicited potent CD8+ T cell responses that provided long-lasting protection against
responses (36) and hence an indispensable component of rationally designed vaccines, we inserted the gene encoding the murine granulocyte-macrophage colony-stimulating factor (GM-CSF) between the MHV vector-borne replicase and spike genes (Fig. 1A). To propagate the MHV-based viral vectors to high titers, we produced a packaging cell line based on murine 17clone1 (Fig. 1A). To propagate the MHV-based viral vectors to high titer was encoded by the same vector that expressed the GP-EGFP protein.

**DC-specific delivery of coronavirus vector-encoded antigens and cytokines.** To assess MHV vector growth kinetics on cells that lack the E protein prevents MHV propagation in primary cells (Fig. 1D). Most importantly, however, MHV vector transduction did not result in particle release in peritoneal macrophages and DCs, demonstrating that the bone marrow-derived CD11c+ DCs, or peritoneal macrophages with the MHV vectors and compared growth kinetics to that of wild-type MHV-A59. In accordance with reports on E gene-deficient MHV-A59 (37), the growth of the MHV-based vector was greatly impaired in L929 cells (Fig. 1D). In disseminated tumors, we used to simultaneously express antigens and immunostimulatory cytokines in target cells.

**FIG 3** Evaluation of antiviral CD8+ T cell responses. (A) B6 mice were immunized i.v. with either 200 PFU LCMV, 106 PFU MHV-GP, or 106 PFU MHV-GM/GP. Splenocytes were analyzed on day 7 postinfection (p.i.) for expression of CD8 and reactivity with H2-D\(^{b}\)/gp33 or H2-K\(^{b}\)/gp34 tetramers, and CD8+ splenocytes were analyzed for gp33- and gp34-specific IFN-\(\gamma\) production. Values in the upper right quadrants represent percentages of Tet+ cells (lower row) or percentages of IFN-\(\gamma\) cells (upper row) in the CD8 T-cell compartment (mean \(\pm\) SEM; 3 mice per group). (B, C) Efficacy of MHV-based vectors in inducing antiviral CD8+ T cell responses. B6 mice were immunized i.v. with the indicated doses of MHV-GP or MHV-GM/GP. Tetramer analysis and IFN-\(\gamma\)-ICS was performed on day 7 postimmunization (mean \(\pm\) SEM; 3 mice per group). (C) Importance of the route of immunization. B6 mice were immunized with 106 PFU MHV-GM/GP. Tetramer analysis and IFN-\(\gamma\)-ICS were performed on day 7 postimmunization (mean \(\pm\) SEM; 2 to 6 mice per group, pooled from 2 different experiments). (D) Duration of vector-induced CD8+ T cell response. B6 mice were immunized with 106 PFU MHV-GM/GP or MHV-GP. Tetramer analysis and IFN-\(\gamma\)-ICS were performed at the indicated time points (mean \(\pm\) SEM; 3 mice per group). (E) In vivo restimulation of MHV vector-induced CD8+ memory T cells. B6 mice were immunized with 106 PFU MHV-GM/GP or injected with PBS. Mice were boosted on day 65 i.v. with 106 PFU MHV-GM/GP or injected with PBS. Tetramer analysis and IFN-\(\gamma\)-ICS were performed on day 4 after the booster immunization (mean \(\pm\) SEM; 4 mice per group). i.n., intranasal.
tein (MHV-GM/GP), was the maximal immune response induced (Fig. 54B). Furthermore, MHV-GM/GP-induced GM-CSF expression did not alter the cellular composition in spleens (see Fig. S5 in the supplemental material). These results support the notion that vector-encoded GM-CSF acts locally in the microenvironment of transduced DCs. However, in vivo transduction with both MHV-GP and MHV-GM/GP mediated DC maturation, with upregulation of CD86 and CD40 (Fig. 2C). Notably, application of MHV-GP vectors led to a less efficient transduction of DCs in vivo, which lasted only for roughly 18 h (Fig. 2A), suggesting that GM-CSF supplied by MHV vectors fosters DC survival and, to a lesser extent, DC maturation and thereby potentiates their antigen presentation function.

