Prevention and treatment of DNA vaccine encoding cockroach allergen Bla g 1 in a mouse model of allergic airway inflammation

B. Zhou1, M. Ensell1, Y. Zhou1, U. Nair1, J. Glickstein1, M. H. Kermany1, Q. Cai1, C. Cai1, W. Liu1, Y.-P. Deng2, A. Kakigi3, M. Barbieri4, M. Mora4, S. Kanangat1 & T. J. Yoo1

1Department of Medicine, University of Tennessee Health Science Center, Memphis, TN; 2Department of Anatomy & Neurobiology, University of Tennessee Health Science Center, Memphis, TN, USA; 3Department of Otolaryngology, University of Tokyo, Tokyo, Japan; 4Department of Otolaryngology, San Martino Hospital University of Genoa, Genoa, Italy


Abstract

Background: One-fourth of the US population is sensitized to the German cockroach. Primary German cockroach allergen Bla g 1 is detected in 63% of homes and 52% of childcare facilities in the United States. No effective treatment or vaccination strategies are yet available.

Objectives: We evaluated the prophylactic and therapeutic efficacy of a plasmid DNA-mediated vaccination using the Bla g 1 gene in a mouse model of allergic inflammatory airway disease.

Methods: A plasmid DNA vector coding for the Bla g 1 allergen controlled by cytomegalovirus promoter was constructed. To estimate the protective efficacy, BALB/c mice were given three injections of plasmid DNA–Bla g 1 prior to sensitization with two priming doses of recombinant Bla g 1 (rBla g 1) antigens, followed by nebulized rBla g 1 challenge. In the therapeutic approach, sensitization was followed by administering Bla g 1 DNA vaccine.

Results: Bla g 1 vaccination significantly reduced allergen-induced airway inflammation, even after mice were presensitized and a Th2-dominant response was established. The Bla g 1 vaccination significantly reduced total inflammatory cell infiltrate, eosinophilia, secretion of Th2 cytokines IL-4 and IL-5 in bronchoalveolar lavage fluid, allergen-induced inflammatory infiltrates in the lungs, and Bla g 1-specific IgE in serum upon challenge with rBla g 1. Importantly, Bla g 1 DNA vaccination was able to induce IL-10-secreting regulatory T cells that could suppress the allergen-specific Th2 cells.

Conclusion: DNA vaccination showed protective and therapeutic efficacy against a clinically relevant allergen Bla g 1.

Exposure to cockroaches is one of the major contributing factors for asthma exacerbation among inner-city children (1–3) where infestations of the German cockroach are common (4–6). It was found using skin test that 26.1% of the US population is sensitized to the German cockroach (7). The primary German cockroach allergen, Bla g 1, was detectable in 63% of homes and 52% of childcare facilities in the United States (8, 9). Bla g 1 from German cockroach is cross-reactive with an American cockroach allergen, Per a 1 (10–12).

Prominent characteristics of allergic airway inflammation (AAI) include reduced airflow, airway hyper-responsiveness, mast cell activation, influx of eosinophils into the lung, and hyperplasia of goblet cells with excessive mucus secretion (13, 14). These are associated with secretion of cytokines and chemokines that stimulate a Th2- and IgE-dominated response.

Glucocorticoids are currently the most effective drugs for treating asthma. However, side-effects remain a significant problem (15). Allergen-specific immunotherapy (SIT) is the only curative approach toward allergic diseases (16, 17) and appears to reduce the incidence of asthma in later life. However, SIT also has undesirable side-effects and requires intensive medical supervision (17, 18).

DNA-based vaccine strategies have been proven to induce protective cellular and humoral immune responses.
against many parasitic, bacterial, and viral diseases (19–21).
Intramuscular injection of plasmid DNA-encoding allergens is thought to lead to the recruitment of allergen-specific Th1 cells, which secrete interferon (IFN)-γ and possibly protect against allergic responses by inhibiting the proliferation and development of Th2 cells and IgE production (19–21).

Regulatory T (Treg) cells secreting IL-10 (Tr1) have been shown to have a major role in allergen tolerance (22–25). The number of Treg cells is decreased in allergic individuals and presumably results in AAI. Both SIT and glucocorticoids have been shown to induce CD4+CD25+Foxp3+ Treg cells and IL-10-producing Tr1 cells (26, 27).

Our results show that Bla g 1 DNA vaccination has both prophylactic and therapeutic effects. Notably, the suppression of AAI by Bla g 1 vaccine was associated with an induction of CD25+CD4+Foxp3+ Treg cells secreting IL-10, which could suppress the in vivo-induced Th2 responses in an in vitro coculture assay.

Material and methods

Molecular cloning, expression, and purification of Bla g 1–recombinant plasmid

Bla g 1-encoding fragment (GenBank accession code AF072220) was amplified from German cockroach cDNA by PCR with Bla g 1-specific primers (5′-CACCATGCCCATAAGATT CCTCAATAA-3′, forward, and 5′-TTATGTTGTATAATCTCTTT-3′, reverse; including Sac I and Xba I sites). The sequence of the amplified Bla g 1 was verified by DNA sequencing, and the Bla g 1-encoding fragment was subcloned into pCI-neo expression vector. Expression of the Bla g 1 in mammalian cells was verified by transfecting 1·10⁶ mouse macrophage cell line AMJ2-C8 (ATCC, Manassas, VA, USA) with 30 μg of Bla g 1 vaccine by LipofectAMINE™ 2000 (Promega, Madison, WI, USA). Two days after culture in RPMI-1640 medium, culture supernatants and cell lysates from transfected cells were analyzed by Western blotting (Invitrogen, Carlsbad, CA, USA) with anti-cockroach-Bla g 1 monoclonal antibody (Indoor Biotechnologies, Charlottesville, VA, USA). Large-scale purification of the expression vectors was conducted with Endo Free Plasmid Maxi kits (Qiagen, Valencia, CA, USA).

Sensitization, airway challenge, and vaccination

Female BALB/c mice, 6 weeks old (Jackson Laboratory, Bar Harbor, ME, USA), were intraperitoneally injected with 10 μg of cockroach allergen Bla g 1 (rBla g 1; Indoor Biotechnologies) together with 4 mg Al(OH)₃ (Serva, Heidelberg, Germany) in a total volume of 150 μl sterile phosphate buffer saline (PBS). The experimental mice subsequently received a daily airway exposure to an aerosol of 1% rBla g 1 in PBS for 30 min over five consecutive days. The DNA vaccination was given intramuscularly with 100 μg of plasmid DNA in a total volume of 150 μl sterile PBS.

Experimental design

The design to evaluate the prophylactic effect of DNA vaccination is shown in Fig. 2A. Mice were given three intra-
muscular injections (days 0, 7, and 14) of Bla g 1 vaccine. On days 28 and 35 after the first injection, mice were sensitized with rBla g 1. The design to study the therapeutic efficacy of DNA vaccination is shown in Fig. 3A. Mice were given two intraperitoneal injections (days 0 and 7) of rBla g 1; the nonsensitized mice received PBS instead of rBla g 1/alum solution. Subsequently, the nonsensitized and sensitized mice were treated with Bla g 1 vaccine on days 21, 28, and 35. On days 51–55 after the first injection, all mice were challenged with rBla g 1 by exposing them to an aerosol of 1% rBla g 1 in phosphate buffer saline for 30 min. Mice were killed 24 h after the last challenge, and serum and bronchoalveolar lavage (BAL) fluids were collected for IgE and cytokine measurements and cellular analysis. (B–E) BAL fluid was collected and centrifuged. Cells were resuspended to count total cells (B), and eosinophils (C) were determined using Wright–Giemsa stain; *P < 0.005 vs control DNA mice; supernatant was collected for enzyme-linked immunosorbent assay (ELISA) of IL-4 (D) and IL-5 (E). Serum Bla g 1-specific IgE (F) was determined by ELISA (modified BD OptEIA ELISA kit; BD Pharmingen, San Jose, CA, USA). The data represent the means ± SE from five mice/group. P value vs control DNA mice.