Antiviral CTL responses following coronavirus vector immunization. Infection with LCMV is characterized by a vigorous expansion of antiviral CTL and persistence of protective memory CTL (38), which are directed against several epitopes. Two different epitopes are present in the gp33-41 region of the LCMV GP that are presented by H2-Db (39) and the H2-Kb-binding gp34-41 (40). On day 7 after LCMV infection, significant numbers of CD8⁺ T cells could be detected by MHC tetramer analysis and intracellular cytokine secretion (ICS) assay (Fig. 3A). It is important to note that the gp33 ICS records both gp33- and gp34-specific CD8⁺ T cells. It appears that the processing of the GP-EGFP transgene in the MHV-GP and MHV-GM/GP vectors permitted the preferential generation of gp34-specific CD8⁺ T cells to a magnitude comparable to that seen during acute LCMV infection (Fig. 3A). The GM-CSF-encoding MHV vector proved to be highly efficient in the induction of antiviral CTL, even at rather low doses of 10³ and 10⁴ PFU (Fig. 3B). Since the intermediate dose of 10⁵ PFU of MHV-GM/GP led to optimal induction of gp34-specific CTL, we used this dose to assess whether application via different routes would influence the induction of transgene-specific CTL. As shown in Fig. 3C, all routes of immunization elicited robust CD8⁺ T cell responses, with intraperitoneal (i.p.), subcutaneous (s.c.), intramuscular (i.m.), and i.v. injection being the most efficient means of application. Both MHV-GP- and MHV-GM/GP-induced CD8⁺ T cells displayed full effector function because gp34 peptide-loaded target cells were rapidly eliminated in immunized hosts (see Fig. S6A in the supplemental material). MHV vector-induced CD8⁺ T cell responses were detectable for more than 65 days after immunization (Fig. 3D) and, most importantly, could be restimulated in vivo by immunization with the same vector (Fig. 3E).

Protection against LCMV challenge requires high levels of appropriately activated CD8⁺ T cells (38). In order to assess the efficacy of MHV vector-based immunization for protection against viral challenge, B6 mice were immunized with graded doses of the MHV-GP or MHV-GM/GP vector and challenged 7 days later with LCMV. Comparable to the high level of CD8⁺ T cell induction (Fig. 3), complete protection was observed in mice after immunization with a dose of 10⁵ PFU (Fig. 4A). Remarkably, as few as 10⁴ PFU of MHV-GM/GP still led to a >3-log reduction of LCMV titers in spleens on day 4 postchallenge (Fig. 4A). Protection against LCMV was long-lasting because mice were still protected after more than 2 months following immunization with 10⁵ PFU MHV-GM/GP via the i.v. route (Fig. 4B), s.c., or i.m. route (see Fig. S6B in the supplemental material). Moreover, the MHV-GM/GP vaccine elicited complete protection against i.p. challenge with LCMV GP recombinant vaccinia virus (VV) (Fig. 4C). Likewise, immunization with MHV-GP provided substantial protection against this heterologous viral infection (Fig. 4C). It is important to indicate that the MHV-based vaccine, even when GM-CSF was encoded by the vector, provided specific protection because replication of the unrelated recombinant vaccinia virus expressing vesicular stomatitis virus (VSV) Indiana glycoprotein (VV-INVDG) was not affected (Fig. 4C). Taken together, these results revealed that the MHV-based vector system is highly efficient in generating protective antiviral immunity and that the incorporation of GM-CSF into the vaccine significantly augmented its protective capacity.