In vitro cytokine production and lymphocytes proliferation

Splenocytes (3 × 10^5 cells/well) were cultured in 96-well flat-bottomed plates (Costar, Corning, NY, USA) in RPMI 1640 complete medium supplemented with 5% fetal calf serum (Gibco, Paisley, UK) and stimulated with rBla g 1 (10 μg/ml). Positive control wells contained 2 μg/ml anti-mouse CD3 (eBioscience, San Diego, CA, USA), and negative control wells contained only PBS. Supernatants were harvested after 48 h for their cytokine measurement (R&D Systems). Cells were pulsed with [3H]-thymidine during the last 18 h of the 72-h assay, harvested, and counted for [3H]-thymidine incorporation (Packard Instrument, Boston, MA, USA).

Flow cytometry

The CD4^+ CD25^+ Foxp3^+ -expressing T cells were identified by staining splenocytes with PE-labeled anti-CD4 and APC-labeled anti-CD25 (eBioscience). For intracellular
staining of Foxp3, cells were fixed and permeabilized before incubation with FITC-labeled anti-mouse Foxp3 (eBioscience). Appropriate isotype-matched control antibodies were used to determine nonspecific staining. Samples were analyzed on a FACScan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

**In vitro** suppression assay

CD4+ CD4+CD25+ and CD4+ CD25+ T cells were isolated using a mouse Treg isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Proliferation assays were performed by culturing CD4+ CD25+ cells (responder, 5 x 10^4 from splenocytes of rBla g 1-sensitized mice) and CD4+ CD25+ T cells (suppressor, 5 x 10^4 from splenocytes of Bla g 1-vaccinated mice, DNA control mice, PBS-treated mice, the nonsensitized mice received Bla g 1 DNA, and naïve mice) in 96-well plates with irradiated antigen-presenting cells (APCs, 5 x 10^4 from splenocytes of normal BALB/c mice) for 72 h at 37°C in complete medium. Cultures were stimulated with rBla g 1 (10 µg/ml), and some cocultures were treated with anti-IL-10 antibodies (10 µg/ml; eBioscience). After 3 days, 1 µCi/well [3H]-thymidine was added. Incorporation of [3H]-thymidine was assessed after 18 h of additional culturing.

**Statistical analysis**

Statistical analyses were performed using ANOVA or Student’s t-test.

**Results**

**Expression of Bla g 1 DNA vector**

The plasmid DNA vector, the Bla g 1 DNA fragment, and the expressed 55-kD protein of Bla g 1 are shown in Fig. 1. The expression levels of the Bla g 1 DNA construct were tested in AMJ2-C8 cells. Culture supernatants and cell lysates from transfected cells were analyzed by Western blotting using Bla g 1-specific antibody. The protein was detected in the culture supernatants (Fig. 1C-2). No Bla g 1 protein was present in control samples transfected with the empty vector (Fig. 1C-1).
Bla g 1 vaccine prevents cockroach allergen–induced pulmonary manifestations

In the prophylactic approach (Fig. 2A), BAL fluid showed that the infiltration of total cells (Fig. 2B) and eosinophils (Fig. 2C) was significantly inhibited at a Bla g 1 vaccine dose of 100 μg and reached maximal inhibition at 200 μg. Doses of 25 and 50 μg, however, exhibited a minimal or low effect on inhibition of infiltration, respectively. In contrast, the control mice exhibited a high level of infiltration. Moreover, Bla g 1-vaccinated mice showed significantly reduced levels of IL-4 and IL-5 (Fig. 2D,E), which finding correlated with significantly reduced infiltration of total cells and eosinophils in the lungs. Furthermore, Bla g 1 vaccine was able to prevent the induction of Bla g 1-specific IgE (Fig. 2F). In contrast, the levels of Bla g 1-specific IgE were high in the control group.