Prophylactic and therapeutic antitumor immunization. In order to evaluate whether MHV vector-based vaccination elicits prophylactic and therapeutic tumor immunity, we resorted to a rapidly growing B16 melanoma model which provides compatibility with the LCMV GP system through expression of a gp33 minigene (B16F10-GP cells) (41). I.v. injection of 5 × 10⁵ B16F10-GP or parental B16F10 cells in control B6 mice resulted in metastatic growth of tumor cells in lungs (Fig. 5A). Immunization with either 10⁵ PFU MHV-GP or 10⁶ PFU MHV-GM/GP completely blocked the growth of B16F10-GP tumor cells, whereas metastasis formation of the parental B16F10 cells was not affected (Fig. 5A). Application of graded doses of MHV-GP or MHV-GM/GP revealed the high efficacy of this vaccination approach in the prophylactic setting, i.e., 10⁴ MHV-GP or only 10³ PFU MHV-GM/GP were sufficient to prevent the growth of the melanoma cells (Fig. 5B). A long-lasting memory response that provided significant protection against B16F10-GP challenge had been generated following MHV-GM/GP immunization (Fig. 5C). Moreover, the potent CD8⁺ T cell response elicited through MHV-GM/GP immunization mediated therapeutic tumor immunity (Fig. 5D), i.e., the tumor burden in lungs of B6 mice was significantly reduced even when the vaccine was applied after the tumors had started to form metastatic nodules, indicating that CD8⁺ T cell re-
sponses elicited by the novel MHV vectors can exert forceful antitumor activity. To further substantiate the finding that MHV vectors can induce potent and relevant antitumor CD8+ T cell responses, we employed the human HLA-A2-restricted Melan-A/MART1 system, in which immune responses against the Mel-A26-35 analog peptide can be monitored in HLA-A2.1-transgenic (A2DR1) mice (42). A2DR1 mice were immunized i.v. with either 10^5 PFU MHV–Mel-A or 10^5 PFU MHV-GM/Mel-A, and Mel-A-specific CD8+ T cell responses were recorded using tetramer analysis and ICS. As shown in Fig. 6A, both vectors elicited substantial CD8+ T cell responses. Time course experiments following i.v. application of MHV–GM/Mel-A revealed a strong global expansion of CD8+ T cells (nearly 10-fold) and a massive expansion of Mel-A-specific CD8+ T cells, with 2 × 10^5 cells per spleen being tetramer positive and 6 × 10^5 cells per spleen secreting gamma interferon (IFN-γ) (Fig. 6B). The Mel-A-specific CD8+ T cell population showed a typical contraction after day 7, and a stable mature DCs infected with HCoV–Mel-A or HCoV–GP–EGFP revealed that high levels of EGFP expression can be reached as early as 9 h postinfection (see Fig. 5 in the supplemental material). Finally, mature DCs infected with HCoV–Mel-A or HCoV–GP–EGFP were employed to stimulate Mel-A26-35-specific human T cells. As shown in Fig. 7C, we observed efficient stimulation of the tumor-specific T cells by HCoV–Mel-A, indicating that HCoV-229E, with its pronounced tropism for human CD13 (43), is expressed on human DCs (31). Furthermore, we have demonstrated previously that HCoV-229E-based vectors can transduce immature and mature human DCs with reporter genes such as the firefly luciferase gene (30). Here we assessed whether HCoV-229E-mediated delivery of the Mel-A26-35 analog peptide to HLA-A2+ human DCs would specifically stimulate human Mel-A-specific CD8+ T cells. To this end, we generated two recombinant human coronaviruses, designated HCoV–Mel-A and HCoV–GP–EGFP, through replacement of HCoV-229E accessory gene 4 with the gene for the EGFP–Mel-A26-35 or gp33–EGFP fusion protein, respectively (Fig. 7A). As shown in Fig. 7B, EGFP expression was readily detectable in both human immature and mature DCs after HCoV–Mel-A infection. Time course analysis of human memory population had been established by day 28 postimmunization (Fig. 6B). During the acute phase following MHV-GM/GP immunization, >90% of the Mel-A-specific CD8+ T cells had downregulated CD62L (Fig. 6C). As expected, memory CD8+ T cells reacquired CD62L expression, indicating establishment of a central memory CD8+ T cell population. Overall, these results underline that the coronavirus-based vaccination approach, particularly in combination with the immunostimulatory cytokine GM-CSF, provides efficient means for the induction of CD8+ T cell responses against a human tumor antigen.