Bla g 1 vaccine suppresses ongoing cockroach allergen–induced pulmonary manifestations indicating therapeutic potentials

In the therapeutic approach (Fig. 3A), Bla g 1-vaccinated mice had markedly lower cellular infiltrates in BAL compared with those in control mice (Fig. 3B). The number of cells recovered from the BAL of Bla g 1-vaccinated mice was comparable to the number recovered from normal, untreated mice and the nonsensitized mice that received Bla g 1 DNA. Moreover, there was no detectable eosinophilic infiltration in the mice treated therapeutically with Bla g 1 DNA (Fig. 3C). This result was also reflected by a decrease in IL-4 and IL-5 levels in the lungs of the vaccinated mice (Fig. 3D,E). Histological analysis of the lungs showed that the cockroach-sensitized and cockroach-challenged mice that received control DNA or PBS (Fig. 4B,C) demonstrated the cardinal features...
of AAI, including clusters of inflammatory cells surrounding the airways and bronchiolar epithelium showing columnar epithelial cell hyperplasia, while the Bla g 1-vaccinated mice nearly restored these features (Fig. 4D); lungs of naive (untreated, unchallenged) mice (Fig. 4A), the nonsensitized mice that received Bla g 1 DNA (Fig. 4E), and Bla g 1-vaccinated mice (Fig. 4D) were indistinguishable, demonstrating the therapeutic effects of the Bla g 1 vaccine.

To investigate whether Bla g 1 vaccine induced T-cell and B-cell responses, lymphoproliferation assays were performed 24 h after the last challenge. The Bla g 1-specific proliferative response was elevated five- to six-fold in Bla g 1-vaccinated mice compared with that of control mice (Fig. 5A). The culture supernatants of Bla g 1-vaccinated mice produced significant levels of IFN-γ compared with those in control DNA mice (Fig. 5B), and Bla g 1-vaccinated mice (Fig. 4D) were indistinguishable, demonstrating the therapeutic effects of the Bla g 1 vaccine.

To investigate whether Bla g 1 vaccine induced T-cell and B-cell responses, lymphoproliferation assays were performed 24 h after the last challenge. The Bla g 1-specific proliferative response was elevated five- to six-fold in Bla g 1-vaccinated mice compared with that of control mice (Fig. 5A). The culture supernatants of Bla g 1-vaccinated mice produced significant levels of IFN-γ compared with those in control DNA mice (Fig. 5B), and Bla g 1-vaccinated mice (Fig. 4D) were indistinguishable, demonstrating the therapeutic effects of the Bla g 1 vaccine.

Treg cells were recruited by Bla g 1 vaccine

Previous studies have demonstrated that Treg cells can confer significant protection against AAI by suppressing the Th2 responses (26–28). Therefore, we compared the proportion and suppressive activity of Treg cells between experimental and control groups. The Bla g 1-vaccinated group had a significantly higher percentage of CD4+CD25+Foxp3+ cells (11.5 ± 1.72%) than did the DNA control group (6.6 ± 0.49%, P = 0.02). Moreover, we examined the suppressive activity of Bla g 1-specific Treg cells on allergen-induced Th2 cells obtained from rBla g 1-sensitized mice. Figure 6B shows that CD4+CD25+ T cells from Bla g 1-vaccinated mice suppressed the proliferation of rBla g 1-activated CD4+CD25+ T cells, and this effect was significantly reversed by anti-IL-10 antibodies. Thus, the Bla g 1 vaccine could be inducing Treg cells secreting IL-10, which suppresses the allergen-induced Th2 activity.

Discussion

There are no specific therapeutic strategies to treat Bla g 1-mediated AAI. In this study, we tested the efficacy of a DNA vaccine against the common household cockroach allergen,
Figure 6 Induction of Treg cells with suppressive functions after Bla g 1 vaccination. Splenocytes were harvested 24 h after the last challenge. (A) Frequency of CD4+ CD25+ Foxp3+ T cells was assessed by flow cytometry. Live lymphocytes from the spleen of mice were gated. The cell surfaces were stained with CD4 (PE) and CD25 (APC) Ab in accordance with the manufacturer’s recommended protocols. After permeabilization buffer treatment, the cells were stained with anti-mouse Foxp3 (FITC) Ab. Treg cell populations in the spleen were significantly increased by Bla g 1 vaccine. (B) The suppressive function of CD4+ CD25+ Treg cells. CD4+ CD25+ T cells purified from splenocytes of Bla g 1-vaccinated mice (B), DNA control mice (D), phosphate buffer saline-treated mice (P), the nonsensitized mice that received Bla g 1 DNA (S), and naive mice (N) by magnetic antibody cell sorting (MACS). CD4+ CD25+ T cells isolated from rBla g 1-sensitized mice as responders. CD4+ CD25+ T cells alone, CD4+ CD25+ T cells alone, and CD4+ CD25+ T cells cocultured with CD4+ CD25+ T cells stimulated with allergen-rBla g 1 in the presence of antigen-presenting cells, and some cocultures treated with neutralizing antibodies to IL-10 (anti-IL-10). After 3 days, 1 μCi/well [3H]-thymidine was added. Incorporation of [3H]-thymidine was assessed after 16 h of additional culturing. Values are the means ± SE of five mice/group. P = 0.006 vs control DNA mice; P = 0.012 vs Bla g 1 vaccination group.
eral tolerance in vivo (24–27). The upregulation of Bla g 1-specific IL-10 production suggests the possibility that Bla g 1 vaccine may induce IL-10-producing Treg cells (31, 32) that suppress the effector responses in the lungs. We therefore examined this possibility in vivo and found a significantly elevated percentage of CD4+ CD25+ Foxp3+ cells in Bla g 1-vaccinated mice. Also, CD4+ CD25+ cells isolated from Bla g 1-vaccinated mice showed profound immunosuppressive activities on rBla g 1-specific effector T cells, which were significantly abrogated by anti-IL-10 antibodies. Therefore, Bla g 1 vaccination could induce IL-10-producing Treg cells in allergic mice that suppress effector T cells and mediate T-cell tolerance. Also, IL-10 could exhibit a number of regulatory mechanisms, including downregulation of mast cell function, suppression of IgE, eosinophil-sensitive chemokine production, and reduced eosinophil survival (33, 34). Therefore, the presence of IL-10-producing CD4+ CD25+ Foxp3+ Treg cells might explain the absence of inflammation in the lungs of Bla g 1-vaccinated mice sensitized and challenged with experimental allergen.

Allergic diseases are all characterized by elevated levels of serum IgE and show a positive, close relationship with clinical symptoms. Bla g 1 vaccination after systemic allergen sensitization significantly reduced Bla g 1-specific IgE production, whereas vaccination before sensitization almost completely diminished Bla g 1-specific IgE production. Also, Treg cells can directly inhibit B-cell response and Ig class switch recombination (35). In contrast to CD4+ CD25+ Foxp3+ Treg cells, Tr1 cells also cross-talk to B cells by producing IL-10, which leads to the regulation of antibody production. Therefore, IL-10 not only generates T-cell tolerance but also regulates specific Ig isotype formation of B cells.

In summary, the current study demonstrates that Bla g 1 vaccine, beyond its protective properties, also displays a therapeutic potential, suggesting that DNA vaccines may provide a novel therapeutic approach for allergic diseases. Mechanistically, our results indicate that Bla g 1 vaccine might be inducing IL-10-producing Bla g 1-specific CD4+ CD25+ Foxp3+ Treg cells through which both prophylactic and therapeutic potentials are mediated.

Acknowledgments

Supported by the Allergy support Fund, project no. R073375003.

Author’s contributions

BZ and ME conceived of the study, participated in its design and coordination, and helped to draft the manuscript. BZ, YZ, UN, and YPD participated in the analysis and interpretation of data and performed the statistical analyses. BZ, ME, YZ, JG, MHK, QC, CC, and WL carried out the assays. AK, MB, MM, and SK drafted the article or revised it critically for important intellectual content. TJY provided final approval of the version to be published.

Conflict of interests

The authors declare that they have no competing interests.