Delivery of tumor antigen to human DCs. Human coronavirus-based vectors permit specific transfer of multiple genes to DCs because the receptor of HCoV-229E, human aminopeptidase N (hAPN or CD13) (43), is expressed on human DCs (31). Furthermore, we have demonstrated previously that HCoV-229E-based vectors can transduce immature and mature human DCs with reporter genes such as the firefly luciferase gene (30). Here we assessed whether HCoV-229E-mediated delivery of the Mel-A26-35 analog peptide to HLA-A2+ human DCs would specifically stimulate human Mel-A-specific CD8+ T cells. To this end, we generated two recombinant human coronaviruses, designated HCoV–Mel-A and HCoV–GP–EGFP, through replacement of HCoV-229E accessory gene 4 with the gene for the EGFP–Mel-A26-35 or gp33–EGFP fusion protein, respectively (Fig. 7A). As shown in Fig. 7B, EGFP expression was readily detectable in both human immature and mature DCs after HCoV–Mel-A infection. Time course analysis of human memory population had been established by day 28 postimmunization (Fig. 6B). During the acute phase following MHV-GM/GP immunization, >90% of the Mel-A-specific CD8+ T cells had downregulated CD62L (Fig. 6C). As expected, memory CD8+ T cells reacquired CD62L expression, indicating establishment of a central memory CD8+ T cell population. Overall, these results underline that the coronavirus-based vaccination approach, particularly in combination with the immunostimulatory cytokine GM-CSF, provides efficient means for the induction of CD8+ T cell responses against a human tumor antigen.

**DISCUSSION**

This study describes a novel vaccine approach that facilitates delivery of viral or tumor antigens to DCs in vivo. Concomitant immunostimulation—here via the cytokine GM-CSF—was achieved through targeted transduction by the same viral vector. Single immunization with only 10^4 to 10^5 MHV-based particles...
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FIG 6  Assessment of anti-Melan-A/MART1 CD8+ T cells in A2DR1 mice. (A) Transgenic mice expressing the human HLA-A2.1 molecule were immunized i.v. with 10^9 PFU MHV–Mel-A or MHV-GM/Mel-A. At day 7 postinfection, splenocytes and mononuclear blood cells were analyzed for expression of CD8 and reactivity with HLA-A2/Mel-A26–35 tetramers and for Mel-A26–35-specific IFN-γ and TNF-α production. Values in the upper right quadrants represent mean percentages of Tet+ cells ± SEM in blood and spleen, percentages of IFN-γ+ cells ± SEM, or percentages of TNF-α+ cells ± SEM in the CD8+ T-cell compartment (3 mice per group). (B) Time course of Mel-A26–35-specific CD8+ T-cell responses in A2DR1 mice following i.v. immunization with 10^9 PFU MHV–GM/Mel-A. Total numbers of CD8+ T cells, tetramer-binding Mel-A26–35-specific CD8+ T cells, and Mel-A26–35-specific IFN-γ+ CD8+ T cells were determined at the indicated time points postimmunization (means ± SEM; 3 mice per group). (C) Differentiation of tetramer-binding Mel-A26–35-specific CD8+ T-cells as determined by CD62L expression at the indicated time points postimmunization (means ± SEM; 3 mice per group). Data are from one representative experiment out of three. hi, high-level expression.

was sufficient to elicit (i) vigorous expansion and optimal differentiation of CD8+ T cells, (ii) protective and long-lasting antiviral immunity, and (iii) prophylactic and therapeutic tumor immunity. Moreover, highly efficient antigen delivery to human DCs with recombinant HCoV-229E and specific stimulation of human CD8+ T cells revealed that this approach is exceptionally well suited for translation into human vaccine studies.

Targeting of antigen to DCs in vivo can be achieved by several means (5). The use of viral vectors appears to be the superior strategy for eliciting innate activation of the immune system and optimal induction of CD8+ T cells (24). However, many virus vector systems are still limited in their ability to induce broad and long-lasting immune responses. For example, recombinant adenoviruses have been studied intensively as vaccine candidates mainly because they can be produced to high titers. Nevertheless, high doses of recombinant adenovirus vectors have to be applied to induce an immune response, most probably because they target antigens mainly to non-lymphoid organs, such as the liver (25, 44). In contrast to viral vectors based on DNA viruses (45), positive-stranded RNA virus-based vectors that replicate in the cytoplasm are considered safe vectors. The safety is well documented for vectors based on widely used vaccine strains, such as poliovirus (46), or virus-like particles (VLPs) that contain replicon RNAs devoid of structural genes (47). It should be noted that application of the MHV-based vectors described in this study was always well tolerated and did not result in any sign of disease or adverse side effect irrespectively of dosage and route of MHV-based vector application, even in immunodeficient mouse strains. Finally, it is noteworthy that although some RNA virus-based vectors are able to target DCs (21), their cloning capacity is, in sharp contrast to that of coronavirus-based vectors, generally restricted and the expression of multiple antigens and/or immunostimulatory cytokines is limited.

The novel coronavirus-based vaccine platform described here displayed a high immunogenicity. It is very likely that it is the pronounced tropism of the MHV-based vectors for DCs and macrophages within secondary lymphoid organs that guarantees efficient activation of primary CD8+ T cell responses. In vivo imaging studies have shown that CD169+ CD11c+ DCs at the subcapsular sinus of lymph nodes efficiently present viral antigen to naïve T cells (48). Likewise, CD169+ macrophages at the same location are able to collect viral antigen from the lymph and present antigen to follicular B cells (49). Moreover, a recent study from our laboratory has revealed that type I IFN-mediated protection of DCs and macrophages from cytopathic effects of MHV infection is essential to buy time for mounting a protective CD8+ T cell response (50). It remains to be resolved which factors—besides the presence of the MHV receptor on DCs (32)—confers the preferential infection of the relevant antigen-presenting cells within secondary lymphoid organs. For further adaptation of the coronavirus-based vectors to the human system, some of the essential parameters have been clarified; i.e., the receptor of HCoV-229E is expressed mainly on monocytes and DCs within secondary lymphoid organs. For further adaptation of the coronavirus-based vectors to the human system, some of the essential parameters have been clarified; i.e., the receptor of HCoV-229E is expressed mainly on monocytes and DCs within secondary lymphoid organs (31), and recombinant HCoV-229E efficiently targets human DCs irrespective of their maturation status. It is important to add that HCoV-229E is one of at least four human coronaviruses that are transmitted via mucosal surfaces and are associated with mild upper respiratory tract infections (common cold). Furthermore, the low pathogenic potential of...
HCoV-229E is corroborated by the fact that HCoV-229E was passaged in volunteers in the 1960s without any serious side effects. These features, together with the pronounced DC-targeting capabilities demonstrated in this report, should encourage the development of coronavirus-based vectors for human use.

A second major advantage of the coronavirus-based vaccination strategy is the large cloning capacity of the vectors, which offers the possibility of incorporating immunostimulatory cytokines. GM-CSF-encoding MHV vectors led to strong production of this cytokine in both macrophages and DCs in vitro. Interestingly, GM-CSF expression in vivo appeared to be largely restricted to locally transduced cells, as no gross differences in GM-CSF levels in spleen or serum were observed after MHV-GM/GP vector immunization. It is most likely that GM-CSF-induced changes within the microenvironment of transduced macrophages and DCs are decisive for the optimal induction of effector and memory CD8+ T cell responses. Indeed, it is the optimally stimulated expression of costimulatory molecules on antigen-presenting cells, together with sufficient innate immune stimulation, that determines the primary expansion and the maintenance of antiviral CD8+ T cells (51). Accordingly, such non-TCR signals ("signals 2 and 3") are considered key components of rationally designed vaccines (4).

The lack of such optimally composed stimuli in a vector-based vaccine most likely requires very elaborate prime-boost immunization regimes, as was recently shown for a combined adenovirus/MVA vaccination approach (52), and/or substantially increased vector doses to achieve efficacy, as for example in vaccination with the latest versions of DC-adapted lentivirus vectors, where application of 5 × 10^7 to 10 × 10^7 particles was required to achieve significant expansion of ovalbumin-specific CD8+ T cells in mice (53).

In conclusion, we provide here a versatile vaccine platform based on coronaviruses that achieved the efficient generation of protective immunity, shown as long-lasting memory against viral challenge and induction of both prophylactic and therapeutic tumor immunity. The biology of coronaviruses and the rational modification of these viral RNA vectors harbor significant potential for future development.

**MATERIALS AND METHODS**

**Ethics statement.** Experiments were performed in accordance with federal and kantonal guidelines under permission numbers SG07/62, SG07/63, 07/64, and 07/71 following review and approval by the Kantonal Veterinary Office (St. Gallen, Switzerland).

**Mice, cells, and viruses.** C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). A2DR1 mice were kindly supplied by F. A. Lemonnier (Pasteur Institute, Paris, France) (42). All mice were maintained in individually ventilated cages and were used at between 6 and 9 weeks of age. J929 and CV-1 cells were purchased from the European Collection of Cell Cultures. MC57 and BSC40 cells were purchased from R. M. Zinkernagel (University of Zürich, Switzerland). D980R cells were a kind gift from S. G. Sawicki (Medical University of Ohio, Toledo, OH). Huh7 cells were a kind gift from V. Lohmann (University of Heidelberg, Germany). The LCMV WE strain was obtained from R. M. Zinkernagel (Universität Zürich, Switzerland). Recombinant vaccinia virus expressing vesicular stomatitis virus Indiana glycoprotein (VV-INDG) was originally obtained from R. M. Zinkernagel (University of Zürich, Switzerland). D980R cells were a kind gift from S. G. Sawicki (Medical University of Ohio, Toledo, OH). Huh7 cells were a kind gift from V. Lohmann (University of Heidelberg, Germany). The LCMV WE strain was obtained from R. M. Zinkernagel (Universität Zürich, Switzerland). Recombinant vaccinia virus expressing LCMV glycoprotein 2 (VV-G2) was originally obtained from B. H. Bishop (University of Oxford, United Kingdom), and recombinant vaccinia virus expressing vesicular stomatitis virus Indiana glycoprotein (VV-INDG) was originally obtained from B. Moss (National Institutes of Health, Bethesda, MD). Titration and determination of antiviral protection assays have been performed as described previously (7). Further details can be found in Text S1 in the supplemental material.

**Cloning and generation of recombinant MHV-based vectors and human coronaviruses.** Genomic RNA of recombinant MHV-based vectors and recombinant HCoVs were produced from cloned cDNA using purified vaccinia virus DNA as the template for in vitro transcription as described previously (54). A detailed description of all cloning steps, sequence information, and production of recombinant coronavirus particles can be found in Text S1 in the supplemental material.

**Isolation of dendritic cells and macrophages, flow cytometry, immunofluorescence.** Generation of bone marrow-derived DCs and flow cytometric analysis were performed as described previously (50). Further information on these procedures can be found in Text S1 in the supplemental material. For immunofluorescence analysis of viral replication, 2 × 10^6 L929 cells or peritoneal macrophages were seeded in slide chambers and trans-
duced with MHV-GP or MHV-GM/GP (MOI = 1). At the time points indicated in Fig. 1, the slides were acetone fixed, blocked with the Fc-blocking antibody 2.4G2, and stained with anti-EGFP Alexa 488 (BioLegend). Images were acquired using a Leica DMRA microscope and processed using Adobe Photoshop (Adobe Systems).

Tetramer analysis and intracellular cytokine staining. Peptide-specific CD8+ T cells and ex vivo production of IFN-γ or tumor necrosis factor alpha (TNF-α) were determined by tetramer staining, and intracellular cytokine staining was performed as described previously (24, 28).

Detailed information on these procedures can be found in Text S1 in the supplemental material.

Generation of human DCs and ICS. DCs were generated from monocytes isolated from peripheral blood mononuclear cells as described previously (55). Mature DCs were infected with Mel-A/EGFP-recombinant lentivirus (see Supplemental material). Images indicated in Fig. 1, the slides were acetone fixed, blocked with the Fc-blocking antibody 2.4G2, and stained with anti-EGFP Alexa 488 (BioLegend). Images were analyzed with the nonpaired distribution. A P value of <0.05 was considered significant.

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