References


Dear Author,
During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper’s edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

<table>
<thead>
<tr>
<th>Query reference</th>
<th>Query</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AUTHOR: An Copyright Transfer Agreement has not yet been received for this paper. Please go to: <a href="http://www.wiley.com/go/ctaaglobal">www.wiley.com/go/ctaaglobal</a> and download a form and return it to Fax No +44 131 226 3803 with your corrections to the proofs. We cannot publish your paper until the Copyright Transfer Agreement is received.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AUTHOR: Please check that authors and their affiliations are correct.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AUTHOR: Please define PE.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AUTHOR: Please define FITC.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AUTHOR: Kindly check whether “pCI-Bla g 1” can be changed to “pCI-neo–Bla g 1” in the sentence “BALB/c mice were…pCI-neo blank vector.”</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AUTHOR: Figure 1 has been saved at a low resolution of 160 dpi. Please resupply at 600 dpi. Check required artwork specifications at <a href="http://authorservices.wiley.com/submit_illust.asp?site=1">http://authorservices.wiley.com/submit_illust.asp?site=1</a></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AUTHOR: Figure 2 has been saved at a low resolution of 190 dpi. Please resupply at 600 dpi. Check required artwork specifications at <a href="http://authorservices.wiley.com/submit_illust.asp?site=1">http://authorservices.wiley.com/submit_illust.asp?site=1</a></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AUTHOR: Figure 3 is of poor quality. Please check required artwork specifications at <a href="http://authorservices.wiley.com/submit_illust.asp?site=1">http://authorservices.wiley.com/submit_illust.asp?site=1</a></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AUTHOR: Figure 5 is of poor quality. Please check required artwork specifications at <a href="http://authorservices.wiley.com/submit_illust.asp?site=1">http://authorservices.wiley.com/submit_illust.asp?site=1</a></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AUTHOR: Figure 6 is of poor quality. Please check required artwork specifications at <a href="http://authorservices.wiley.com/submit_illust.asp?site=1">http://authorservices.wiley.com/submit_illust.asp?site=1</a></td>
<td></td>
</tr>
</tbody>
</table>
AUTHOR: All colour figures will appear in colour online when published on Early View. If you wish the figure to appear in colour in print please complete the Colourwork agreement form and return it with your corrections. The form and charge information can be found online: www.onlinelibrary.wiley.com/journal/10.1111/(ISSN)1398-9995/homepage/ForAuthors.html.
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: [http://get.adobe.com/reader/](http://get.adobe.com/reader/)

Once you have Acrobat Reader open on your computer, click on the **Comment** tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the **Annotations** section, pictured opposite. We’ve picked out some of these tools below:

1. **Replace (Ins)** Tool – for replacing text.
   - Strikethrough (Del) Tool – for deleting text.
   - **Add note to text** Tool – for highlighting a section to be changed to bold or italic.
   - **Add sticky note** Tool – for making notes at specific points in the text.

**How to use it**

- Highlight a word or sentence.
- Click on the **Replace (Ins)** icon in the Annotations section.
- Type the replacement text into the blue box that appears.

- Highlight a word or sentence.
- Click on the **Strikethrough (Del)** icon in the Annotations section.
- Strikethrough (Del) Tool – for deleting text.

- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

- Click on the **Add note to text** icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

- Click on the **Add sticky note** icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.
5. **Attach File Tool** – for inserting large amounts of text or replacement figures.

   Inserts an icon linking to the attached file in the appropriate pace in the text.

   **How to use it**
   - Click on the Attach File icon in the Annotations section.
   - Click on the proof to where you’d like the attached file to be linked.
   - Select the file to be attached from your computer or network.
   - Select the colour and type of icon that will appear in the proof. Click OK.

6. **Add stamp Tool** – for approving a proof if no corrections are required.

   Inserts a selected stamp onto an appropriate place in the proof.

   **How to use it**
   - Click on the Add stamp icon in the Annotations section.
   - Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
   - Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. **Drawing Markups Tools** – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

   Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

   **How to use it**
   - Click on one of the shapes in the Drawing Markups section.
   - Click on the proof at the relevant point and draw the selected shape with the cursor.
   - To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
   - Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: